Journal of Organometallic Chemistry 769 (2014) 46–57

Contents lists available at ScienceDirect

Journal of Organometallic Chemistry

journal homepage: www.elsevier.com/locate/jorganchem

Alkenyl-substituted titanocene dichloride complexes: Stability studies, binding and cytotoxicity

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ARTICLE INFO

Article history:
Received 25 February 2014
Received in revised form
10 June 2014
Accepted 23 June 2014
Available online 18 July 2014

Keywords:
Titanocene derivatives
Cyclic voltammetry
Cytotoxicity
Anticancer drugs
Hydrolysis
UV–vis spectroscopy

ABSTRACT

Four alkenyl-substituted titanocene dichloride complexes [Ti(η^5-C_5H_5){η^5-C_5H_4(CMeR(CH_2CH_2CH=CH_2))}Cl_2] (R = Me (**8**), Ph (**9**)) and [Ti(η^5-C_5H_5){η^5-C_5H_3(CMeR(CH_2CH_2CH=CH_2))($SiMe_3$)}] (R = Me (**10**), Ph (**11**)) have been synthesized and characterized.

The cytotoxic activity of **8**–**11** has been tested against human tumour cell lines from four different tissue origins [8505C (anaplastic thyroid cancer), DLD-1 (colon cancer), FaDu (head and neck cancer), A2780 (ovarian cancer) and A549 (lung carcinoma)] and compared with those of the reference complexes [Ti(η^5-C_5H_5)$_2Cl_2$] and cisplatin. The majority of the studied titanocene compounds are more active than the reference complex [Ti(η^5-C_5H_5)$_2Cl_2$] indicating that the presence of alkenyl substituents leads to an increase in the cytotoxic activity. In addition, the presence of a trimethylsilyl group on the cyclopentadienyl ring also leads to an increase in the cytotoxic activity of **10** with respect to **8**. The contrary is observed for **9** and **11** (except on the DLD-1 cell line) with **9** (without –$SiMe_3$) being more active than **11** (with –$SiMe_3$). However, all synthesized complexes, exhibited lower cytotoxic activity than cisplatin.

Stability and binding studies based on cyclic voltammetry and UV–visible spectroscopy have been carried out in order to explore possible interactions between titanocene derivatives and various intracellular molecules, such as the nitrogenous bases cytosine and thymine, the nucleotides adenosine and guanosine, and single-strand fish sperm DNA (FS-DNA). These experiments have allowed us to construct models to examine the interactions and action mechanisms of titanocene complexes inside the cells. In addition, this is one of the first studies on the interactions of titanocene derivatives with DNA fragments using cyclic voltammetry.

1. Introduction

A large number of metal compounds have shown antiproliferative activity against tumour cells in animals or *in vitro* cell cultures. However, in spite of their severe side effects, only platinum-based compounds have had a clinical significance in chemotherapy. Thus, many groups have focused their research on finding alternatives to platinum-based drugs, in order to minimize these negative side effects, by studying metal complexes of Au, Co, Ga, Ge, Pd, Ru, Sn and Ti [1–12].

In recent years, titanocenes have been tested in the treatment of certain types of cancer [13–15]. Cytotoxicity of titanocene dichloride was evaluated against animal and human tumours [16], and in spite of the stability problems in aqueous solutions of titanocene derivatives the high cytotoxicity of these compounds led to titanocene dichloride being tested in phase II clinical trials [17]. Unfortunately, titanocene dichloride is not effective against advanced renal and breast metastatic carcinomas [18]. Despite

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http://dx.doi.org/10.1016/j.jorganchem.2014.06.031
0022-328X/

these drawbacks, many research groups, including our own, have focused their efforts on the synthesis of titanocene complexes incorporating different substituents and polar functional groups on the cyclopentadienyl rings [19–22], with the purpose of improving the cytotoxic activity of the corresponding compounds.

Further studies have revealed that titanium is able to bind to nucleic acids of tumour cells [23], hampering DNA replication and cancer propagation [24]. This fact suggests that DNA is probably one of the molecular targets of this type of drugs inside cells. Sadler and co-workers [25], found that titanium could enter into the cells assisted by the major iron transport protein, "transferrin", although transport into cancer cells *via* albumin interactions is also possible [26].

In this paper, we present the synthesis and characterization of alkenyl-substituted titanocene dichloride complexes. The cytotoxic activity has been tested against the human tumour cell lines, 8505C, DLD-1, FaDu, A2780 and A549. Hydrolysis and binding studies by UV–visible spectroscopy and cyclic voltammetry methods have been carried out in order to shed light on the possible action mechanism of the cytotoxic active titanocene complexes. The work reported here constitutes one of the first studies on the interactions of titanocene derivatives with DNA fragments using cyclic voltammetry.

2. Experimental

2.1. General manipulations

All reactions were performed using standard Schlenk tube techniques in an atmosphere of dry nitrogen. Solvents were distilled from the appropriate drying agents and degassed before use. Cyclopentadiene dimer, pyrrolidine, Li^{n}Bu (1.6 M in hexane), LiMe (1.6 M in Et_2O), LiPh (1.8 M in dibutylether), $SiMe_3Cl$ and $CH_3COCH_2CH_2CH{=}CMe_2$ were purchased from Aldrich and used directly. (C_5H_4)=CMe($CH_2CH_2CH{=}CH_2$) (**1**) and Li{C_5H_4(CMe_2($CH_2CH_2CH{=}CH_2$))} (**2**) and [Ti(η^5-C_5H_5){η^5-C_5H_4(CMe_2($CH_2CH_2CH{=}CH_2$))}Cl_2] (**8**) were synthesized as previously described by us [27]. [Ti(η^5-C_5H_5)Cl_3] was prepared by the reaction of $TiCl_4$ with $C_5H_5SiMe_3$ [28]. IR spectra were recorded on a Thermo Nicolet Avatar 330 FT-IR spectrophotometer. ^{1}H NMR and ^{13}C{^{1}H} NMR spectra were recorded on a Varian Mercury FT-400 spectrometer. Microanalyses were carried out with a Perkin–Elmer 2400 microanalyzer. EI-MS spectroscopic analyses were performed on a MASPEC II system [II32/A302] (m/z 50–1000).

2.2. Synthesis of compounds

2.2.1. Synthesis of Li{C_5H_4($CMePh(CH_2CH_2CH{=}CH_2)$)} (**3**)

LiPh (10.75 mL, 19.35 mmol, 1.80 M in dibutylether) was added dropwise to (C_5H_4)=CMe($CH_2CH_2CH{=}CH_2$) (**1**) (2.57 g, 17.60 mmol) in hexane at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 4 h. The solvent was removed *in vacuo* and the resulting white solid washed with hexane (2 × 50 mL) and dried under vacuum to yield a free-flowing white powder. Yield: 2.47 g, 78%. ^{1}H NMR (400 MHz, THF-d_8, 25 °C): δ = 1.62 (s, 3H, C*Me*Ph), 1.80–2.14 (m, 4H, $CH_2CH_2CH{=}CH_2$), 4.81 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{cis}, $^2J_{gem}$ = 2.4 Hz, $^3J_{cis}$ = 10.4 Hz), 4.97 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{trans}, $^2J_{gem}$ = 2.4 Hz, $^3J_{trans}$ = 17.2 Hz), 5.56 (m, 4H, C_5H_4), 5.80 (m, 1H, $CH_2CH_2CH{=}CH_2$), 6.98 (t, 1H, *H* in *para* position of Ph), 7.13 (m, 2H, *H* in *meta* position of Ph), 7.35 (d, 2H, *H* in *ortho* position of Ph) ppm. ^{13}C{^{1}H} NMR (100 MHz, THF-d_8, 25 °C): δ = 27.5 and 30.1 ($CH_2CH_2CH{=}CH_2$), 42.7 (C*Me*Ph), 43.3 (CpC), 101.3, 101.5 and 153.4 (C_5H_4), 112.6 ($CH_2CH_2CH{=}CH_2$), 124.1, 126.7, 127.2 and 128.7 (C_6H_5), 140.4 ($CH_2CH_2CH{=}CH_2$) ppm. $C_{17}H_{19}Li$ (230.3): calcd C 88.67, H 8.32; found C 88.21, H 8.47%.

2.2.2. Synthesis of C_5H_4(CMe_2($CH_2CH_2CH{=}CH_2$))($SiMe_3$) (**4**)

$SiMe_3Cl$ (1.30 mL, 10.26 mmol) was added dropwise to a solution of Li{C_5H_4(CMe_2($CH_2CH_2CH{=}CH_2$))} (**2**) (1.40 g, 8.34 mmol) in tetrahydrofuran (THF) at 0 °C for 5 min. The reaction mixture was warmed to room temperature and stirred for 6 h. The solvent was then removed *in vacuo* to give an oily solid, which was extracted with hexane (2 × 50 mL). The mixture was filtered and hexane removed from the filtrate to give a yellow oily solid. Yield: 1.73 g, 89%. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, for the predominant isomer): δ = −0.03 (s, 9H, Si*Me*$_3$), 1.18 (s, 6H, C*Me*$_2$), 1.59 (t, 2H, C*H*$_2$$CH_2CH{=}CH_2$), 1.94 (m, 2H, $CH_2CH_2CH{=}CH_2$), 3.24 (s, 1H, *H*C_5), 4.90 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{cis}, $^2J_{gem}$ = 1.2 Hz, $^3J_{cis}$ = 10.2 Hz), 4.97 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{trans}, $^2J_{gem}$ = 1.2 Hz, $^3J_{trans}$ = 17.2 Hz), 5.82 (m, 1H, $CH_2CH_2CH{=}CH_2$), 6.06, 6.45 and 6.59, (m, each 1H, C_5H_3) ppm. ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$, 25 °C, for the predominant isomer): δ = −1.7 (Si*Me*$_3$), 28.3 and 29.6 ($CH_2CH_2CH{=}CH_2$), 35.4 (C*Me*$_2$), 42.4 (CpC), 50.6 (C^1–C_5H_4), 113.8 ($CH_2CH_2CH{=}CH_2$), 140.0 ($CH_2CH_2CH{=}CH_2$), 125.3, 130.3, 133.9 and 154.1 (C_5H_4) ppm. $C_{15}H_{26}Si$ (234.5): calcd C 76.84, H 11.18; found C 76.54, H 11.09%.

2.2.3. Synthesis of C_5H_4($CMePh(CH_2CH_2CH{=}CH_2)$)($SiMe_3$) (**5**)

The preparation of **5** was carried out in an identical manner to **4**. $SiMe_3Cl$ (0.98 mL, 7.73 mmol) and Li{C_5H_4($CMePh(CH_2CH_2CH{=}CH_2)$)} (**3**) (1.48 g, 6.44 mmol). Yield: 1.78 g, 93%. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, two principal isomers): δ = 0.00 and 0.01 (s, each 9H, Si*Me*$_3$), 1.54 and 1.55 (s, each 3H, C*Me*Ph), 1.84–2.07 (m, 8H, C*H*$_2$C*H*$_2$CH=CH_2), 3.26 and 3.28 (s, each 1H, *H*C_5), 4.90–5.00 (m, 4H, $CH_2CH_2CH{=}CH_2$), 5.80 (m, 2H, $CH_2CH_2CH{=}CH_2$), 6.19–7.29 (m, 16H, C_5H_3 and C_6H_5) ppm. ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$, 25 °C, two principal isomers): δ = −1.6 and −1.5 (Si*Me*$_3$), 26.6, 26.9, 29.4 and 29.9 ($CH_2CH_2CH{=}CH_2$), 40.8 and 40.9 (C*Me*Ph), 43.5 and 43.5 (CpC), 50.4 and 50.5 (C^1–C_5H_4), 114.1 and 114.2 ($CH_2CH_2CH{=}CH_2$), 139.6 and 139.6 ($CH_2CH_2CH{=}CH_2$), 125.7, 125.7, 131.5, 131.6, 133.8, 133.9, 153.5 and 153.6 (C_5H_4), 126.2, 126.3, 126.6, 126.8, 127.0, 127.2, 128.0 and 128.2 (C_6H_5) ppm. $C_{20}H_{28}Si$ (296.5): calcd C 81.01, H 9.52; found C 80.88, H 9.49%.

2.2.4. Synthesis of Li{C_5H_3(CMe_2($CH_2CH_2CH{=}CH_2$))($SiMe_3$)} (**6**)

Li^{n}Bu (5.10 mL, 8.16 mmol, 1.60 in hexane) was added dropwise to a solution of C_5H_4(CMe_2($CH_2CH_2CH{=}CH_2$))($SiMe_3$) (**4**) (1.73 g, 7.42 mmol) in hexane at −78 °C. The mixture was warmed to 25 °C and stirred for 16 h. Solvent was then removed *in vacuo* giving a white solid, which was washed with hexane (2 × 50 mL) and dried under vacuum to yield the title compound. Yield: 1.59 g, 89%. ^{1}H NMR (400 MHz, THF-d_8, 25 °C): δ = 0.10 (s, 9H, Si*Me*$_3$), 1.21 (s, 6H, C*Me*$_2$), 1.58 (m, 2H, C*H*$_2$$CH_2CH{=}CH_2$), 1.92 (m, 2H, CH_2C*H*$_2$CH=CH_2), 4.77 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{cis}, $^2J_{gem}$ = 2.4 Hz, $^3J_{cis}$ = 10.0 Hz), 4.88 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{trans}, $^2J_{gem}$ = 2.4 Hz, $^3J_{trans}$ = 19.2 Hz), 5.77–5.84 (m, 4H, $CH_2CH_2CH{=}CH_2$ and C_5H_3) ppm. ^{13}C{^{1}H} NMR (100 MHz, THF-d_8, 25 °C): δ = 0.8 (Si*Me*$_3$), 30.1 and 30.3 (*C*$H_2CH_2CH{=}CH_2$), 34.60 (C*Me*$_2$), 45.5 (CpC), 104.0, 106.6, 107.4, 110.1 and 132.0 (C_5H_3), 112.3 ($CH_2CH_2CH{=}CH_2$), 140.8 ($CH_2CH_2CH{=}CH_2$) ppm. $C_{15}H_{25}LiSi$ (240.4): calcd C 74.95, H 10.48; found C 74.51, H 10.22%.

2.2.5. Synthesis of Li{C_5H_3($CMePh(CH_2CH_2CH{=}CH_2)$)($SiMe_3$)} (**7**)

The preparation of **7** was carried out in an identical manner to **6**. Li^{n}Bu (4.13 mL, 6.61 mmol, 1.60 M in hexane) and C_5H_4($CMePh(CH_2CH_2CH{=}CH_2)$)($SiMe_3$) (**5**) (1.78 g, 6.01 mmol). Yield: 1.14 g, 63%. ^{1}H NMR (400 MHz, THF-d_8, 25 °C): δ = 0.09 (s, 9H, Si*Me*$_3$), 1.62 (s, 3H, C*Me*Ph), 1.90 (m, 2H, C*H*$_2$$CH_2CH{=}CH_2$), 2.11 (m, 2H, CH_2C*H*$_2$CH=CH_2), 4.81 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{cis}, $^2J_{gem}$ = 2.4 Hz, $^3J_{cis}$ = 10.0 Hz), 4.91 (dd, 1H, $CH_2CH_2CH{=}CH_2$, H_{trans}, $^2J_{gem}$ = 2.4 Hz, $^3J_{trans}$ = 17.0 Hz), 5.70–5.83 (m, 4H, $CH_2CH_2CH{=}CH_2$ and C_5H_3), 6.98 (t, 1H, *H* in *para* position of Ph), 7.15 (t, 2H, *H* in *meta*

position of Ph), 7.36 (d, 2H, *H* in *ortho* position of Ph) ppm. $^{13}C\{^1H\}$ NMR (100 MHz, THF-d_8, 25 °C): δ = 1.5 (Si*Me*$_3$), 28.2 and 30.8 (*CH*$_2$*CH*$_2$CH=CH$_2$), 43.4 (C*Me*Ph), 44.3 (Cp*C*), 106.4, 107.4, 109.1, 111.0 and 133.1 (*C*$_5$H$_3$), 113.5 (CH$_2$CH$_2$CH=*C*H$_2$), 141.1 (CH$_2$CH$_2$*C*H=CH$_2$), 125.0, 127.5, 128.1 and 153.5 (*C*$_6$H$_5$) ppm. $C_{20}H_{27}LiSi$ (302.4): calcd C 79.42, H 9.00; found C 78.99, H 8.91%.

2.2.6. Synthesis of [Ti(η^5-C$_5$H$_5$){η^5-C$_5$H$_4$(CMePh(CH$_2$CH$_2$CH=CH$_2$))}Cl$_2$] (**9**)

A solution of Li{C$_5$H$_4$(CMePh(CH$_2$CH$_2$CH=CH$_2$))} (1.00 g, 4.35 mmol) in THF (50 mL) was added dropwise during 15 min to a solution of [Ti(η^5-C$_5$H$_5$)Cl$_3$] (0.91 g, 4.15 mmol) in THF (50 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 2 h. Solvent was removed in vacuum and a toluene/hexane 9:1 mixture (50 mL) added to the resulting solid. The suspension was filtered and the filtrate concentrated (20 mL) and cooled to −30 °C to give the title compound as a red solid. Yield: 1.70 g, 81%. FT-IR (KBr): $\bar{\nu}$ = 3096(m) for ν_{CH}, 1638 (m) for $\nu_{C=C}$, cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$, 25 °C): δ = 1.81 (s, 3H, C*Me*Ph), 2.08 (m, 2H, CH$_2$*CH*$_2$CH=CH$_2$), 2.36 (m, 2H, *CH*$_2$CH$_2$CH=CH$_2$), 4.92 (m, 2H, CH$_2$CH$_2$CH=*CH*$_2$), 5.75 (m, 1H, CH$_2$CH$_2$*CH*=CH$_2$), 6.08 and 6.44 (m, each 2H, C$_5$*H*$_4$), 6.15 (s, 5H, C$_5$*H*$_5$), 6.86 (t, 1H, *H* in *para* position of Ph), 7.26 (m, 2H, *H* in *meta* position of Ph), 7.37 (d, 2H, *H* in *ortho* position of Ph) ppm. $^{13}C\{^1H\}$ NMR (100 MHz, CDCl$_3$, 25 °C): δ = 23.7 and 28.7 (*CH*$_2$*CH*$_2$CH=CH$_2$), 42.2 (C*Me*Ph), 44.1 (Cp*C*), 113.8 (CH$_2$CH$_2$CH=*C*H$_2$), 114.5, 117.2 and 144.9 (*C*$_5$H$_4$), 120.5 (*C*$_5$H$_5$), 121.9, 126.7, 127.4 and 128.4 (*C*$_6$H$_5$), 138.3 (CH$_2$CH$_2$*C*H=CH$_2$) ppm. EI-MS (*m*/*z* (relative intensity)): 371 (5) [M$^+$−Cl], 341 (100) [M$^+$−Cp], 148 (30) [M$^+$−Cp−Cl−CMePh(CH$_2$CH$_2$CH=CH$_2$)], 91 (35) [C$_7$H$_7^+$], 65 (36) [Cp$^+$]. $C_{22}H_{24}Cl_2Ti$ (406.9): calcd. C 64.89, H 5.94; found C 64.72, H 5.89%.

2.2.7. Synthesis of [Ti(η^5-C$_5$H$_5$){η^5-C$_5$H$_3$(CMe$_2$(CH$_2$CH$_2$CH=CH$_2$))(SiMe$_3$)}Cl$_2$] (**10**)

The preparation of **10** was carried out in an identical manner to **9**. Li{C$_5$H$_3$(CMe$_2$(CH$_2$CH$_2$CH=CH$_2$))(SiMe$_3$)} (**6**) (1.50 g, 6.25 mmol) and [Ti(η^5-C$_5$H$_5$)Cl$_3$] (1.30 g, 5.93 mmol). Yield: 0.29 g, 12%. FT-IR (KBr): $\bar{\nu}$ = 3019(m) for ν_{CH}, 1640 (m) for $\nu_{C=C}$, cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$, 25 °C): δ = 0.27 (s, 9H, Si*Me*$_3$), 1.32 and 1.34 (s, each 3H, C*Me*$_2$), 1.50–1.85 (m, 4H, *CH*$_2$*CH*$_2$CH=CH$_2$), 4.87–4.92 (m, 2H, CH$_2$CH$_2$CH=*CH*$_2$), 5.65 (m, 1H, CH$_2$CH$_2$*CH*=CH$_2$), 6.58 (s, 5H, C$_5$*H*$_5$), 6.69, 6.81 and 7.05 (m, each 1H, C$_5$*H*$_3$) ppm. $^{13}C\{^1H\}$ NMR (100 MHz, CDCl$_3$, 25 °C): δ = 0.0 (Si*Me*$_3$), 26.5 and 29.1 (*CH*$_2$*CH*$_2$CH=CH$_2$), 37.4 (C*Me*$_2$), 46.4 (Cp*C*), 114.5 (CH$_2$CH$_2$CH=*C*H$_2$), 119.7, 123.6, 126.3, 131.9 and 150.6 (*C*$_5$H$_3$), 120.4 (*C*$_5$H$_5$), 138.7 (CH$_2$CH$_2$*C*H=CH$_2$) ppm. EI-MS (*m*/*z* (relative intensity)): 416 (1) [M$^+$], 351 (94) [M$^+$−Cp], 183 (10) [M$^+$−C$_5$H$_3$(CMe$_2$(CH$_2$CH$_2$CH=CH$_2$))(SiMe$_3$)], 73 (100) [SiMe$_3^+$]. $C_{20}H_{30}Cl_2SiTi$ (416.9): calcd C 57.56, H 7.25; found C 57.42, H 7.10%.

2.2.8. Synthesis of [Ti(η^5-C$_5$H$_5$){η^5-C$_5$H$_3$(CMePh(CH$_2$CH$_2$CH=CH$_2$))(SiMe$_3$)}Cl$_2$] (**11**)

The preparation of **11** was carried out in an identical manner to **9**. Li{C$_5$H$_3$(CMePh(CH$_2$CH$_2$CH=CH$_2$))(SiMe$_3$)} (**7**) (1.00 g, 3.31 mmol) and [Ti(η^5-C$_5$H$_5$)Cl$_3$] (0.69 g, 3.14 mmol). Yield: 0.34 g, 23%. $\bar{\nu}$ = 3099(m) for ν_{CH}, 1636 (m) for $\nu_{C=C}$, cm^{-1}. ^{1}H NMR (400 MHz, CDCl$_3$, 25 °C, two isomers): δ = 0.22 and 0.26 (s, each 9H, Si*Me*$_3$), 1.68 and 1.80 (s, each 3H, C*Me*Ph), 1.50–2.20 (m, 8H, *CH*$_2$*CH*$_2$CH=CH$_2$), 4.90 (dd, 1H, CH$_2$CH$_2$CH=*CH*$_2$, H$_{cis}$, $^2J_{gem}$ = 1.8 Hz, $^3J_{cis}$ = 10.2 Hz), 4.96 (dd, 1H, CH$_2$CH$_2$CH=*CH*$_2$, H$_{trans}$, $^2J_{gem}$ = 1.8 Hz, $^3J_{trans}$ = 16.8 Hz), 5.75 (m, 2H, CH$_2$CH$_2$*CH*=CH$_2$), 6.05 and 6.17 (s, each 5H, C$_5$*H*$_5$), 5.97, 6.37, 6.69, 6.71, 6.74 and 6.83 (m, each 1H, C$_5$*H*$_3$), 7.20–7.46 (m, 10H, C$_6$*H*$_5$) ppm. $^{13}C\{^1H\}$ NMR (100 MHz, CDCl$_3$, 25 °C, two isomers): δ = 0.2 and 0.3 (Si*Me*$_3$), 23.6, 26.3, 28.9 and 29.2 (*CH*$_2$*CH*$_2$CH=CH$_2$), 41.4 and 44.3 (C*Me*Ph), 44.5 and 44.9 (Cp*C*), 114.8 and 114.9 (CH$_2$CH$_2$CH=*CH*$_2$), 120.8 and 120.9 (*C*$_5$H$_5$), 138.4 and 138.6 (CH$_2$CH$_2$*C*H=CH$_2$), 119.8, 120.4, 122.4, 125.0, 125.8, 127.0, 127.2, 127.8, 128.7, 128.8, 129.5, 130.6, 132.0, 138.0, 142.7, 144.8, 147.4 and 148.4 (*C*$_5$H$_3$ and *C*$_6$H$_5$) ppm. EI-MS (*m*/*z* (relative intensity)): 443 (5) [M$^+$−Cl], 413 (80) [M$^+$−Cp], 91 (11) [C$_7$H$_7^+$], 73 (100) [SiMe$_3^+$]. $C_{25}H_{32}Cl_2SiTi$ (478.9): calcd C 62.64, H 6.73; found C 62.22, H 6.67%.

2.3. DFT calculations for structural studies

All DFT calculations were performed by employing the Gaussian 09 program package [29] using the B3LYP functional [30–33]. The 6-31G** basis set was used for all atoms [34–37]. The appropriateness of the chosen functional and basis set for titanium complexes has been stated elsewhere [38,39]. All systems have been optimized without symmetry restrictions. The resulting geometries were characterized as equilibrium structures by the analysis of the force constants of normal vibrations (see Supplementary material).

2.4. Stability studies

2.4.1. Stability studies monitored by UV–vis spectroscopy

A 5 mL solution of the titanocene complex in dimethylsulfoxide (DMSO) (1.67×10^{-4} M) was prepared and its UV–vis spectrum was recorded. Afterwards, tris(hydroxymethyl)aminomethane (TRIS) buffer solution (50 mM NaCl–5 mM Tris–HCl, pH 7.4) was added (approx. 9:1 DMSO, Ti/H$_2$O) and the corresponding UV–vis spectrum was recorded at different time intervals (0, 12, 24, 36 and 48 h).

2.4.2. Stability studies monitored by ^{1}H NMR spectroscopy

To evaluate the stability in DMSO-d_6 or in DMSO-d_6/D$_2$O of the titanocene compounds, 20 mg of the corresponding complex was dissolved in 1 mL of DMSO-d_6 (or solution DMSO-d_6/D$_2$O 9:1) and their ^{1}H NMR spectra were recorded at room temperature at different time intervals (0, 0.5, 4 and 24 h).

2.5. DNA binding experiments monitored by UV–vis spectroscopy

Fish sperm DNA (FS-DNA) was kindly provided by the Departamento de Ciencias de la Salud of the Universidad Rey Juan Carlos (Spain). The spectroscopic titration of FS-DNA was carried out in the TRIS buffer solution (50 mM NaCl–5 mM Tris–HCl, pH 7.4) at room temperature. A solution of FS-DNA in the buffer gave a ratio of UV absorbance 1.8–1.9:1 at 260 and 280 nm, indicating that the DNA was sufficiently free of protein [40]. Milli-Q water was used to prepare the solutions. The DNA concentration per nucleotide was determined by absorption spectroscopy using the known molar extinction coefficient value of 6600 M^{-1} cm^{-1} at 260 nm [41]. Absorption titrations were performed by using a fixed titanium(IV) complex concentration (2.5×10^{-4} M) to which increments of the DNA stock solution were added. Complex–DNA adducts solutions were incubated at 37 °C for 30 min before the absorption spectra were recorded.

2.6. In vitro studies

2.6.1. Preparation of drug solutions

A solution of the studied titanium complexes was prepared in dimethyl sulphoxide (DMSO, Sigma Aldrich) at a concentration of 20 mM, filtered through Millipore filter, 0.22 μm, before use, and diluted by nutrient medium to various working concentrations. Nutrient medium was RPMI-1640 (PAA Laboratories) supplemented with 10% foetal bovine serum (Biochrom AG) and penicillin/streptomycin (PAA Laboratories).

2.6.2. Cell lines and culture conditions

The cell lines 8505C, FaDu, A549, A2780 and DLD-1 are included in this study. All these cell lines were kindly provided by Dr. Thomas Mueller, Department of Hematology/Oncology, Martin-Luther University of Halle-Wittenberg, Halle (Saale), Germany. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat inactivated foetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% (v/v) CO_2.

2.6.3. Cytotoxicity assay

The cytotoxic activities of the titanium complexes **8**–**11**, were evaluated using the sulforhodamine-B (SRB, Sigma Aldrich) microculture colorimetric assay [42]. In short, exponentially growing cells were seeded into 96-well plates on day zero at the appropriate cell densities to prevent confluence of the cells during the period of the experiment. After 24 h, the cells were treated with serial dilutions of the studied compounds for 96 h. Final concentrations achieved in treated wells were 1, 10, 20, 50, 100, 150 and 200 μM. Each concentration was tested in three triplicates on each cell line. The final concentration of DMSO solvent never exceeded 0.5%, which was non-toxic to the cells. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. After 96 h treatment, the supernatant medium from the 96 well plates was thrown away and the cells were fixed with 10% TCA. For a thorough fixation, plates were then allowed to stand at 4 °C for approximately 2 h. After fixation the cells were washed in a strip washer. The washing was carried out four times with water using alternate dispensing and aspiration procedures. The plates were then dyed with 100 μL of 0.4% SRB for about 45 min. After dyeing, the plates were again washed to remove the dye with 1% acetic acid and allowed to air dry overnight. Subsequently, 100 μL of 10 mM Tris base solution was added to each well of the plate and absorbance was measured at 570 nm using a 96 well plate reader (Tecan Spectra, Crailsheim, Germany). IC_{50} values, defined as the concentrations of the compound at which 50% of cell inhibition occur ± SD were calculated using four-parameter logistic functions and presented as mean from three independent experiments.

2.7. Cyclic voltammetry studies

Interaction studies between titanocene complexes **8** and [Ti(η^5-$C_5H_5)_2Cl_2$] with several biological molecules (FS-DNA, nitrogenous bases cytosine and thymine, and nucleotides adenosine and guanosine) were carried out at physiological pH using a TRIS buffer solution (50 mM NaCl–5 mM Tris–HCl, pH 7.4). The cyclic voltammograms were taken with a potentiostat/galvanostat Autolab PGSTAT302 Metrohm. All experiments were carried out using a conventional three electrode cell. Glassy carbon was used as working and Ag/AgCl as reference electrode. A Pt wire was also used as the auxiliary electrode. Electrochemical data were obtained using 0.1 M solutions of NaCl in DMSO/H_2O as supporting electrolyte. All solutions were previously deoxygenated by bubbling high purity nitrogen. To measure and obtain the corresponding voltammogram, solutions of the titanocene derivatives were prepared, due to solubility factors, using a DMSO/H_2O (1:9) mixture and adding NaCl 0.1 M.

With respect to the biological molecules, stock solutions (0.05 M) of cytosine, thymine, adenosine and guanosine were prepared. From these solutions, different concentrations (10, 20, 30 and 50 mM) were prepared and used during the electrochemical assays.

To prepare the FS-DNA solution, 100 mg was dissolved in buffer solution (100 mL, pH = 7.4). To determine DNA concentration (c) in solution, the Lambert–Beer law was applied ($A = \varepsilon \times b \times c$), where A is the measured absorbance in spectrophotometer, ε is the molar absorptivity (6600 M^{-1} for DNA) [41] and b is the path length (1 cm). From this stock solution, different samples (5×10^{-4}, 7.5×10^{-4}, 1×10^{-3} and 2×10^{-3} M) were prepared to measure the interaction with the titanocene complexes, **8** and [Ti(η^5-$C_5H_5)_2Cl_2$].

Initially, the voltammogram corresponding to the metal complex in the absence of biological molecules was obtained, using five different scan rates (100, 200, 300, 400, 500 and 1000 mV/s). Subsequently, the voltammogram corresponding to the metal complex in the presence of biological molecules (with concentrations increasing from 10 to 50 mM) was also recorded.

3. Results and discussion

3.1. Synthesis, characterization and structural studies of alkenyl-substituted titanocene complexes

3.1.1. Synthesis and characterization

Compounds **1**–**11** were prepared as indicated in Scheme 1.

Methyl and phenyl lithium react with the fulvene (C_5H_4)=CMe(CH_2CH_2CH=CH_2) (**1**) *via* nucleophilic addition at the exocyclic double bond to give the lithium cyclopentadienyl compounds Li{C_5H_4(CMe_2(CH_2CH_2CH=CH_2))} (**2**) and Li{C_5H_4(CMePh(CH_2CH_2CH=CH_2))} (**3**), respectively.

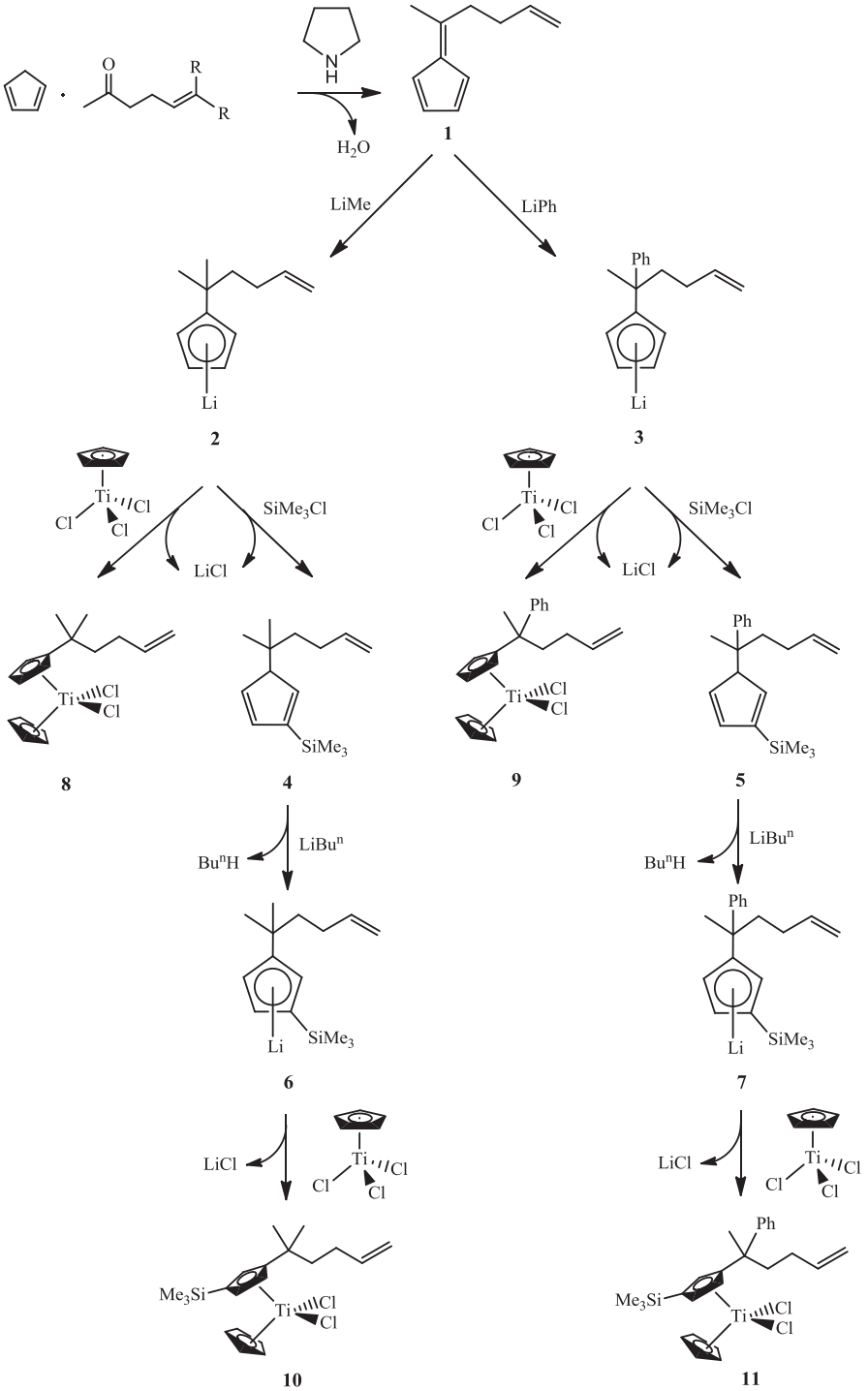
Compound **3** was characterized by ^{1}H and ^{13}C NMR spectroscopy (see Experimental section). A multiplet was observed at 5.56 ppm in the ^{1}H NMR spectrum of **3**, which was assigned to the cyclopentadienyl ring protons. In addition, a singlet due to the methyl group substituting the carbon atom adjacent to the cyclopentadienyl ring was observed at 1.62 ppm. The alkenyl fragment of **3** exhibited, in the ^{1}H NMR spectrum, five sets of signals, two corresponding to the CH_2 methylene protons (a multiplet between 1.80 and 2.14 ppm), one to the alkenyl proton of the C-γ (a multiplet at 5.80 ppm) and a multiplet between 4.79 and 4.94 ppm corresponding to the terminal alkenylic protons. Moreover, the signals due to the aromatic protons of the phenyl ring were observed (a triplet at 6.98 ppm corresponding to the proton in *para* position, a multiplet at 7.13 corresponding to the *meta* protons and a doublet at 7.35 corresponding to the *ortho* protons). Finally, the ^{13}C{^{1}H} NMR spectra of **3** showed the expect signals (see Experimental section).

Compounds **2** and **3** reacted with $SiMe_3Cl$ to give the trimethylsilyl substituted compounds C_5H_4(CMe_2(CH_2CH_2CH=CH_2))($SiMe_3$) (**4**) and C_5H_4(CMePh(CH_2CH_2CH=CH_2))($SiMe_3$) (**5**). **4** and **5** were isolated as mixtures of isomers as confirmed by ^{1}H NMR spectroscopy. Both compounds present the expected signals due to the alkenyl and the trimethylsilyl fragments.

The reaction of **4** and **5** with *n*-butyl lithium gave Li{C_5H_3(CMe_2(CH_2CH_2CH=CH_2))($SiMe_3$)} (**6**) and Li{C_5H_3(CMePh(CH_2CH_2CH=CH_2))($SiMe_3$)} (**7**). These compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, observing the expected signals due to the alkenyl, trimethylsilyl and cyclopentadienyl moieties (see Experimental section).

The titanocene(IV) dichloride derivatives, [Ti(η^5-C_5H_5){η^5-C_5H_3R(CMeR′(CH_2CH_2CH=CH_2))}Cl_2] (R = H, R′ = Me (**8**), Ph (**9**); R = $SiMe_3$, R′ = Me (**10**), Ph (**11**)), were synthesized by the reaction of [Ti(η^5-C_5H_5)Cl_3] and one molar equiv of the corresponding lithium derivative (**2**, **3**, **6** and **7**), respectively. **8**–**11** were isolated as red solids and characterized by FT-IR spectroscopy, ^{1}H and ^{13}C NMR spectroscopy, EI-MS and elemental analysis.

In the ^{1}H NMR spectra of the titanocene complexes one singlet, corresponding to the unsubstituted cyclopentadienyl ring, was observed between 6 and 6.5 ppm. The expected signals



Scheme 1. Synthesis of compounds **1**–**11**.

corresponding to the methyl, phenyl and alkenyl fragments were also present (see Experimental section).

The IR spectra of **8**–**11** showed bands corresponding to the vibration frequency of the C–H bond (ν_{CH}) of the alkenyl fragment (around 3100 cm^{-1}) and the stretching vibration of the C=C bonds ($\nu_{C=C}$) (1600 cm^{-1}) (see Experimental section for assignation of the IR bands of each compound). In the UV–visible spectra of **8**–**11**, two bands at *ca.* 300 and 400 cm^{-1} corresponding to the chromophore groups and the MLCT band of these molecules were observed. **8**–**11** were also characterized by EI-MS and peaks indicative of the loss of cyclopentadienyl, chloride, alkenyl and trimethylsilyl (for **10** and **11**) fragments were observed (see Experimental section for further details and assignation of peaks).

3.1.2. Structural studies

Density functional theory (DFT) calculations were carried out for the novel titanocene derivatives **9**–**11** at the B3LYP level [30–33]. The 6-31G** basis set was used for all atoms and all systems were optimized without symmetry restrictions. Selected bond lengths

Table 1
Selected bond lengths (pm) and angles (°) for **9**–**11**.

	9	10	11
Ti(1)–Cent(1)	213.8	215.4	214.5
Ti(1)–Cent(2)	209.9	212.2	210.6
Ti(1)–Cl(1)	234.9	236.5	234.5
Ti(1)–Cl(2)	234.9	237.0	235.4
C(1)–C(11)	153.3	153.1	153.4
C(11)–C(12)	156.4	157.2	156.5
C(12)–C(13)	153.4	154.1	153.4
C(13)–C(14)	150.9	151.8	150.9
C(14)–C(15)	133.4	135.0	133.4
Si(1)–C(6)		191.2	190.0
Cent(1)–Ti(1)–Cent(2)	132.10	132.65	132.20
Cl(1)–Ti(1)–Cent(1)	105.88	105.04	106.56
Cl(1)–Ti(1)–Cent(2)	104.94	106.60	105.78
Cl(2)–Ti(1)–Cent(1)	104.93	104.93	103.76
Cl(2)–Ti(1)–Cent(2)	106.09	105.03	106.17
Cl(1)–Ti(1)–Cl(2)	97.92	97.18	97.00
C(12)–C(13)–C(14)	115.12	115.32	115.12
C(13)–C(14)–C(15)	127.40	127.30	127.43

Cent(1) and Cent(2) are the centroids of C(1)–C(5) and C(6)–C(10), respectively.

and angles of the optimized structure of the titanocenes are listed in Table 1. The calculated structures of **9**–**11** are presented in Fig. 1.

The bond lengths between titanium and the centroid of the cyclopentadienyl rings oscillate between 209.9 and 215.4 pm. The shortest distances are found for the unsubstituted cyclopentadienyl rings or those with a trimethylsilyl group, while longer distances are found for the C5 rings with the bulky substituent containing the alkenyl fragment. The Ti–Cl bond lengths between 234.5 and 237.0 pm are in the expected range for titanocene dichloride compounds [22].

The Cent–Ti–Cent and Cl(1)–Ti(1)–Cl(2) angles of about 132° and 97° for all the compounds, respectively, are in the same range as the distances found for other DFT-calculated substituted titanocene derivatives and comparable with those recorded for the X-ray crystal structures of related compounds [22].

The C–C bond lengths for the double bonds of the alkenyl chains show similar distances between 133.4 and 135.0 pm which are in agreement with others reported in X-ray crystal structures of metallocene complexes with alkenyl groups [22]. In addition, the angles C(13)–C(14)–C(15) of around 127° show the sp^2 character of the carbon atoms C(14) and C(15), confirming the C=C double bond.

3.2. Stability studies

The stability in water of anticancer drugs is one of the most important factors in understanding the cytotoxic behaviour of these compounds inside the human body. For this reason, hydrolysis experiments monitored by UV–vis and NMR spectroscopy have been carried out.

3.2.1. Stability studies monitored by UV–vis spectroscopy

Stability in physiological medium (TRIS buffer solution) of synthesized titanocene complexes **8**–**11** has been evaluated at different time intervals using UV–vis spectroscopy. At all time intervals, a displacement towards blue region (hypsochromism) of the maximum absorption at 310 nm was observed (Fig. 2 and Figs. S1–S3 of Supplementary material) in the presence of water. For all the studied complexes, this displacement is observed at $t = 0$ h which presumably indicates instant coordination of H_2O to the titanocene complex by displacement of the chlorido ligands to give the species $[Ti(\eta^5\text{-}C_5H_4R)_2(H_2O)Cl]^+$ or $[Ti(\eta^5\text{-}C_5H_4R)_2(H_2O)_2]^{2+}$ (both with a half life of less than 1 h) and subsequent elimination of a cyclopentadienyl ligand. Moreover, a new absorption band appears at 260 nm, tentatively assigned to the hydrolysis product, which becomes more pronounced in the spectrum as time progresses.

3.2.2. Stability studies monitored by ^1H NMR spectroscopy

Stability studies monitored by ^1H NMR spectroscopy at different time intervals have been carried out for **8**–**11** in DMSO-d_6 (Fig. 3) and DMSO-d_6/D_2O (9:1) (Fig. 4).

In reference to the analysis using DMSO-d_6, the decrease of the intensity of the singlet assigned to the unsubstituted cyclopentadienyl ligand is observed in all spectra, indicating that these compounds interact with DMSO. This decrease of intensity can be estimated by comparing the integration of the proton signal of the unsubstituted cyclopentadienyl ligand in each compound (Table 2). On analyzing this signal in the NMR spectra (Fig. 3, Table 2 and Figs. S4–S6 of Supplementary material), **8** and **10** (without phenyl fragment) appear to be more stable in DMSO than **9** and **11** (with phenyl fragment). After 24 h this signal has disappeared indicating the elimination of the unsubstituted cyclopentadienyl ligand. Apart from this aspect, there is no time-dependent appearance of new chemical shifts of any significance in the spectra.

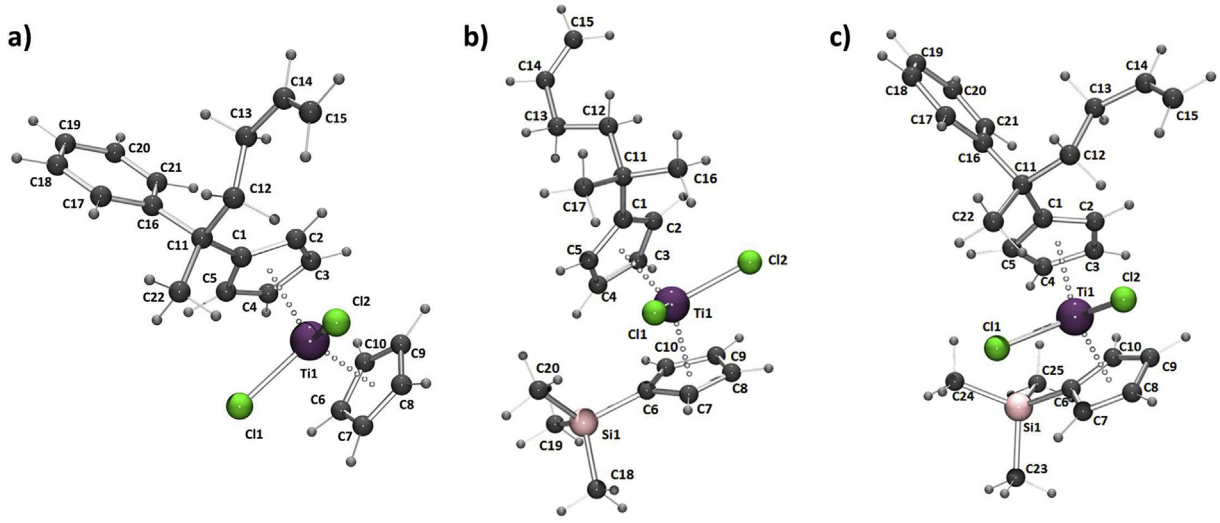


Fig. 1. DFT-calculated structures of **9** (a), **10** (b) and **11** (c).

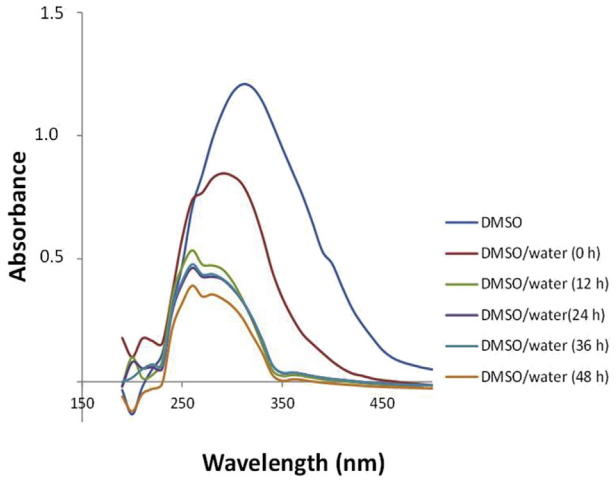


Fig. 2. UV–vis spectra for compound **9** (1.67×10^{-3} M) in DMSO or DMSO/H_2O solutions (9:1) at different time intervals and pH = 7.4.

The experiment was repeated using DMSO-d_6/D_2O (9:1). The ^1H NMR spectra were recorded at different time intervals (0, 0.5, 4 and 24 h; Fig. 4, Table 2 and Figs. S7–S9 of Supplementary material). The first visual observation was the precipitation of a small quantity of fine powder, attributed to the formation of hydrated TiO_2. According to the spectra, **8** is the most stable compound in water, as for **9**–**11** the chemical shift arising from the unsubstituted cyclopentadienyl moiety immediately disappears (or decrease drastically in intensity) at $t = 0$ h. Apart from this aspect, there is no appearance of new signals of any significance in the spectra during 24 h.

3.3. DNA interaction studies

Many studies have shown that DNA is probably one of the most important biological target molecules in many kind of compounds with proven anticancer properties, especially in titanocene complexes [5,23].

For this reason, a qualitative DNA interaction study has been carried out to confirm the possible interactions between the synthesized titanocene complexes and DNA. The absorption spectra of **8**, **9**, **10** and **11** in the absence and presence of DNA have been recorded (Fig. 5) and show a displacement of the maximum absorption at *ca.* 260 nm towards blue region (hypsochromism) and a decrease in the intensities of these bands (hypochromism) in the corresponding UV–vis spectra. This displacement of the bands indicates interaction between titanocene compounds and DNA, through different electrostatic interactions (hydrogen bonds) on active and specific sites in the DNA molecule, such as O atoms of phosphate groups (Ti-phosphate derivatives have previously been reported) [43] or N atom (N7) and O atom (O6) of the bases, especially of deoxyguanosine monophosphate (dGMP) [44].

The intrinsic binding constant of each compound, K_b, was determined using the following equation [45]:

$$\frac{[DNA]}{\varepsilon_a - \varepsilon_f} = \frac{[DNA]}{\varepsilon_0 - \varepsilon_f} + \frac{1}{K_b\left(\varepsilon_0 - \varepsilon_f\right)}$$

where [DNA] is the concentration of DNA in base pairs, ε_a, ε_f and ε_0 correspond to A_{obs}/[Complex], the extinction coefficient of the free titanium complexes and the extinction coefficient of the complexes in the fully bound form, respectively, and K_b is the intrinsic binding constant. The ratio of slope to intercept in the plot of [DNA]/($\varepsilon_a - \varepsilon_f$) *versus* [DNA] gives the value of K_b (see Fig. S15 of Supplementary material).

Thus, the intrinsic binding constants of 6.00×10^4, 5.71×10^4, 6.00×10^4 and 7.50×10^4 M^{-1} for **8**–**11**, respectively, have been successfully calculated, observing that complex **11**, gives a K_b slightly higher than those of the other titanocene derivatives **8**–**10**, indicating a slightly higher affinity from DNA. These values are, however, slightly lower than others found for similar titanocene derivatives [22c].

3.4. Cytotoxic studies

The *in vitro* cytotoxic activities of complexes **8**–**11** against the following human tumour cell lines: 8505C (anaplastic thyroid

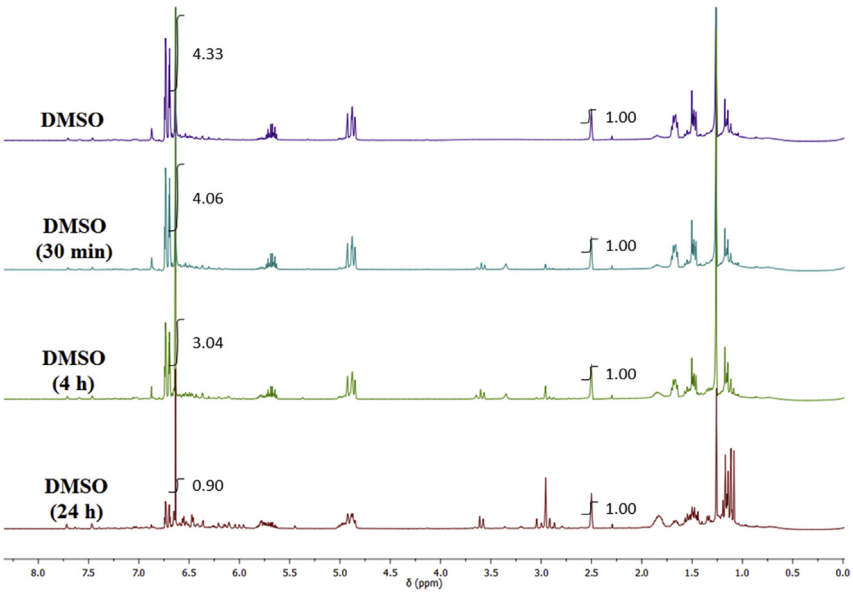


Fig. 3. ^1H NMR spectra for **8** in DMSO-d_6 at different time intervals (0, 0.5, 4 and 24 h). Integration of Cp and residual DMSO of DMSO-d_6 has been included to confirm the decay in the signals of the cyclopentadienyl ligand.

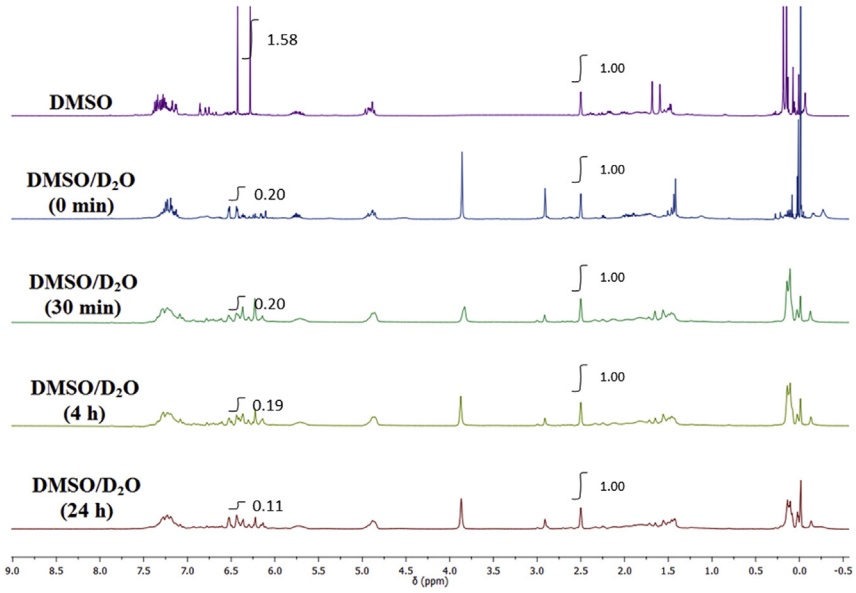


Fig. 4. ^{1}H NMR spectra for **11** in DMSO-d_6/D_2O (9:1) at different time intervals (0, 0.5, 4 and 24 h). Integration of Cp and residual DMSO of DMSO-d_6 has been included to confirm the decay in the signals of the cyclopentadienyl ligand.

carcinoma), A549 (lung adenocarcinoma), A2780 (ovarian cancer), DLD-1 (colon carcinoma) and FaDu (head and neck carcinoma) were determined by SRB assay. The IC_{50} values of the studied compounds, cisplatin and titanocene dichloride are summarized in Table 3.

Complexes were found active against all the analyzed cell lines (except **10** against 8505C cell line, IC_{50} > 200 μM) and showed a dose-dependent antiproliferative effect. In general, all the complexes show a higher cytotoxic activity against A2780 compared to the other cell lines indicating preferential activity under these experimental conditions. This tendency is also observed for cisplatin.

From all the series of titanocene derivatives reported here, **9** presents the highest cytotoxic activity against the studied cancer cell lines, except for DLD-1 where **11** is more active than **9** (Fig. 6).

Table 2
Percentage of unreacted unsubstituted Cp ligand of **8**–**11** titanocene complexes in DMSO and DMSO-d_6/D_2O (9:1) estimated by ^{1}H NMR spectroscopy.

Compounds	Reaction time (h)	Unreacted % of the unsubstituted cyclopentadienyl ligand	
		DMSO-d_6	DMSO-d_6/D_2O (9:1)
8	0	100	55
	0.5	100	53
	4	80	49
	24	23	12
9	0	100	10
	0.5	100	9
	4	34	8
	24	<1	6
10	0	100	5
	0.5	100	3
	4	75	<1
	24	29	<1
11	0	100	13
	0.5	100	10
	4	40	10
	24	<1	7

IC_{50} values of **9** vary from 33.7 ± 2.6 μM to 116.9 ± 2.0 μM. The comparison of the obtained cytotoxic activities for **8**–**11** with that of titanocene dichloride has allowed us to study the relationship between the different substituents on the cyclopentadienyl ring and the cytotoxic activity. As can be observed in Table 3, all the synthesized complexes are more active than titanocene dichloride on 8505C, A549 and DLD-1 cell lines, indicating a positive effect on the final cytotoxic activity. However, all compounds exhibited lower activity than other promising titanocene derivatives such as Tacke's titanocene-Y [4]. In addition, on direct comparison with cisplatin, the cytotoxic activity of all complexes is significantly lower.

3.5. Cyclic voltammetry studies

Electrochemical studies have been shown to be a useful tool in explaining the action mechanism between metal-based drugs and target biomolecules present in the human body [46]. A variation of the oxidation or reduction peak current observed in the corresponding voltammogram may demonstrate the binding of drugs to these biological molecules.

Cyclic voltammetry studies have therefore been carried out with **8** (as an example of the alkenyl titanocenes) and the reference complex [Ti(η^5-C_5H_5)$_2$$Cl_2$] in order to shed light on the possible

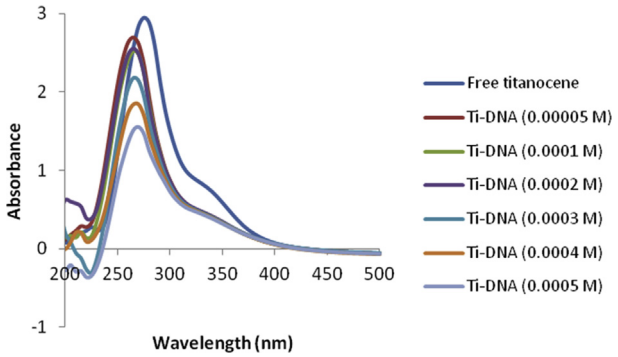


Fig. 5. UV–vis spectra of titanocene **9** (2.5×10^{-4} M) before and after the addition of different concentrations of DNA.

Table 3
IC_{50} (μM) for the 96 h of action of **8**–**11**, titanocene dichloride and cisplatin on 8505C (anaplastic thyroid cancer), A549 (lung carcinoma), A2780 (ovarian cancer), DLD-1 (colon carcinoma) and FaDu (head and neck cancer) determined by sulforhodamine-B microculture colorimetric assay (SRB assay).

Compounds	Studied cell lines				
	8505C	A549	A2780	DLD-1	FaDu
8[a]	103 ± 2	96 ± 3	n.d.	71 ± 2	n.d.
9	73 ± 1	73 ± 3	34 ± 3	117 ± 2	112 ± 2
10	>200	143 ± 4	62 ± 2	131 ± 9	181 ± 2
11	86 ± 3	87 ± 4	51 ± 3	103 ± 1	122 ± 1
Cisplatin	5.02 ± 0.23	1.51 ± 0.02	0.55 ± 0.03	5.14 ± 0.12	1.21 ± 0.14
$[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$	>200	167 ± 3	125 ± 5	>200	n.d.

[a] Data for IC_{50} values for **8** against 8505C, A549 and DLD-1 cell lines [22c].

interaction of the titanocene complexes with some important biological molecules present inside the cells. These studies should allow us to observe the relation between the level of interaction of the synthesized compounds with these biological molecules and the resulting cytotoxic activity. The biological molecules used in these electrochemical experiments were cytosine, thymine, adenosine, guanosine and FS-DNA. All voltammograms were obtained using linear cyclic voltammetry with a glassy carbon electrode as working electrode, an Ag/AgCl reference electrode and a Pt auxiliary electrode.

3.5.1. Interaction studies of $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$

3.5.1.1. Electrochemical characterization of $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$. According to data in the literature [47], $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$ in aqueous solution is rapidly hydrolyzed giving the cationic species $[Ti(\eta^5\text{-}C_5H_5)_2(H_2O)Cl]^+$ through the loss of a chlorido ligand. Due to the fact that the half life of this cationic species is about 50 min, this cationic species is considered to be the electroactive species in the electrochemical process.

When scanning from 0 V to more negative values with a scan rate of 100 mV/s, the titanocene derivative shows a reduction process corresponding to [Ti(IV)/Ti(III)] with a value of $E_p^c = -0.459$ V and oxidation due to the inverse process [Ti(III)/Ti(IV)] with a value of $E_p^a = -0.343$ V (Fig. 7a).

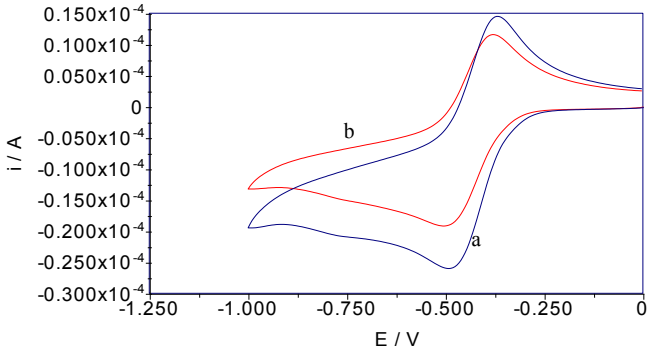


Fig. 7. Cyclovoltammogram of a 10 mM solution of $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$ in H_2O/DMSO, 10% using NaCl (0.1 M) as supporting electrolyte (pH = 7.4, ν = 100 mV/s). (a) Before and (b) after addition of guanosine 10 mM.

The obtained $\Delta E_p = 116$ mV and I_p^c/I_p^a values slightly lower than one indicate quasi-reversibility of the electrochemical process. There exists a chemical reaction associated to the electrochemical reduction. However, it should be noted that the electrogenerated Ti(III) species is not chemically stable and evolves within the solution.

A linear relationship is observed when representing I_p^c values *versus* $\nu^{1/2}$, indicating that the process is controlled by diffusion. The electroactive species transport mechanism occurs from solution to the working electrode surface, as reported by Wang and co-workers (Fig. 8) [48].

The diffusion coefficient for $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$ can be estimated using the Randles–Sevcik equation, given below ($D = 5.61 \times 10^{-6}$ cm^2/s).

$$i_p = 2.686 \times 10^5 \cdot n^{3/2} \cdot A \cdot c \cdot D^{1/2} \cdot \nu^{1/2}$$

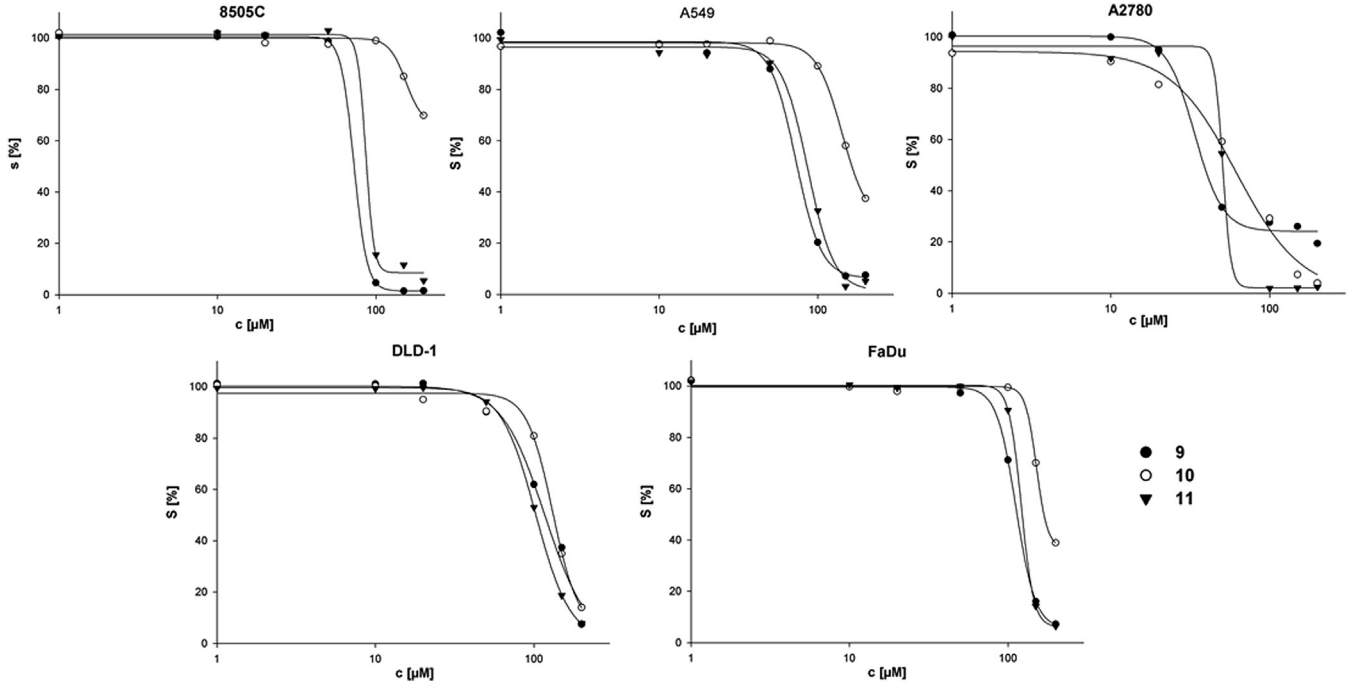


Fig. 6. Representative graphs showing survival of 8505C, A549, A2780, DLD-1 and FaDu cells grown for 96 h in the presence of increasing concentrations (logarithmic scale) of the novel compounds **9**–**11**. Standard deviations (all of them less than 10%) are omitted for clarity.

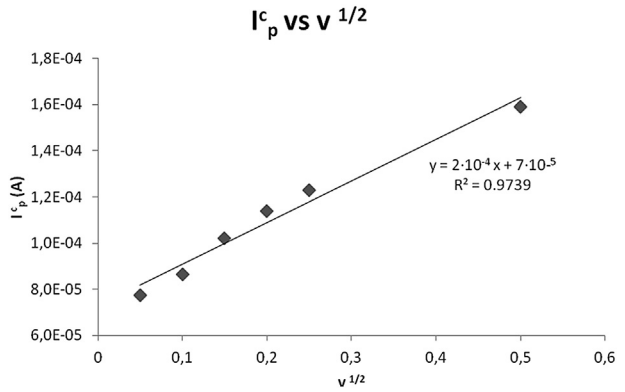


Fig. 8. I_p^c *versus* $\nu^{1/2}$ for a 10 mM solution of [Ti(η^5-C_5H_5)$_2$Cl$_2$] in H_2O/DMSO, 10%.

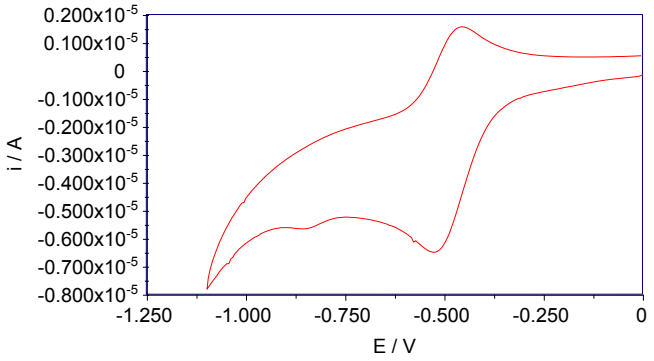


Fig. 10. Cyclovoltammogram of a 10 mM solution of **8** in H_2O/DMSO, 10% using NaCl (0.1 M) as supporting electrolyte (pH = 7.4, ν = 100 mV/s).

where, i_p is the peak intensity (in A), n is the number of electrons transferred during the redox process, A is the working electrode surface area (in cm^2), c is the electroactive species concentration (in mol/cm^3), D is the diffusion coefficient (in cm^2/s) and ν is the scan rate (in V/s).

3.5.1.2. Interaction studies of [Ti(η^5-C_5H_5)$_2$Cl$_2$] with nitrogenous bases and nucleotides. The addition of different biological molecules to a solution of [Ti(η^5-C_5H_5)$_2$Cl$_2$] causes a decrease in the intensity of the reduction peak and a shift in the reduction potential towards more negative values (see Fig. 7 for the interaction between [Ti(η^5-C_5H_5)$_2$Cl$_2$] and guanosine; see Fig. S16 of Supplementary material for the interaction between [Ti(η^5-C_5H_5)$_2$Cl$_2$] and thymine).

This behaviour can be explained by considering an interaction between the titanocene derivative and the biological molecule. This interaction should lead to a larger complex with a lower diffusion rate (i.e., a lower value for the diffusion coefficient, D). Using the same method as before, we have calculated the diffusion coefficient for the [Ti(η^5-C_5H_5)$_2$Cl$_2$]–guanosine adduct ($D = 6.88 \times 10^{-7}$ cm^2/s).

3.5.1.3. Interaction studies of [Ti(η^5-C_5H_5)$_2$Cl$_2$] with FS-DNA. The voltammograms for [Ti(η^5-C_5H_5)$_2$Cl$_2$] (Fig. 9) in the absence and presence of DNA ([DNA]/[Ti] = 0.1) at pH = 7.4 show that the addition of DNA does not modify the value of the reduction peak. In addition, a decrease in intensity of the peak was not observed. The other parameters also remained unchanged; the value of the formal electrode potential at −0.43 V, the potential difference between cathodic and anodic peaks (ΔE) from 107 to 195 mV depending on scan rate and I_p^c/I_p^a values slightly lower than 1. The diffusion coefficient for this assay was 1.11×10^{-6} cm^2/s, which is lower than the value of the diffusion coefficient for the electroactive especies generated by [Ti(η^5-C_5H_5)$_2$Cl$_2$] ($D = 5.61 \times 10^{-6}$ cm^2/s) and higher than the value for [Ti(η^5-C_5H_5)$_2$Cl$_2$]/guanosine ($D = 6.88 \times 10^{-7}$ cm^2/s).

These results are in agreement with a previously published study which at physiological pH values showed that the absolute value of the peak current did not decrease [47]. From our results one can conclude that the interaction of [Ti(η^5-C_5H_5)$_2$Cl$_2$] with DNA is weaker than the interaction of [Ti(η^5-C_5H_5)$_2$Cl$_2$] with nitrogenous bases and nucleotides. Quantitative comparison of the results is difficult since the peak value intensity also depends on the concentration and different scale units have been used in the experimental studies due to solubility aspects. Thus, on the basis of the lower calculated diffusion coefficient a strong interaction of [Ti(η^5-C_5H_5)$_2$Cl$_2$] with guanosine can be assumed.

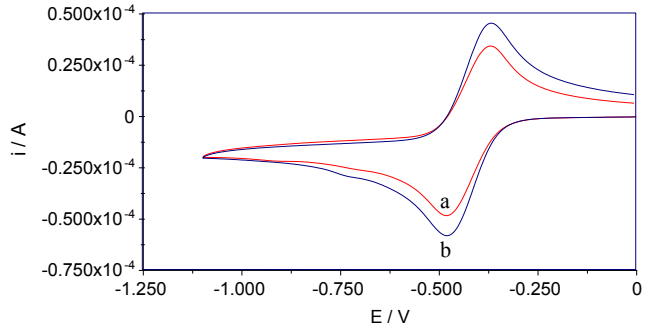


Fig. 9. Cyclovoltammogram of a 10 mM solution of [Ti(η^5-C_5H_5)$_2$Cl$_2$] in H_2O/DMSO, 10% using NaCl (0.1 M) as supporting electrolyte (pH = 7.4, ν = 100 mV/s). (a) Before and (b) after addition of DNA 1 mM.

*3.5.2. Interaction studies of **8***

*3.5.2.1. Electrochemical characterization of **8**.* The electrochemical characterization for **8** in the presence and absence of biological molecules has been carried out using a glassy carbon electrode as the working electrode in an aqueous solution of DMSO (10%) with NaCl 0.1 M as electrolyte. Ag/AgCl was the reference electrode and Pt used as an auxiliary electrode.

Fig. 10 shows the CV response of complex **8** at 100 mV/s, the cathodic sweep reveals one reduction process associated to Ti(IV)/Ti(III) reduction, at −0.52 V. In the reverse scan an associated re-oxidation peak is observed. Higher stability for the Ti(III) electrogenerated species within the solution with respect to cationic species, formed by hydrolysis of the titanocene derivative, is observed.

This may be due to the coordination of the double bond of the alkenyl fragment to the Ti atom which gives Ti(III) cationic species

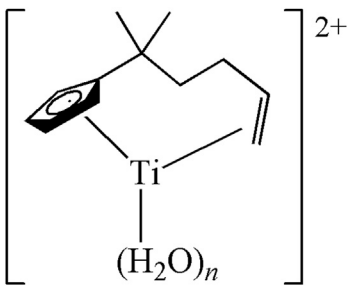


Fig. 11. Proposed Ti(III) cationic species produced by reduction of **8**.

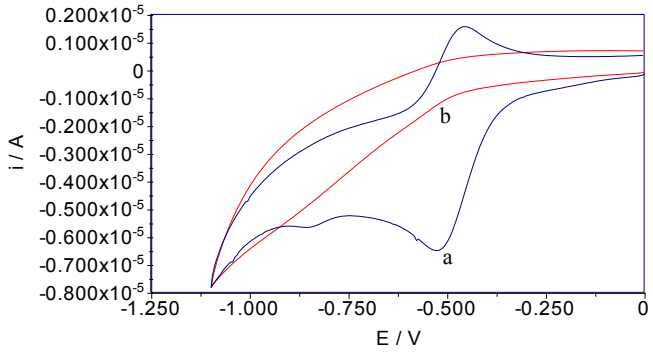


Fig. 12. Cyclovoltammogram of a 10 mM solution of **8** in H_2O/DMSO, 10% using NaCl (0.1 M) as supporting electrolyte (pH = 7.4, ν = 100 mV/s). (a) Before and (b) after addition of adenosine.

that are more stable than the analogous species generated from [Ti(η^5-C_5H_5)$_2$$Cl_2$] (Fig. 11).

The voltammogram for **8** also shows a separation between peaks 51 and 66 mV, depending on scan rate and I_p^c/I_p^a values close to 1. The formal potential value (−0.49 V) is shifted towards more negative values with respect to [Ti(η^5-C_5H_5)$_2$$Cl_2$]. In addition, the calculated diffusion coefficient for **8** is 1.26×10^{-7} cm^2/s.

In this particular case, the introduction of an alkenylic fragment on one of the cyclopentadienyl rings suggests that this derivative is less readily reduced compared with [Ti(η^5-C_5H_5)$_2$$Cl_2$]. This fact is probably due to the electron donor properties of the alkenyl fragment as well as the possible coordination of the double bond to titanium after the hydrolysis and the application of the potential.

*3.5.2.2. Interaction studies of **8** with nitrogenous bases and nucleotides.* The interactions between **8** and different biological molecules led to a very notable decrease in the intensity of the reduction peak, as can be observed in the corresponding voltammograms obtained with adenosine 50 mM (Fig. 12), cytosine 50 mM (Fig. S17 of Supplementary material) and guanosine 30 mM (Fig. S18 of Supplementary material) at pH = 7.4.

Unfortunately, the calculation of the diffusion coefficient of the processes corresponding to the interaction between **8** and nitrogenous bases and nucleotides proved to be unsuccessful. It seems that the interaction between complex **8** and the other biological molecules is so high that the measurement of the peak intensity to subsequently calculate *D* is not possible.

*3.5.2.3. Interaction studies of **8** with FS-DNA.* With respect to DNA addition, according to the voltammogram (Fig. 13 and Fig. S19 of Supplementary material) for **8** in the presence of FS-DNA ([DNA]/[Ti] = 0.1 and pH = 7.4), there is no significant variation neither in the formal potential value (E^o = −0.51 V) nor in the intensity of the reduction peak. However, a slight decrease of the calculated diffusion coefficient is observed ($D = 2.77 \times 10^{-9}$ cm^2/s), indicating a low degree of interaction with DNA, even lower than that observed for [Ti(η^5-C_5H_5)$_2$$Cl_2$]. These results confirm that, although the degree of interaction of the substituted titanocene derivatives is lower than that of [Ti(η^5-C_5H_5)$_2$$Cl_2$], this is not the determinant step for the enhancement of the cytotoxicity. Thus, it is possible that other steps such as hydrolysis of the complexes or solvent interactions, amongst others, have greater importance in the cytotoxic process.

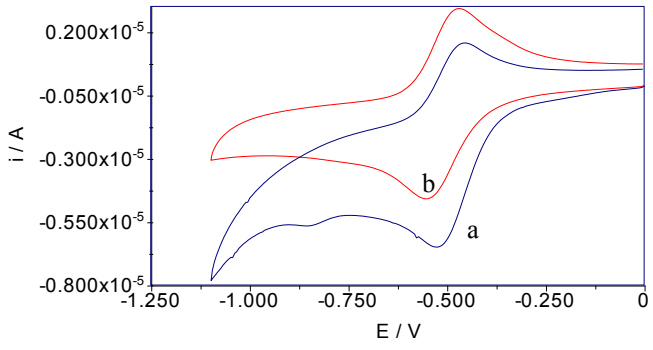


Fig. 13. Cyclovoltammogram of a 10 mM solution of **8** in H_2O/DMSO, 10% using NaCl (0.1 M) as supporting electrolyte (pH = 7.4, ν = 100 mV/s). (a) Before and (b) after addition of DNA 1 mM.

4. Conclusions and outlook

Several new titanocene(IV) dichloride complexes with alkenyl substituents on the cyclopentadienyl rings have been synthesized and characterized.

The cytotoxic activity of these compounds (**8**–**11**) has been tested against human tumour cell lines and compared with the reference complexes [Ti(η^5-C_5H_5)$_2$$Cl_2$] and cisplatin. The results show a dose-dependent cytotoxicity for all the studied complexes. The highest cytotoxicity was observed (33.71 ± 2.61) for **9** against A2780 cell line. In addition, all complexes are more active than reference compound [Ti(η^5-C_5H_5)$_2$$Cl_2$] on all the studied cell lines. Thus, we can confirm that the incorporation of alkenyl substituents on the cyclopentadienyl ring, gives rise to increased cytotoxic activity.

The results of hydrolysis studies of **8**–**11** indicate a higher stability for **8** and **10** in DMSO with respect to **9** and **11**, but in DMSO/D_2O there is fast hydrolysis of cyclopentadienyl ligands in all the complexes. The stability in water (physiological medium) of the synthesized complexes is probably related to their cytotoxic activity. The most unstable complex in water, **10**, is also the most inactive compound in all the studied cell lines. These results suggest that the degree of stability of these compounds in physiologic medium and the effectiveness of transport processes inside cells play more determining roles than that associated to the binding ability of these compounds to nuclear DNA.

Moreover, according to the results obtained from cyclic voltammetric studies, **8** and [Ti(η^5-C_5H_5)$_2$$Cl_2$] show interactions with the studied molecules (cytosine, thymine, adenosine and guanosine). However, only very weak interactions were observed for these complexes with FS-DNA, which show again the importance of the stability in physiologic medium and the transport processes of the active species to the cell nucleus.

Thus, we observed that the diffusion coefficients of the different titanocene derivatives were not very different; indicating that, in contrast to many studies which are focused on the binding properties, it seems that the most important steps associated to the cytotoxic activity of this kind of compounds are related to the stability of these compounds in physiologic medium and to the transport processes of the active species to the cell nucleus.

Acknowledgements

We gratefully acknowledge financial support from the Ministerio de Economía y Competitividad, Spain (Grant no. CTQ2012-30762) from the Ministry of Science and Technological Development of the Republic of Serbia (Grant No. 173013). V. Clausse and I. García-Benito are gratefully acknowledged for their help in the cyclic voltammetry experiments.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jorganchem.2014.06.031.

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