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Novel enantiopure cyclopentadienyl Ti(IV) oximato compounds as potential anticancer agents

Isabel de la Cueva-Alique[‡], Laura Muñoz-Moreno[⊥], Yosra Benabdelouahab[‡], Benelita T. Elie^{‡,†}, Mohammed Amin El Amrani^{||}, Marta E. G. Mosquera[‡], María Contel^{‡,†}, Ana M. Bajo[⊥], Tomás Cuenca[‡], and Eva Royo^{‡,*}

[‡]Departamento de Química Orgánica y Química Inorgánica, Facultad de Biología, Ciencias Ambientales y Química, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain

[⊥]Departamento de Biología de Sistemas, Facultad de Biología, Ciencias Ambientales y Química, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain

[‡]Department of Chemistry, Brooklyn College, The City University of New York, Brooklyn, New York, NY 11210, USA

[†]Chemistry and Biology PhD Programs, The Graduate Center, The City University of New York, 365 Fifth Avenue, New York, NY 10016, USA

^{||}Université Abdelmalek Essaadi, Departement de Chimie-Laboratoire de Chimie Organique Appliquée, Mhannech II, B.P. 2121 Tétouan, Morocco

Abstract

The synthesis and characterization of new enantiopure cyclopentadienyl titanium oximato compounds (*S,R*)-[(η^5 -C₅H₅)Ti{ κ^2 NO,(R)NH·HCl}Cl₂] (R = Ph (phenyl) **1a**·HCl, Bn (benzyl) **1b**·HCl, 2-pic (2-picolyl) **1c**·HCl), (*S,R*)-[(η^5 -C₅H₅)TiCl₂{ κ^2 NO,(Ph)NH}] (**1a**) and (*S,R*)-[(η^5 -C₅H₅)₂TiCl{ κ^2 NO,(R)NH}] (R = Ph **2a**, Bn **2b**, 2-pic **2c**), along with studies on their behaviour in D₂O at different pD values are reported. The structure of previously described ammonium-oxime (2*S*,5*R*)-{NOH,(Bn)NH·HCl} (**b**·HCl) and novel titanium derivative **1a** have been determined by single crystal X-ray crystallography. The effect of the compounds on cytotoxicity, cell adhesion and migration of the androgen-independent prostate cancer PC-3 cells has been assessed. Compounds **2b** and **2c** are more cytotoxic than additive doses of titanocene dichloride and free oxime proligand, probing the synergistic effect of these novel compounds. The cytotoxicity of **2b** and **2c** has been further evaluated against human renal Caki-1, colon DLD-1 and triple negative breast MDA-MB-231 cancer cell lines. The activity found for **2c** on PC-3 and Caki-1 is higher than that of highly active Titanocene Y (bis-[(p-methoxybenzyl)cyclopentadienyl]titanium(IV) dichloride), while showing selectivity against renal cancer when compared to a non-tumorigenic human renal (HEK-293T) cell line. Compounds **2b** and especially **2c** are apoptotic in Caki-1 cancer cell lines. Cell adhesion and wound-healing assays confirmed that derivatives **1c**·HCl, **2b**

*Corresponding author: ; Email: eva.royo@uah.es. Phone Nr. (+34) 918854629; Fax Nr. (+34) 918854683

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and **2c** affect the adhesion and migration patterns of the PC-3 cell line. Interactions of the novel compounds with plasmid (pBR322) DNA have also been studied, showing that the oximato Ti(IV) derivatives have a weak or no interaction with DNA at physiological pH.

Graphical abstract

Novel easily accessible enantiopure cyclopentadienyl titanium oximato compounds are reported. Two of them are strongly cytotoxic against PC-3 and Caki-1 cancer cells while inducing apoptosis and showing selectivity when compared to a non-tumorigenic renal cell line. They also affect the adhesion and migration ability of human prostate cancer cells.

Keywords

Cyclopentadienyl titanium compounds; chirality; PC-3 and Caki-1 cell lines; DNA; cell adhesion; cell migration

Introduction

Although cisplatin and follow-on derivatives are still widely used in the clinic, their side effects, general low solubility and intrinsic or acquired resistance in some cancer types have encouraged research for the discovery of new metal compounds with improved properties [1–6]. Within this context, titanium derivatives play a central role. Budotitane [*cis*-diethoxybis(1-phenylbutane-1,3-dionato)titanium(IV)] [7–9] and titanocene dichloride ($[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$, TDC) [10,11] were the first reported Ti(IV) compounds which showed promising anticancer properties. Budotitane was the first non-platinum complex to enter clinical trials for its activity towards colon tumour cells, but the compound did not progress beyond phase I due to formulation problems [7,8,12–15]. TDC exhibited significant antitumor activity, *in vitro* and *in vivo*, against cisplatin-resistant cells and tumours generally difficult to treat [10,11,13–16]. Based on these encouraging results, TDC entered clinical trials as well [17]. Although the clinical response was not significant enough to justify continuing trials, the absence of bone marrow toxicity was thought to be promising and the interest in the potential of superior titanium compounds as anticancer agents remains since then [5,18]. The instability and insolubility of TDC in water and lack of standard formulation were contributing factors to discontinuing from clinical trials. Thus, a plethora of modified titanium-based compounds have been synthesized and studied as potential antitumor agents, especially during the last decade [5,6,14,15,18–32]. Substituted titanocenes such as Titanocene Y [33,34] (Ti-Y, Fig. 1), Titanocene Y* [35,36] (Ti-Y*, bis-[(*p*-diethylaminobenzyl)cyclopentadienyl]titanium(IV) dichloride, Fig. 1) and Titanocene T [37] (Ti-T, bis-[(1-methyl-3-diethylaminomethyl)indol-4-yl]methylcyclopentadienyl]titanium(IV) dichloride, Fig. 1), developed by the group of M.

Tacke, or titanium compounds with salan ligands (Ti-salan, Fig. 1) described by the group of E. Tshuva [14,38–40] have shown great therapeutic promise and are undergoing further development [36,40,41]. Heterometallic Ti-Au derivatives have also demonstrated to be promising drug candidates for the treatment of renal cancer [30]. A recently published comparison between the biological behaviour of Ti-Y and Ti-salan derivatives demonstrate that they do not follow analogous bio-distribution patterns, showing that the ligands bound to the Ti(IV) centre assume an important relevance for the activity of these metal-based drugs [41].

In this context, oxime-containing ligands offer significant advantages for biological applications. Some oxime organic derivatives have been reported to have biological effects such as endothelium-independent relaxation in blood-vessels, an increase in the targeting of specific nuclear bases of DNA and oxidative DNA cleavage [42,43]. Oxime-containing Pt(II) compounds have been reported as a novel class of nonclassic platinum-based complexes with interesting antitumor properties, different DNA binding behaviour and a different pattern of protein interaction from those found in classical cisplatin [44,45]. The study on a variety of Rh(III) and Ir(III) compounds with oximato ligands show a strong cytotoxic effect toward HeLa and HL60 cancer lines, whereas the compounds do not modify DNA in a similar way to that of cisplatin [46]. DNA-binding studies of oxime-containing ruthenium compounds have been previously investigated, but only a weak, non-intercalative in nature interaction was found [47]. We reported recently a new family of chiral, water soluble and stable *p*-cymene ruthenium(II) compounds with amino-oxime ligands. The compounds show significant effects on cytotoxicity, cell adhesion to collagen and migration of androgen-independent prostate cancer cells while they do not exhibit strong interactions with plasmid (pBR322) DNA [48].

Although oxime and oximato metal derivatives have been extensively studied [49–51], oximato titanium compounds have received little attention. The first titanocene oximato derivatives, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{Ti}(\text{H}_2\text{O})(\kappa^2\text{O}=\text{NCRR}')^+]$, were reported by Thewalt *et al* [52] and described as surprisingly stable against air and water. More recently, the synthesis of a novel family of titanocene compounds of general formula $[(\eta^5\text{-C}_5\text{H}_5)_2\text{Ti}(\kappa^2\text{O}=\text{NCRR}')\text{Cl}]$ [53–55] and several oximato titanium derivatives $[\text{Ti}(\text{OR}^1)_{4-n}(\kappa^2\text{O}=\text{NCR}^2\text{R}^3)_n]$ (where $n = 1\text{--}4$) have also been published [56–61]. Oxime groups are presented as excellent chemical modifiers with a wide versatility of coordination modes going from mono- κN , κO to di-hapto $\kappa^2\text{N,O}$, either with *side on* or bridging coordination, depending on the nature of the ligand and the metal centre [49–51,62]. Some titanium oximato compounds have shown interesting antibacterial properties [60,61], however, their anticarcinogenic effects have not been reported before.

Encouraged by our previous results on the enantiomerically pure amino-oxime ruthenium compounds [48], we hypothesized that oximato ligands could increase the water solubility and stability of the resulting titanium derivatives [52]. Thus, we explored reactions of $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ and $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ with the previously described enantiopure amino-oxime derivatives (2*S,R*)-{NOH,(*R*)NH} (*R* = phenyl **a**, benzyl **b**, 2-picoyl **c**, see Fig. 2) [48,63,64], derived from *R*-limonene. This naturally occurring terpene is an inexpensive

starting reagent, commercially available in optically pure form and easily tailored by stereoselective functionalization [63–65].

The effect of stereochemistry on biological activity is of great importance in medicinal chemistry, as many of the biological targets are chiral [66–69]. Although various examples of chiral anticancer titanium compounds have been reported [39,70–72], examples of enantiopure titanium anticancer compounds are scarce [67]. Manna et al [67] have shown that stereochemistry plays an important role in determining cytotoxicity and should be considered in the design, modification and improvement of active compounds. In this work we report on the synthesis and characterization of a novel family of chiral cyclopentadienyl ammonium- or amino-oximate titanium derivatives (Fig 2). We describe their *in vitro* activity against human androgen-independent prostate cancer PC-3 cell line as well as the study of their interactions with plasmid (pBR322) DNA. The cytotoxicity of selected compounds has been evaluated on renal cancer Caki-1, colon cancer DLD-1, triple negative breast cancer MDA-MB-231 and non-tumorigenic human embryonic kidney HEK-293T cell lines. The cell death type as well as the effects on cell migration of selected compounds is also described.

2. Experimental Section

2.1. Chemicals and synthesis

Synthesis of titanium complexes were performed at an argon/vacuum manifold using standard Schlenk techniques or in a glove-box MBraun MOD System. Solvents were dried by known procedures and used freshly distilled. (2*S*,5*R*)-{NOH,(R)NH}, (R = Ph **a**, Bn **b**, 2-pic **c**) [63,64], corresponding adducts (2*S*,5*R*)-{NOH,(R)NH·HCl} (R = Ph **a**·HCl, Bn **b**·HCl, 2-Pic **c**·HCl) [63,64], [(η⁵-C₅H₅)TiCl₃] [73] and Titanocene Y [74] were prepared according to previous reports. NEt₃, HCl (1M in ether), DCl (35% wt in D₂O), NaOD (40% wt in D₂O), NaCl, KCl, Na₂HPO₄, KHPO₄ and [(η⁵-C₅H₅)₂TiCl₂] were purchased from Sigma-Aldrich. NEt₃ was stored under molecular sieves prior use. Commercially available reagents were used without further purification. NMR spectra were recorded on a Bruker 400 Ultrashield. ¹H and ¹³C chemical shifts are reported relative to tetramethylsilane. ¹⁵N chemical shifts are reported relative to liquid ammonia (25 °C). ³¹P chemical shifts are reported relative to phosphoric acid (85% wt in D₂O, 25 °C). Coupling constants *J* are given in Hertz. Elemental analyses were performed in our laboratories (UAH) on a Perkin Elmer 2400 CHNS/O Analyzer, Series II.

2.1.1. (S,R)-[(η⁵-C₅H₅)Ti{κ²NO,(Ph)NH·HCl}Cl₂] (1a·HCl)—A diethylether (10 mL) solution of (2*S*,5*R*)-{NOH,(Ph)NH} (0.52 g, 2.01 mmol) and [(η⁵-C₅H₅)TiCl₃] (0.44 g, 2.01 mmol) was warmed up in a J. Young valved ampule for 3 h at 55 °C. The resulting yellow suspension was filtered to isolate the yellow solid which precipitates from the mixture and washed twice (2×5 mL) with hexane. The solid was dried under vacuum and identified as derivative **1a**·HCl. Yield: 0.64 g, 1.34 mmol, 66%. Solubility in H₂O at 24 °C (mM): 2.1 ± 0.2. Value of pH ([2.0 mM]) in H₂O at 24 °C: 2.40. Anal. Calcd for C₂₁H₂₇N₂OTiCl₃: C, 52.80; H, 5.70; N, 5.86%; Found: C, 53.29; H, 5.45; N, 6.09%. FTIR (KBr): 3090–3120 (br, NH·HCl); 1640, 1570 (w-m, C=N). ¹H NMR (plus HSQC, plus HMBC, plus COSY,

400.1 MHz, 293 K, CDCl₃): δ 11.62 (br, 2H, -NH·HCl), 7.21 (m, 5H, -C₆H₅), 6.63 (s, 5H, C₅H₅), 4.86, 4.80 (both s, each 1H, =CH₂), 3.19, 3.18 (both m, overlapped, 2H, -CH₂⁶), 3.07 (m, 1H, -CH₂³), 2.45 (m, 1H, -CH₂⁴), 2.16 (m, 1H, -CH-C=), 1.82 (m, 1H, -CH₂³), 1.44 (m, 1H, -CH₂⁴), 1.77, 1.76 (both s, each 3H, NC-CH₃ + CH₃C=). ¹³C-NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K, CDCl₃): δ 154.1 (–, Cq=N), 147.0 (–, =Cq-Me), 132.1 (–, C_{ipso}Ph), 129.6, 129.5, 126.1 (+, C₆H₅), 121.7 (+, C₅H₅), 111.7 (–, =CH₂), 66.7 (–, Cq-NH), 46.3 (+, -CH⁵), 38.9 (–, -CH₂³), 32.9 (–, -CH₂⁶), 25.5 (–, -CH₂⁴), 21.9, 21.0 (both +, CH₃-CNH + CH₃-C=). ¹⁵N NMR (gHMBC, 40.5 MHz, 293 K, CDCl₃): δ 333.6 (C=N), 79.5 (NH·HCl).

2.1.2. (S,R)-[(η^5 -C₅H₅)Ti(κ^2 NO,(Bn)NH·HCl)Cl₂] (1b·HCl)—A diethylether (10 mL) solution of (2*S*,5*R*)-{NOH,(Bn)NH} (0.42 g, 1.54 mmol) and [(η^5 -C₅H₅)TiCl₃] (0.34 g, 1.54 mmol) was stirred for 30–45 min at room temperature. The resulting yellow suspension was filtered to isolate the yellow solid which precipitates from the mixture and washed twice (2×5 mL) with hexane. The solid was dried under vacuum and identified as derivative **1b·HCl**. Yield: 0.58 g, 1.18 mmol, 77%. Solubility in H₂O at 24 °C (mM): 8.1 ± 0.4. Value of pH ([2.0 mM]) in H₂O at 24 °C: 2.16. Anal. Calcd for C₂₂H₂₉N₂OTiCl₃: C, 53.74; H, 5.94; N, 5.70; Found: C, 53.85; H, 5.81; N, 5.62. FTIR (KBr): 3100–3200 (br, NH·HCl); 1638, 1572 (both m, C=N). ¹H NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, CDCl₃): δ 10.35, 9.84 (br, each 1H, -NH·HCl), 7.66 (m, 2H, -C₆H₅), 7.19 (m, 3H, -C₆H₅), 6.84 (s, 5H, C₅H₅), 4.84, 4.78 (both m, each 1H, CH₂-Ph), 4.84, 4.78 (both s, each 1H, =CH₂), 2.80, 2.44 (both m, each 1H, -CH₂⁶), 2.91 (m, 1H, -CH₂³), 2.64 (m, 1H, -CH₂⁴), 2.05 (m, 1H, -CH-C=), 1.75 (m, 1H, -CH₂³), 1.58 (m, 1H, -CH₂⁴), 2.08, 1.77 (both s, each 3H, NC-CH₃ + CH₃C=). ¹³C-NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K, CDCl₃): δ 155.1 (–, Cq=N), 146.9 (–, =Cq-Me), 130.9 (–, C_{ipso}Ph), 131.7, 129.3, 128.7 (+, C₆H₅), 122.1 (+, C₅H₅), 111.7 (–, =CH₂), 63.5 (–, Cq-NH), 47.6 (–, -CH₂Ph), 46.2 (+, -CH⁵), 39.0 (–, -CH₂³), 32.4 (–, -CH₂⁶), 25.8 (–, -CH₂⁴), 21.4, 21.0 (both +, CH₃-CNH + CH₃-C=). ¹⁵N NMR (gHMBC, 40.5 MHz, 293 K, CDCl₃): δ 331.8 (C=N), 67.3 (NH·HCl).

2.1.3. (S,R)-[(η^5 -C₅H₅)Ti(κ^2 NO,(2-pic)NH·HCl)Cl₂] (1c·HCl)—A diethylether (10 mL) solution of (2*S*,5*R*)-{NOH,(2-pic)NH} (0.40 g, 1.46 mmol) and [(η^5 -C₅H₅)TiCl₃] (0.32 g, 1.46 mmol) was stirred for 5 min at room temperature. The resulting yellow suspension was filtered to isolate the yellow solid which precipitates from the mixture and washed twice (2×5 mL) with hexane. The solid was dried under vacuum and identified as derivative **1c·HCl**. Yield: 0.49 g, 1.00 mmol, 70%. Solubility in H₂O at 24 °C (mM): 8.6 ± 0.4. Value of pH ([2.0 mM]) in H₂O at 24 °C: 2.21. FTIR (KBr): 3150–3200 (br, NH·HCl); 1643, 1572 (both m, C=N). Anal. Calcd for C₂₁H₂₈N₃OTiCl₃: C, 51.19; H, 5.73; N, 8.53; Found: C, 51.16; H, 5.43; N, 9.03. ¹H NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, CDCl₃): δ 10.43 (br, 2H, -NH·HCl), 8.40, 7.70, 7.25, 7.31 (all m, each 1H, -C₅NH₄), 6.76 (s, 5H, C₅H₅), 4.77, 4.72 (both s, each 1H, =CH₂), 4.40 (m, 2H, CH₂^{pic}), 2.97, 2.56 (both m, each 1H, -CH₂⁶), 2.76 (m, 1H, -CH₂³), 2.18 (m, 1H, -CH-C=), 2.08 (m, 1H, -CH₂⁴), 1.88 (overlapped, 5H, -CH₂³ + -CH₂⁴ + NC-CH₃), 1.73 (s, 3H, CH₃C=). ¹³C-NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K, CDCl₃): δ 152.8 (–, Cq=N), 150.4 (–, C_{ipso}C₅NH₄), 147.3, 138.9, 124.1, 122.9 (all +, -NC₅H₄), 146.3 (–, =Cq-Me), 122.0 (+, C₅H₅), 111.0 (–, =CH₂), 61.6 (–, Cq-NH), 44.5 (–, -CH₂-NC₅H₄), 44.2 (+, -CH⁵), 37.9 (–, -

CH_2^3), 31.5 (–, $-\text{CH}_2^6$), 25.5 (–, $-\text{CH}_2^4$), 21.2, 20.3 (both +, $\text{CH}_3\text{-CNH} + \text{CH}_3\text{-C=}$). ^{15}N NMR (gHMBC, 40.5 MHz, 293 K, CDCl_3): δ 335.0 (C=N), 284.9 (NC_5H_4), 56.3 (NH-HCl).

2.1.4. (S,R)-[($\eta^5\text{-C}_5\text{H}_5$)Ti($\kappa^2\text{NO, (Ph)NH}$)Cl₂] (1a)—A toluene (5 mL) solution of **1a**·HCl (0.20 g, 0.42 mmol) and NEt_3 (0.058 mL, 0.42 mmol) was stirred for 30 min at room temperature. The resulting orange suspension was filtered to separate the solution from insoluble $\text{NEt}_3\cdot\text{HCl}$. The orange solution was evaporated to dryness and the resulting yellow solid washed twice with hexane (2×5 mL) and dried under vacuum. Yield: 0.13 g, 0.29 mmol, 69%. CHN analysis data found did not agree with theoretical data calculated for $\text{C}_{21}\text{H}_{26}\text{N}_2\text{OTiCl}_2$, due to impurification of the solid with $\text{NEt}_3\cdot\text{HCl}$. Recrystallization of the solid from toluene at -20°C affords crystals suitable for X-ray analysis. FTIR (KBr): 3374 (m, NH); 1641, 1601 (both m, C=N). ^1H NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, CDCl_3): δ 7.10 (m, 2H, $-\text{C}_6\text{H}_5$), 6.72 (m, 3H, $-\text{C}_6\text{H}_5$), 6.82 (s, 5H, C_5H_5), 4.77, 4.74 (both s, each 1H, $=\text{CH}_2$), 3.71 (br, 1H, NH), 2.95, 2.49 (m, 1H, $-\text{CH}_2^6$), 2.22 (m, 1H, $-\text{CH-C=}$), 2.12, 1.86 (m, 1H, $-\text{CH}_2^3$), 1.87, 1.75 (m, 1H, $-\text{CH}_2^4$), 1.85, 1.73 (both s, each 3H, $\text{NC-CH}_3 + \text{CH}_3\text{C=}$). ^{13}C - NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K, CDCl_3): δ 162.8 (–, Cq=N), 147.4 (–, $=\text{Cq-Me}$), 145.5 (C_{ipsoPh}), 129.2, 118.6, 115.2 (+, C_6H_5), 121.2 (+, C_5H_5), 110.2 (–, $=\text{CH}_2$), 57.7 (–, Cq-NH), 46.1 (+, $-\text{CH}^5$), 43.3 (–, $-\text{CH}_2^3$), 31.2 (–, $-\text{CH}_2^6$), 26.3 (–, $-\text{CH}_2^4$), 23.2, 20.7 (both +, $\text{CH}_3\text{-CNH} + \text{CH}_3\text{-C=}$). ^{15}N NMR (gHMBC, 40.5 MHz, 293 K, CDCl_3): δ 319.0 (C=N), 85.1 (NHPh).

2.1.5. (S,R)-[($\eta^5\text{-C}_5\text{H}_5$)₂Ti($\kappa^2\text{NO, (Ph)NH}$)Cl] (2a)—A toluene (5 mL) solution of [$(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2$] (0.25 g, 1.00 mmol), (2*S*,5*R*)-{NOH,(Ph)NH} (0.26 g, 1.00 mmol) and NEt_3 (0.12 mL, 0.95 mmol) was warmed up to 50°C and stirred overnight. The resulting orange suspension was filtered, to eliminate insoluble $\text{NEt}_3\cdot\text{HCl}$, and the final orange solution evaporated to dryness to afford a yellow solid which was washed twice with hexane (2×5 mL) and dried under vacuum. Yield: 0.28 g, 0.59 mmol, 59%. Solubility in H_2O at 24°C (mM): 2.7 ± 0.6 . Value of pH ([2.0 mM]) in H_2O at 24°C : 4.34. Anal. Calcd for $\text{C}_{26}\text{H}_{31}\text{N}_2\text{OTiCl}$: C, 66.32; H, 6.64; N, 5.95; Found: C, 66.62; H, 6.89; N, 5.99. FTIR (KBr): 3370 (mr, NH); 1644, 1602 (both m, C=N). ^1H NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, CDCl_3): δ 7.15 (m, 2H, $-\text{C}_6\text{H}_5$), 6.73 (m, 3H, $-\text{C}_6\text{H}_5$), 6.40, 6.41 (both s, each 5H, C_5H_5), 4.75, 4.72 (both s, each 1H, $=\text{CH}_2$), 3.53 (br, 1H, NH), 2.83 (m, 1H, $-\text{CH}_2^6$), 2.15 (m, 1H, $-\text{CH-C=}$), 2.01 (m, 1H, $-\text{CH}_2^3$), 1.83 (m, 2H, overlapped $-\text{CH}_2^{4+6}$), 1.73 (m, 1H, $-\text{CH}_2^3$), 1.63 (m, 1H, $-\text{CH}_2^4$), 1.76, 1.56 (both s, each 3H, $\text{NC-CH}_3 + \text{CH}_3\text{C=}$). ^{13}C - NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K, CDCl_3): δ 160.5 (–, Cq=N), 149.2 (–, $=\text{Cq-Me}$), 147.0 (C_{ipsoPh}), 129.4, 118.3, 115.8 (+, C_6H_5), 117.2, 117.1 (+, C_5H_5), 109.5 (–, $=\text{CH}_2$), 57.4 (–, Cq-NH), 46.3 (+, $-\text{CH}^5$), 44.0 (–, $-\text{CH}_2^3$), 28.0 (–, $-\text{CH}_2^6$), 26.6 (–, $-\text{CH}_2^4$), 23.7, 21.2 (both +, $\text{CH}_3\text{-CNH} + \text{CH}_3\text{-C=}$). ^{15}N NMR (gHMBC, 40.5 MHz, 293 K, CDCl_3): δ 400.2 (C=N), 86.1 (NHPh).

2.1.6. (S,R)-[($\eta^5\text{-C}_5\text{H}_5$)₂Ti($\kappa^2\text{NO, (Bn)NH}$)Cl] (2b)—An analogous procedure to that described for **2a** was followed, starting from [$(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2$] (0.20 g, 0.80 mmol), (2*S*,5*R*)-{NOH,(Bn)NH} (0.22 g, 0.80 mmol) and NEt_3 (0.11 mL, 0.80 mmol). Compound **2b** was obtained as a yellow solid. Yield: 0.35 g, 0.72 mmol, 88%. Solubility in H_2O at 24°C (mM): 6.6 ± 0.2 . Value of pH ([2.0 mM]) in H_2O at 24°C : 5.54. Anal. Calcd for

$C_{27}H_{33}N_2OTiCl$: C, 66.88; H, 6.86; N, 5.78; Found: C, 67.29; H, 6.62; N, 5.85. FTIR (KBr): 3370 (m, NH); 1646, 1601 (both m, C=N). 1H NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, $CDCl_3$): δ 7.32 (m, 5H, $-C_6H_5$), 6.39, 6.39 (both s, each 5H, C_5H_5), 4.76, 4.74 (both s, each 1H, $=CH_2$), 3.76, 3.55 (both m, each 1H, $-CH_2Ph$), 2.92 (m, 1H, $-CH_2^6$), 2.05 (m, 1H, $-CH-C=$), 1.90 (m, 1H, $-CH_2^3$), 1.72 (m, 1H, $-CH_2^6$), 1.68 (m, 2H, $-CH_2^4$), 1.59 (m, 1H, $-CH_2^3$), 1.56 (m, 1H, $-CH_2^4$), 1.25 (br, 1H, NH), 1.47, 1.25 (both s, each 3H, $NC-CH_3 + CH_3C=$). ^{13}C -NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K, $CDCl_3$): δ 159.2 (–, Cq=N), 149.3 (–, $=Cq-Me$), 141.6 ($C_{ipso}Ph$), 128.7, 128.7, 127.2 (+, C_6H_5), 117.1, 117.1 (+, C_5H_5), 109.4 (–, $=CH_2$), 57.1 (–, Cq-NH), 47.3 (–, $-CH_2Ph$), 45.6 (+, $-CH^5$), 41.2 (–, $-CH_2^3$), 27.8 (–, $-CH_2^6$), 26.2 (–, $-CH_2^4$), 23.9, 21.3 (both +, $CH_3-CNH + CH_3-C=$). ^{15}N NMR (gHMBC, 40.5 MHz, 293 K, $CDCl_3$): δ 398.9 (C=N), 60.0 (NHBn).

2.1.7. $(S,R)-[(\eta^5-C_5H_5)_2Ti\{\kappa^2NO,(2-pic)NH\}Cl]$ (2c)—An analogous procedure to that described for **2a** was followed, starting from $[(\eta^5-C_5H_5)_2TiCl_2]$ (0.30 g, 1.20 mmol), $(2S,5R)-\{NOH,(2-pic)NH\}$ (0.33 g, 1.20 mmol) and NEt_3 (0.11 mL, 1.20 mmol). Compound **2c** was obtained as a yellow-orange solid. Yield: 0.28 g, 0.61 mmol, 61%. Solubility in H_2O at 24 °C (mM): 15.7 ± 1.7 . Value of pH ([2.0 mM]) in H_2O at 24 °C: 5.22. Anal. Calcd for $C_{26}H_{32}N_3OTiCl$: C, 64.27; H, 6.64; N, 8.65; Found: C, 64.41; H, 6.47; N, 8.89. FTIR (KBr): 3304 (m, NH); 1640, 1591, 1569 (both s, C=N). 1H NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, $CDCl_3$): δ 8.50, 7.60, 7.30, 7.12 (all m, each 1H, $-C_5NH_4$), 6.38, 6.38 (both s, each 5H, C_5H_5), 4.77, 4.74 (both s, each 1H, $=CH_2$), 3.91, 3.70 (both m, each 1H, $CH_2-C_5NH_4$), 2.84 (m, 1H, $-CH_2^6$), 2.07 (m, 1H, $-CH-C=$), 1.98 (m, 2H, overlapped $-CH_2^{3+6}$), 1.78 (s, 3H, $CH_3C=$), 1.64 (m, 1H, $-CH_2^3$), 1.62 (m, 2H, $-CH_2^4$), 1.60 (m, 1H, $-CH_2^4$), 1.48 (br, 4H, $NC-CH_3 + NH$). ^{13}C -NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K, $CDCl_3$): δ 157.6 (–, Cq=N), 148.1 (–, $=Cq-Me$), 160.2 ($C_{ipso}C_5NH_4$), 149.3, 136.7, 122.9, 122.9 (+, C_5NH_4), 117.1, 117.1 (+, C_5H_5), 109.6 (–, $=CH_2$), 48.5 (–, $-CH_2-C_5NH_4$), 45.3 (+, $-CH^5$), 41.1 (–, $-CH_2^3$), 27.7 (–, $-CH_2^6$), 26.2 (–, $-CH_2^4$), 23.9, 21.3 (both +, $CH_3-CNH + CH_3-C=$). ^{15}N NMR (gHMBC, 40.5 MHz, 293 K, $CDCl_3$): δ 402.1 (C=N), 312.5 (C_5NH_4), 52.6 (NHpic).

2.1.8. 1H NMR experiments at various pH—Titanium compounds were dissolved in 2000 μL of D_2O , 1,4-dioxane ([Ti]:[1,4-dioxane] = 1:3) was then added as internal reference and time-dependent 1H NMR spectra of 500 μL aliquots of final solutions were carried out (no changes on measured pD values were observed before and after 1,4-dioxane addition). Phosphate buffer saline (PBS) was prepared according to Cold Spring Harbor Protocols (<http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8247>) using NaCl, KCl, Na_2HPO_4 and KH_2PO_4 in D_2O . Adjustment of pD ($pD = pH^* + 0.4$, where $pH^* = pH$ meter reading in D_2O) was carried out using a solution of DCl (0.01M) or NaOD (0.01M) in D_2O , with the help of a HANNA HI208 pHmeter. Titanium compounds were then dissolved in 2000 μL of the freshly prepared PBS, final pD measured (7.30–7.38) and time-dependent 1H NMR spectra of 500 μL aliquots of final solutions were carried out at 25 °C. Alternatively, titanium compounds were dissolved in 2000 μL of D_2O , 1,4-dioxane ([Ti]:[1,4-dioxane] = 1:3) was then added as internal reference, final pD adjusted to 7.4 using a solution of NaOD (0.01M) in D_2O , and time-dependent 1H NMR spectra of 500 μL aliquots of final solutions were

carried out. The percentage of metal bound cyclopentadienyl was estimated by integration of the resonance due to 1,4 dioxane (δ 3.75 in D₂O) relative to the observed metal bound C₅H₅ singlets. Alternatively, the percentage of metal bound cyclopentadienyl was estimated by integration of the signals arising from the aromatic rings of the Ph, Bn or 2-pic substituent of the free ammonium-oxime **a**·HCl-**c**·HCl that is formed in D₂O right after dilution of the compounds versus the observed metal bound C₅H₅ singlets.

2.1.9 Single-crystal X-ray structure determination—Suitable single crystals of **1a** and **b**·HCl for the X-ray diffraction study were selected. Data collection was performed at 200(2) K, with the crystals covered with perfluorinated ether oil. The crystals were mounted on a Bruker-Nonius Kappa CCD single crystal diffractometer equipped with a graphite-monochromated Mo-K α radiation (λ = 0.71073 Å). Multiscan [75] absorption correction procedures were applied to the data. The structures were solved, using the WINGX package [76], by direct methods (SHELXS-97) and refined using full-matrix least-squares against F^2 (SHELXL-97) [77]. All non-hydrogen atoms were anisotropically refined. Hydrogen atoms were geometrically placed and left riding on their parent atoms except for the hydrogen atoms H1, H2 and H10 in compound **b**·HCl and H2, H3, H81 and H82 in **1a** that were found in the Fourier map and refined. Full-matrix least-squares refinements were carried out by minimizing $\sum w(F_o^2 - F_c^2)^2$ with the SHELXL-97 weighting scheme and stopped at shift/err < 0.001. The final residual electron density maps showed no remarkable features. *Crystal data for b*·HCl: (C₁₇H₂₅ClN₂O), FW = 308.84, Orthorhombic, space group $P2_12_12_1$, crystal dimensions (mm³) 0.50 × 0.48 × 0.47, a = 9.0817(6), b = 9.2452(5), c = 19.9983(9) Å, V = 1679.10(16) Å³, Z = 4, ρ_{calcd} = 1.222 g cm⁻³, μ = 0.229 mm⁻¹, $F(000)$ = 664, θ range = 3.001 to 27.498, deg, no. of rflns collected = 13323, no. of indep rflns / R_{int} = 3847 / 0.0653, no. of data/restraints/params = 3847 / 0 / 202, $R1/wR2$ ($I > 2\sigma(I)$) = 0.0409 / 0.0961, $R1/wR2$ (all data) = 0.0599 / 0.1047, GOF (on F^2) = 0.967, Absolute structure parameter = -0.05(3). Final difference Fourier maps did not show peaks higher than 0.303 nor deeper than -0.275 eÅ⁻³. *Crystal data for 1a*: (C₂₁H₂₆Cl₂N₂OTi), FW = 441.24, Orthorhombic, space group $P2_12_12_1$, crystal dimensions (mm³) 0.42 × 0.29 × 0.28, a = 10.4465(16), b = 12.885(2), c = 16.065(3) Å, V = 2162.4(6) Å³, Z = 4, ρ_{calcd} = 1.355 g cm⁻³, μ = 0.656 mm⁻¹, $F(000)$ = 920, θ range = 3.16–27.51 deg, no. of rflns collected = 17759, no. of indep rflns / R_{int} = 4956 / 0.0700, no. of data/restraints/params = 4956 / 0 / 260, $R1/wR2$ ($I > 2\sigma(I)$) = 0.0394 / 0.0813, $R1/wR2$ (all data) = 0.0767 / 0.0949, GOF (on F^2) = 1.059, Absolute structure parameter = -0.03(3). Final difference Fourier maps did not show peaks higher than 0.315 nor deeper than -0.347 eÅ⁻³. CCDC-1413283 (**1a**), -1413284 (**b**·HCl).

CCDC-1413283 and 1413284 contain the supplementary crystallographic data for this paper. Copies of these data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk)

2.2. Cell culture, inhibition of cell growth and cell death analysis

2.2.1. Cell culture and MTT assays for PC-3 cells—The androgen-unresponsive cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA) and may be related to recurrent prostate cancers that have achieved androgen independence [78]. All culture media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/

streptomycin/amphoterycin B (Life Technologies, Barcelona, Spain). The culture was performed in a humidified 5% CO₂ environment at 37 °C. After the cells reached 70–80% confluence, they were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin/0.2% EDTA and seeded at 30,000–40,000 cells/cm². The culture medium was changed every 3 days.

PC-3 (1×10^5) cells were grown in 96-well plates. After 24 h, cells with RPMI-1640 medium (Roswell Park Memorial Institute) (Mediatech Inc., Manassas, VA) containing 10% FBS and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B) were treated with the compounds at 24 and/or 72 h. Stock solutions of $[(\eta^5\text{-C}_3\text{H}_5)\text{TiCl}_3]$, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ and ammonium-oxime proligands were freshly prepared in 1% of DMSO in water, while oximate titanium compounds were dissolved in water. The stock solutions were then diluted in complete medium and used for sequential dilutions to desired concentrations. The final concentration of DMSO in the cell culture medium did not exceed 0.1%. Control groups with and without DMSO (0.1%) were included in the assays.

The cytotoxic activity of the chemical compounds was screened against PC-3 cell line within a wide concentration range. Cell growth was determined by a tetrazolium assay, which measures the reduction of substrate MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a dark blue formazan product by mitochondrial dehydrogenases in living cells. Isopropanol was added to each well to dissolve the formazan precipitate and absorbance values were read at 570 nm using the plate reader Multiskan FC (ThermoScientific, Waltham, Massachusetts, USA) with a reference wavelength at 620 nm [79,80]. Each experiment was repeated at least three times and each concentration was tested in at least six replicates. Results are expressed as a percentage of survival with respect to control cells in the absence of the compound. IC₅₀ values (half-inhibitory concentration) were calculated from curves constructed by plotting cell survival (%) *versus* compound concentration (M). The IC₅₀ values were calculated with the GraphPad Prism software.

2.2.2. Cell culture and XTT assays for Caki-1, DLD-1, MDA-MB 231 and HEK-293T cells—Human renal carcinoma cells Caki-1, human breast adenocarcinoma cells MDA-MB-231, as well as the human colorectal adenocarcinoma cells DLD-1 in comparison with healthy human embryonic kidney cells HEK-293T, were used to study **2b** and **2c**'s cytotoxic activity. The cells were all obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). All the cells were grown adherently. The Caki-1 and DLD-1 cells were cultured in RPMI-1640 media containing 10% FBS, Life Technologies, Grand Island, NY), 1% Minimum Essential Media (MEM) nonessential amino acids (NEAA, Mediatech) and 1% penicillin-streptomycin (Pen Strep, Mediatech). The HEK-293T and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech) supplemented with 10% FBS, 1% Minimum Essential Media (MEM) nonessential amino acids (NEAA, Mediatech) and 1 % penicillin-streptomycin (Pen Strep, Mediatech). All cells were cultured at 37° C and 5% CO₂ and 95% air in a humidified incubator.

For evaluation of cell viability, cells were seeded at a concentration of 5×10^3 cells/well in 90 µl of DMEM or RPMI-1640 without phenol red and without antibiotics, supplemented

with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLite Microwell Plate, Fisher Scientific, Waltham, Massachusetts, USA) and grown for 24 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. Cisplatin was dissolved in H₂O. All titanium compounds were dissolved in 1% of DMSO in water before addition to cell culture. The final concentration of DMSO in the cell culture medium did not exceed 0.1%. The intermediate dilutions of the compounds were added to the wells to obtain concentrations of 1 μ M, 10 μ M and 100 μ M, and the cells were incubated for 72 h. Control groups with and without DMSO (0.1%) were included in the assays. Following 72 h drug exposure, 50 μ L per well of 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2Htetrazolium-5-carboxanilide (XTT) (Roche Diagnostics Indianapolis, Indiana, USA) labelling mixture was added to the cells at a final concentration of 0.3 mg mL⁻¹ and incubated for 4 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. The optical absorbance of each well in a 96-well plate was quantified using BioTek ELx808 absorbance microplate reader (BioTek Winooski, VT) set at 450 nm wavelength. The percentage of surviving cells was calculated from the ratio of absorbance of treated to untreated cells. The IC₅₀ value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean ($\pm$ S.E.M) of at least two independent experiments each with triplicate measurements.

2.2.3. Cell viability for Compounds 2b and 2c, cisplatin and Titanocene Y.

ApoTox-Glo Triplex Assay—For apoptosis, viability, and cytotoxicity assays, the Caki-1 cells were seeded in 96-well opaque-walled tissue culture plates with clear bottoms (Thermo Scientific Nunc; Somerset, NJ, USA) at an initial density of 5×10^4 cells/mL in DMEM without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM Lglutamine. Following 24 h incubation, cells were treated with 5 μ M of compounds **2b**, **2c** or 30 μ M of cisplatin, Titanocene Y for 72 h. The cells were then assayed using the ApoTox-Glo Triplex Assay (Promega GmbH, High-Tech-Park, Mannheim, Germany). 20 μ L of viability/cytotoxicity reagent containing both glycyphenylalanyl-aminofluorocoumarin (GF-AFC) and bis-alanylalanyl-phenylalanyl-rhodamine 110 (bis-AAF-R110) substrates were added to each well, and they were briefly mixed by orbital shaking at 200 rpm for 30 seconds and then incubated at 37 °C for 2 h. Fluorescence was measured at a 400 nm for excitation/505 nm for emission (viability) and 485 nm for excitation/520 nm for emission (cytotoxicity/necrosis) using a BioTek Fluorescence Microplate Reader (BioTek U.S., Winooski, VT). Next, 100 μ L of Caspase-Glo 3/7 reagent was added to each well, and the samples were briefly mixed by orbital shaking at 200 rpm for 30 seconds and then incubated at room temperature for 1 hour. Luminescence was measured for 1 second and is proportional to the amount of caspase activity present (BioTek U.S., Winooski, VT). The results for each treatment were expressed as fold change between non-treated (0.1% DMSO) and treated samples. ApoTox-Glo Triplex assays were repeated twice ($n = 2$), and each repetition was run in quadruplicate. The average of the four values was used for statistical calculations. The data are presented as the mean value.

2.3. Cell adhesion assay

Concentrated type-I collagen solution (8 μ g/cm²) was diluted in 10 mM glacial acetic acid and coated onto 96-well plates for 1 h at 37 °C. Plates were washed twice with PBS (pH

7.4). PC-3 cells were harvested with 0.25% trypsin/0.2% EDTA and collected by centrifugation. They were suspended in RPMI-1640 medium/ 0.1% (w/v) bovine serum albumin (BSA) (pH 7.4) and treated with the complexes for 30 min. Then, cells plated at 2.5×10^4 cells per 100 μL . The assay was terminated at 40 min by aspiration of the wells. Cell adhesion was quantified by MTT colorimetric assay as described above.

2.4. Wound-healing assay

PC-3 cells were seeded in 24-well plates at a density of 1.5×10^5 cells in complete media (10% FBS and 1% penicillin/streptomycin/amphoterycin b), and then grown to confluence for 24 h. A small wound area was made with a scraper in the confluent monolayer. The cells were then incubated in either the absence or presence of the complexes. Four representative fields of each wound were captured using a Nikon D1aphot 300 inverted microscopy at different times (0–6 h). Wound areas of samples at time 0 were averaged and assigned a value of 100%.

2.5. Data analysis

Data were subjected to one-way anova and differences were determined by bonferroni's multiple comparison test. Each experiment was repeated at least three times. Data are shown as the means of individual experiments and presented as the mean $\pm$ S.E.M; $P < 0.05$ was considered statistically significant.

2.6. Mobility shift assay

2 μL aliquots of pBR322 plasmid DNA (100 $\mu\text{g/mL}$) in buffer (5 mM Tris/HCl, 50 mM NaClO_4 , pH = 7.49) were incubated with different concentrations (0.3 mM stock solutions, within the range 0.25–2.0 metal complex:DNAbp) of the ligands **a-HCl-c-HCl**, and compounds **1a-HCl-1c-HCl**, **2a-2c** at 37 °C for 20 h in the dark in a total volume of 12 μL . Samples of cisplatin-, TDC-, and $(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3\text{-DNA}$ were prepared as controls. After the incubation period, the samples were loaded onto the 1 % agarose gel. The samples were separated by electrophoresis for 1.5 h at 83 V in Tris-acetate/EDTA buffer (TAE). Afterwards, the gel was stained for 30 min with a solution of GelRed Nucleic Acid stain.

3. Results and Discussion

3.1. Syntheses and characterization of titanium compounds

Reactions of $(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3$ with enantiopure amino-oxime derivatives (2*S*,5*R*)-{NOH, (R)NH} (R = Ph **a**, Bn **b**, 2-pic **c**) [63,64] affords new chiral-at-ligand ammonium-oximate titanium compounds (*S,R*)- $(\eta^5\text{-C}_5\text{H}_5)\text{Ti}(\kappa^2\text{NO},(\text{R})\text{NH}\cdot\text{HCl})\text{Cl}_2$ (R = Ph **1a-HCl**, Bn **1b-HCl**, 2-pic **1c-HCl**) respectively (Fig. 3). The elimination of HCl takes place with subsequent formation of ammonium RNH $\cdot$ HCl fragments within the ligand. Milder reactions conditions are required depending on the nature of the amine substituent, following the order 2-pic > Bn > Ph. Thus, the reaction proceeds in min at room temperature to afford **1c-HCl** while warming of the solutions at 50 °C is needed for quantitative formation of **1a-HCl**.

Compounds **1a-HCl-1c-HCl** are slightly soluble in toluene, benzene or diethylether, and soluble in dichloromethane or chloroform, which agree well with the presence of the

ammonium moiety. In order to obtain crystals suitable for an X-ray diffraction study, synthesis of more soluble amino-oximato titanium compound $[(\eta^5\text{-C}_5\text{H}_5)\text{Ti}(\kappa^2\text{NO}, (\text{Ph})\text{NH})\text{Cl}_2]$, was attempted. Treatment of **1a**·HCl with NEt_3 , in a stoichiometric ratio 1:1, leads to isolation of pure (*S,R*)- $[(\eta^5\text{-C}_5\text{H}_5)\text{Ti}(\kappa^2\text{NO}, (\text{Ph})\text{NH})\text{Cl}_2]$, **1a**, which is formed in solution with the concomitant precipitation of $\text{Et}_3\text{N}\cdot\text{HCl}$. X-ray quality crystals of enantiomerically pure **1a** were grown from a saturated toluene solution at -20°C ; an ORTEP diagram is shown in Fig. 4. The X-ray diffraction structure confirms a dihapto $\kappa^2\text{NO}$ coordination of the oximato unit to the titanium centre, which is the usual coordination mode found for other titanium(IV) oximato derivatives [52,55–57,59,62,81]. N, O and C atoms of the $\text{ON}=\text{C}$ unit are coplanar, and the plane that contained them bisects the angle $\text{Cl}(2)\text{-Ti-Cl}(3)$ along the Ti-Cp centroid vector. Ti-N and Ti-O bond distances are similar to those observed in oximato titanium alkoxide [56,57,59,62] or cyclopentadienyl titanium(IV) hydroxylaminato compounds [82].

Synthesis of titanocene oximato compounds required the addition of an external base. Thus, reactions of $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ with derivatives **a–c** leads to detection of unreacted starting reagents by ^1H NMR. Treatment of $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ and amino-oxime derivatives **a–c** in the presence of NEt_3 allows isolation of compounds (*S, R*)- $[(\eta^5\text{-C}_5\text{H}_5)_2\text{Ti}(\kappa^2\text{NO}, (\text{R})\text{NH})\text{Cl}]$ ($\text{R} = \text{Ph}$ **2a**, Bn **2b**, 2-pic **2c**) (Fig. 5), which are formed together with $\text{Et}_3\text{N}\cdot\text{HCl}$.

Full NMR spectroscopy characterization of the titanium compounds was achieved with the help of bidimensional ^1H - ^1H COSY, ^{13}C - ^1H HSQC, HMBC and ^{15}N - ^1H HMBC experiments.

Disappearance of the downfield $=\text{NOH}$ proton signal observed in the ^1H NMR spectra of free ligands **a–c** confirm oxime deprotonation and oximato coordination to the metal center. Thus, ^1H NMR spectra of **1a**·HCl·**1c**·HCl show broad resonances at δ 11.62 (**1a**·HCl), 9.84, 10.35 (**1b**·HCl) and 10.43 (**1c**·HCl) assigned to $\text{NH}\cdot\text{HCl}$ hydrogen atoms. The association between the resonances at δ 9.84, 10.35 with those due to the $-\text{CH}_2\text{Ph}$ substituent observed in ^1H - ^1H COSY NMR of compound **1b**·HCl, unambiguously confirms the assignation made (see Supplementary data). In contrast, ^1H NMR spectra of **1a**, **2a–2c** show broad resonances at δ 3.71 (**1a**), 3.53 (**2a**), 1.25 (**2b**) and 1.48 (**2c**), assigned to $-\text{NHR}$ protons. Further confirmation of the oximato formation was provided from ^{13}C and ^{15}N - ^1H HMBC NMR data. Oximato coordination shifts the ^{13}C NMR resonance due to the CqNOTi group (δ 152.8–154.5 (**1a**·HCl·**1c**·HCl), 160.5–157.6 (**2a–2c**)) to higher field relative to the signal assigned to CqNOH in the amino-oxime free ligands (δ 164.9 (**a**), 163.0 (**b**), 162.4 (**c**)). The difference between the chemical shifts of the nitrogen signals arising from the oxime $=\text{NOH}$ fragment in proligands **a–c** (δ 343.5 (**a**), 340.0 (**b**), 343.2 (**c**)) and those found in compounds **1a**·HCl·**1c**·HCl, **1a** and **2a–2c** (δ 331.8–335.0, 319.0 and δ 398.9–402.1, respectively) also supports the proposed structure for these Ti(IV) compounds. Amino nitrogen resonances found in ^{15}N - ^1H HMBC NMR spectra of amino-oximato **1a**, **2a–2c** (δ 85.1 (**1a**), 86.1 (**2a**), 60.0 (**2b**), 52.6 (**2c**)) keep almost unaltered relative to those found for free derivatives **a–c** (δ 84.1 (**a**), 60.0 (**b**), 51.8 (**c**)), while signals at δ 79.5 (**1a**·HCl), 67.3 (**1b**·HCl) and 56.3 (**1c**·HCl) are assigned to ammonium nitrogen atoms.

IR spectra of ammonium-oximate **1a·HCl-1c·HCl** exhibit strong, broad $\nu(-\text{NH}, -\text{NH}\cdot\text{HCl})$ bands at 3090–3200 cm^{-1} . In contrast, IR spectra of **1a**, **2a–2c** show sharp absorptions at 3374 (**1a**), 3370 (**2a**), 3370 (**2b**) and 3304 (**2c**) cm^{-1} , assigned to amino $\nu(-\text{NH})$ bands. C=N stretching frequencies of oximate group [54,56,59] are observed in the 1638–1646, 1570–1602 cm^{-1} regions, with similar wavenumbers relative to those bands found in the spectra of organic derivatives **a–c** [48].

We attempted quaternization of the amino group present in compounds **2a–2c** by reaction of the oximate titanium derivatives with HCl (1M solution in diethylether) or iodomethane. Addition of HCl to tetrahydrofuran, chloroform or toluene solutions of **2a–2c** in a Ti:HCl ratio of 1:1 affords corresponding $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ and organic compounds **a–c**, respectively. Reactions of **2a–2c** with iodomethane were monitored by ^1H NMR spectroscopy (CDCl_3 or C_6D_6 in a J. Young valve NMR tube). Starting reactants were detected after 24 h of reaction at room temperature, with no resonances assignable to any new organometallic compound. An increase of the temperature to 50–90°C gave rise to signals that indicated minor to significant decomposition of compounds **2a–c**, while the singlet observed for iodomethane (δ 2.15 (CDCl_3) or 1.43 (C_6D_6)) remained unchanged.

3.2. Hydrolysis behaviour of titanium compounds

In comparison to the limited solubility in water of the starting materials $[(\eta^5\text{-C}_5\text{H}_5)_n\text{TiCl}_{4-n}]$ ($n = 1, 2$), new compounds **1b·HCl**, **1c·HCl**, **2b** and **2c** formed clear yellow solutions (6.6–15.7 mM) after water addition, while derivatives **1a·HCl** or **2a** are only slightly soluble in water after sonication.

The hydrolytic behaviour of the novel oximate titanium compounds was evaluated by ^1H NMR in D_2O and $\text{D}_2\text{O}:[\text{D}_8]\text{THF}$ (1:9). Upon dissolution in D_2O (1,4-dioxane was used as internal reference [83]), monocyclopentadienyl compounds **1a·HCl-1c·HCl** give acidic yellow solutions (2 mM, $\text{pD} = 2.80\text{--}2.56$, $\text{pD} = \text{pH}^* + 0.4$, where $\text{pH}^* = \text{pHmeter reading in } \text{D}_2\text{O}$ [84]), while compounds **2a–2c** afford pD values of 4.74 (**2a**), 5.95 (**2b**) and 5.62 (**2c**), respectively, at similar concentrations of ca. 2 mM. Formation of free proligands (2*S*,5*R*)-{ $\text{NOH}, (\text{R})\text{NH}\cdot\text{HCl}$ } ($\text{R} = \text{Ph } \mathbf{a}\cdot\text{HCl}, \mathbf{Bn } \mathbf{b}\cdot\text{HCl}, 2\text{-pic } \mathbf{c}\cdot\text{HCl}$) was observed right after dilution of mono- or dicyclopentadienyl compounds, and no resonances due to water soluble species with metal bound oximate ligands were apparent from the recorded ^1H NMR spectra of any of the novel derivatives. In this regard, we expected that titanocene oximate hydrolysis would afford free amino-oxime compounds **a–c**, which are insoluble in water. However, resonances due to soluble **a·HCl-c·HCl** are observed within the first 5 min of dilution, which confirm that both, halide and oximate ligands are rapidly hydrolyzed in aqueous medium. White, X-ray quality crystals of **b·HCl** were formed from a solution of compound **2b** in D_2O at room temperature; an ORTEP diagram is shown in Fig. 6. The X-ray molecular structure found confirms the formation of the ammonium-oxime derivative.

The aqueous solution behaviour of $[(\eta^5\text{-C}_5\text{H}_5)_n\text{TiCl}_{4-n}]$ ($n = 1, 2$) has been well documented [83–89]. Rapid hydrolysis of halide ligands occurs through a series of equilibrium reactions in which cyclopentadienyl rings (Cp) remain metal bound at $\text{pH} < 3.0\text{--}3.5$. At higher pH, protonolysis of Cp initiates to give cyclopentadiene as well as insoluble, uncharacterized

titanium hydrolysis products [83,84,87,89]. Accordingly, resonances due to cyclopentadiene (multiplets at δ 6.61, 6.55 and 2.98, see Supplementary data) were observed in the recorded ^1H NMR spectra of more acidic **1a**·HCl-**1c**·HCl or **2a** only after 24 h, while more basic solutions of **2b** and **2c** exhibit, within the first min after dilution, minor multiplets assigned to free cyclopentadiene. Besides those resonances, the ^1H NMR spectra of titanocene derivatives **2a–2c** show, during the first 24 h, metal bound Cp species (ca. 80–90% of metal bound Cp relative to 1,4-dioxane after 1 h and less than 10% after 24 h for **2b** and **2c**) similar to those detected before in D_2O solutions of $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ at pD = 4.1 (singlets at δ 6.60 and 6.50 [83,87], see Supplementary data). Evaluation of cytotoxic compounds (see Cytotoxicity studies, section 3.3.1) **1c**·HCl, **2b** or **2c** in a 1:9 D_2O :[D_8]THF mixture by ^1H NMR lead to similar results. The first spectrum taken following D_2O addition reveal a rapid reaction for all derivatives, with indication of free oxime proligand formation and complete disappearance of cyclopentadienyl resonances corresponding to compounds **1c**·HCl, **2b** or **2c**.

Titanocene compounds **2b** and **2c** were also evaluated in phosphate buffered saline (PBS) solutions in D_2O by ^1H and ^{31}P NMR. Their ^1H NMR spectra showed minor metal bound Cp species only within the first hour (ca. 2% of metal bound Cp relative to free oxime ligand after 1 h, see Supplementary data), observed always with free proligands and minor resonances due to cyclopentadiene. ^{31}P NMR spectra of the **2b** or **2c** solutions in PBS do not revealed important changes compared with that of PBS solution in D_2O while the spectra recorded within 10 min of adding the titanium compounds were the same as those recorded subsequently (only one broaden signal detectable at δ 2.07, assigned to inorganic phosphates present in the PBS solution [90,91]). Similar results were observed when physiological pH was achieved by dissolving the organometallic derivatives in D_2O and adjusting the pD to 7.4 by NaOD addition. Aromatic region of the ^1H NMR spectra obtained following the two different methods were analogous.

The results described herein indicate that the novel oximate titanium compounds are rapidly hydrolysed in water to afford free ammonium-oxime proligands. As already described, cyclopentadienyl ring loss rate depends on pH, but no important differences between our systems and $[(\eta^5\text{-C}_5\text{H}_5)_n\text{TiCl}_{4-n}]$ ($n = 1, 2$) have been found so far at similar pH values.

3.3. *In vitro* cell studies

Titanocene Y has already shown an encouraging activity in PC-3 tumour-bearing mice [92]. Recently, we published a family of cytotoxic ruthenium oxime compounds able to modulate both adhesion and migratory capabilities of PC-3 cells using concentrations of only 5 μM [48]. Thus, we evaluated the effect of the novel chiral cyclopentadienyl oximate Ti(IV) compounds on cytotoxicity, cell adhesion and migration of the human androgen-independent prostate cancer PC-3. In addition and since some titanocene compounds (such as titanocene Y, titanocene Y*, titanocene T and heterometallic titanocene-gold compounds [14,20–30,33,34,36,37]) have shown to be very active against renal cancer both *in vitro* and *in vivo*, we evaluated the cytotoxicity of selected compounds in another three cell lines (human renal Caki-1, human colon DLD-1 and human triple negative breast MDA-MB-231 cancer cell lines). In order to assess the compounds' selectivity for cancerous cells with respect to

normal cell lines, they were also screened for their antiproliferative effects on the non-tumorigenic human embryonic kidney cells HEK-293T. Effects of selected compounds on cell death *in vitro* were also evaluated and the results are discussed in the next sections.

3.3.1. Cytotoxicity studies—The cytotoxicity of the complexes **1a-HCl**, **1c-HCl**, **2a–2c**, **a-HCl-c-HCl** and $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$ on the human prostate cancer (PC-3) cell line was assessed as the IC_{50} values after 72 h of incubation. The results are summarized in Table 1.

Although starting organometallic systems $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ [27] and water soluble proligands **a-HCl-c-HCl** are poorly cytotoxic ($\text{IC}_{50} > 170 \mu\text{M}$), oximato titanium compounds **1c-HCl** and **2c** are more effective than Titanocene Y [27,92], under similar experimental conditions, with an inhibitory activity of 75% at concentrations of 50 μM . Other titanium compounds which have proved their antitumor activity against PC-3 cells line are Schiff-base titanium (IV) derivatives [93] (IC_{50} values within the range 5–18 μM) or heterometallic titanocene-gold compounds (IC_{50} values ranged from 25–40 μM) [27].

The antiproliferative effect of the most active derivatives **1c-HCl**, **2b** and **2c** were also assessed after an exposure time of 24 h, showing inhibitory activities of 20–30% at concentrations of 50 μM (see Supplementary data).

We also studied the effect of the combination of organometallic derivatives $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ or $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$ with proligands **b-HCl** or **c-HCl** on the cellular viability. The additive dose of $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_2]$ and **b-HCl** or **c-HCl** produced lower antiproliferative effects than those observed after treatment with only **2b** or **2c**. This fact indicates that there is indeed a synergistic effect of these novel compounds **2b** and **2c** in their *in vitro* activity on PC-3 cell lines. In contrast, addition of a combined dose consisting of $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$ and proligands **b-HCl** or **c-HCl** did not cause any significant difference in cell viability compared to that found for **1b-HCl** or **1c-HCl**, respectively (see Supplementary data). These findings suggest that the activity of compounds **2b** and **2c** does not result from that of free ligands and titanocene cations following ligand dissociation, as could be inferred from our results on their hydrolytic behaviour, but rather involves an active titanium species whose formation is positively influenced by the presence of the oximato ligand within pro-drugs **2b** or **2c**.

Thus, compounds **2b** and **2c** were selected for further studies *in vitro* on renal cancer (Caki-1), colon cancer (DLD-1), breast cancer (MDA-MB-23) and non-tumorigenic human renal (HEK-293T) cell lines. The cytotoxicity of **2b** and **2c** was assayed by monitoring their ability to inhibit cell growth using the XTT assay (see Experimental Section) after 72 h of incubation with the drug (since we had already seen that the IC_{50} values greatly decreased overtime for PC-3 cell lines). The results are collected in Table 2. In this table we have included IC_{50} values already reported with the same cell lines and incubation times for control compounds TDC, titanocene Y and cisplatin [27,30].

The IC_{50} values obtained show clearly that there is a selectivity of the new titanium compound **2c** for the human prostate and the renal cancer cell lines over the triple negative

breast and the colon cancer cell lines. The values for **2b** and **2c** in Caki-1 cell lines are lower than those reported for cisplatin, TDC and even for titanocene Y, which is a benchmark for titanocene derivatives in this cell line. Very importantly, compounds **2b** and especially **2c** are considerably less toxic to the non-tumorigenic human embryonic kidney cell line (HEK-293T) than to Caki-1 renal cells (**2c** is a 15-fold less toxic). Both compounds display low IC₅₀ values in the range of other improved-titanocene derivatives described so far in Caki-1 cancer cell lines [35,36]. While heterometallic titanocene derivatives have been described recently with IC₅₀ in the low micromolar or sub-micromolar range [27,30], **2b** and especially **2c**, represent good examples of discrete, easily accessible organometallic compounds containing solely a titanium centre. Their cytotoxicity in PC-3 and Caki-1 cancer cell lines, their selectivity and solubility properties in water warrant further exploration of these compounds as potential prostate and renal anticancer agents.

3.3.2. Mechanism of Cell Death—We selected cytotoxic compounds **2b** and **2c** as models to evaluate the mechanism of cell death in renal Caki-1 cancer cells (Fig. 7). We also evaluated the mechanism of cell death in Caki-1 (under the same conditions and using the approximate IC₅₀ concentration) of cisplatin and Titanocene Y. Caki-1 viability, necrosis, and apoptotic activity were analysed by the ApoTox-Glo Triplex (see Experimental Section) which combines three chemical assays to assess viability, cytotoxicity, and caspase activation events within a single well. Viability and cytotoxicity are inversely proportional, such that if the viability of cells is reduced, cytotoxicity is usually stimulated. In general, the cytotoxicity may be due to programmed cell death (apoptosis) or primary necrosis. The treatment of Caki-1 cancer cells with 5 µM of **2b** or **2c** induces apoptosis in 38% and 76% of cells respectively. The treatment of these cells with 30 µM (ca. IC₅₀ value for both cisplatin and Titanocene Y [27]) induced apoptosis in 38% and 33% respectively. Those results clearly point to apoptosis as the principal mode of cell death in Caki-1 cells treated with oximato titanocene compounds **2b** and **2c**. Other titanocenes like TDC, Titanocene C (bis-[N,N-dimethylamino-2(N-methylpyrrolyl)-methyl-cyclopentadienyl]titanium(IV) dichloride), X ([1,2-di(cyclopentadienyl)-1,2-di-(4-N,N-dimethylaminophenyl)ethanediyl]titanium(IV) dichloride) and Y, and heterometallic complexes containing titanocene fragments are also known to induce apoptosis in different cancer cell lines [16,21,27,30,94,95]. Moreover, as shown in Figure 7, compound **2c** in a concentration 6 times lower than that of cisplatin and Titanocene Y (one of the benchmarks for activity in Caki-1 cancer cells and tumours) reduces the viability of cells in a much higher percentage and is more apoptotic.

3.3.3. Cell adhesion—The oximato titanium complexes **1c·HCl**, **2b** and **2c**, which showed the highest antiproliferative effects on the PC-3 cell line, were further evaluated on cell adhesion to type-I collagen in order to determine their effect on metastatic capability of human androgen-independent prostate PC-3 cancer cells. Tumour metastasis is the process by which a tumour cell leaves the primary tumour, travels to a distinct site and establishes a secondary tumour. The disruption of intercellular structures and cell-cell local interaction (cell adhesion) can lead to the dissociation of the primary tumour and an enhanced potential for dissemination and metastatic spread of cancer cells to secondary location [96–100]. To investigate the effect of our compounds on the cell adhesion *in vitro*, we incubated PC-3

cells in the absence or presence of 50 μM of **1c·HCl**, **2b** or **2c** on a collagen plate. In order to establish a comparison, we also performed the experiment on the starting materials $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ and **c·HCl**. PC-3 cells rapidly adhered to collagen basement in 40 min. The adhesion pattern exhibited by organometallic derivative $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ (32%) was similar to that of cyclopentadienyl oximate titanium compounds (**1c·HCl** (30%), **2b** (32%), **2c** (40%), Fig. 8), all of them showing a very significant increase of the adhesion ability relative to that observed for control cells. Monocyclopentadienyl and organic ammonium-oxime derivatives, $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$ (17%) and **c·HCl** (14%), displayed a lower adhesion ability than that of oximate titanium systems [48].

3.3.4. Cell migration—To the best of our knowledge, the effect of organometallic titanium compounds on the migratory capability of cancer cells has been scarcely studied. In this regard, the treatment of human 518A2 melanoma cells with schisandroxymethyltitanocene for 24 h revealed a similar improvement on the cell migration as $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$. Both titanocene derivatives were able to prevent wound healing in the cancer cells colonies, with dose concentrations of 0.1 μM [101]. Very recently, a heterometallic Ti-Au compound described by the group of Contel and co-workers has demonstrated anti-migratory effects on Caki-1 cells with concentrations of 5 μM [30].

The wound-healing assay is a useful method for gauging the anti-migratory activity of drug candidates. In order to evaluate cell migration we performed wound-healing assays in which a small wound area was made on the plate with a confluent monolayer of cells.

After 6 h of incubation, the wound in the control wells was almost fully repopulated with migrated cells (Fig. 9). The cells treated with 50 μM of complexes **1c·HCl**, **2b** and **2c** showed a significant lower migration capability (62–72% of wound healing) than that of control cells, while compounds $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ and **c·HCl** treated cells exhibited a non-significant lower migration ability than that of oximate titanium systems (Fig. 9).

3.4. Interactions with plasmid DNA

DNA is one of the critically important targets in cancer chemotherapy. Although the nature of the active species of titanium anticancer drugs and their mechanism of action remained unresolved, the notion of DNA being their probable target was supported by early works, which found that Ti localizes to the chromatin and inhibits DNA synthesis [1]. However, later investigations indicated that titanium interacts only weakly with DNA at neutral pH. Thus, below pH 5, titanium compounds such as $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ or Ti(IV) hydrolysis species bind to nucleotides, notably to the phosphate units, but binding is generally very weak near pH 7 [15,16,19,84,89,102,103]. Weak DNA interactions between Titanocene Y and DNA double helix have been recently found [36,104].

We performed agarose gel electrophoresis studies to evaluate the effects of the compounds **1a·HCl**-**1c·HCl**, **2a–2c**, **a·HCl**-**c·HCl**, $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ and cisplatin on plasmid (pBR322) DNA. The plasmid has two main forms; form I (supercoiled or covalently closed, CCC) and II (relaxed or open circular, OC). The electrophoretic patterns of plasmid DNA incubated with increasing amounts of cisplatin, organic derivatives **a·HCl**-**c·HCl** and

titanium complexes $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$, **1a**·HCl-**1c**·HCl and **2a**–**2c**, are shown in Fig. 10. Changes in electrophoretic mobility of both forms are usually taken as evidence of metal-DNA binding. Unwinding of the DNA causes a retardation of the fast running band of the CCC form, whereas condensation and cross-linking of DNA accelerates the migration of the OC form. Binding of cisplatin to plasmid DNA results in a decrease in mobility of the CCC form and an increase in mobility of the OC form (see lanes a–d for cisplatin in Figure 10) [27,30,44,105–107]. At physiological pH, none of the tested ammonium-oxime or Ti(IV) derivatives showed any important impact on the DNA mobility. This fact suggests either that the compounds do not interact with DNA or that interaction is too weak to be detected by this technique.

Conclusions

The results presented here and those reported before by us on ruthenium derivatives, support the idea that the use of the enantiopure amino-oxime ligands **a**–**c** is a useful strategy to design easily accessible chiral and water soluble metal compounds with potential as anticancer agents. The novel oximato Ti(IV) compounds exhibit a different DNA binding behaviour from that found in cisplatin. The studies on the hydrolytic behaviour of the new titanium derivatives did not permit the detection of water soluble titanium oximato-containing hydrolysis product. However, titanocene derivatives **2b** and **2c** have demonstrated to have synergistic effects relative to additive doses of TDC and free proligands. This fact suggests the existence of titanium active species whose formation is positively influenced by the presence of the oximato ligands. Compound **2c** displays some of the lower IC₅₀ values described so far in PC-3 and Caki-1 cancer cell lines for titanocene derivatives containing solely a titanium centre, while showing selectivity against renal cancer when compared to the non-tumorigenic human renal HEK-293T cell line. The cell death type for compounds **2b** and specially **2c** is mainly through apoptosis. Furthermore, **2b** and **2c** show interesting *in vitro* results on cell adhesion and migration of the androgen-independent prostate cancer PC-3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	Analysis of Variance
Budotitane	<i>cis</i> -diethoxybis(1-phenylbutane-1,3-dionato)titanium(IV)
Caki-1	Human renal cancer cell line

Cisplatin	(SP-4-2)-diamminedichloroplatinum(II)
COSY	Correlation Spectroscopy
DLD	Human promyelocytic leukemia cell line
DLD-1	Human colon cancer cell line
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Et	ethyl
FBS	Fetal Bovine Serum
FTIR	Fourier Transformed Infrared spectroscopy
HEK-293T	Non-tumorigenic human embryonic kidney cell line
HeLa	Human cervical cancer cell line
HL60	Human promyelocytic leukemia cell line
HSQC	Heteronuclear Single Quantum Coherence spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation spectroscopy
MDA-MB-231	Triple negative human breast cancer cell line
MEM	Minimum Essential Media
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance
NEAA	Nonessential Amino Acids
PBS	Phosphate Buffered Saline
PC-3	Human androgen-independent prostate cancer cell line
RPMI	Roswell Park Memorial Institute medium
TDC	Titanocene dichloride
THF	tetrahydrofuran
SEM	Standard Error of Mean
Titanocene C	bis-[N,N-dimethylamino-2-(N-methylpyrrolyl)-methyl-cyclopentadienyl] titanium(IV) dichloride
Titanocene T	bis-[[(1-methyl-3-diethylaminomethyl)indol-4-yl } methyl cyclopentadienyl]titanium(IV) dichloride
Titanocene X	[1,2-di(cyclopentadienyl)-1,2-di-(4-N,N-dimethylaminophenyl)ethanediyl]titanium(IV) dichloride

Titanocene Y	bis-[(p-methoxybenzyl)cyclopentadienyl]titanium(IV) dichloride
Titanocene Y*	bis-[(p-diethylaminobenzyl)cyclopentadienyl]titanium(IV) dichloride
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfohenyl)-2Htetrazolium-5-carboxanilide

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://>. These data include: Representative NMR spectra of compounds **a–c**, **a·HCl·c·HCl**, **1a·HCl·1c·HCl**, **2a–2c**, representative time dependent ¹H NMR spectra in D₂O and PBS solutions, cell viability of representative compound **2c** after 24 and 72 h, comparison between the cytotoxicity of compounds **1b·HCl**, **1c·HCl**, **2b**, **2c** and [(η⁵-C₅H₅)TiCl₃]+**b·HCl**, **+c·HCl** or [(η⁵-C₅H₅)₂TiCl₂]+**b·HCl**, **+c·HCl** on cell viability of PC-3 cells; bond lengths and angles for X-ray molecular structures of **1a** and **b·HCl**.

Highlights

- We report the synthesis of new enantiopure cyclopentadienyl oximato Ti(IV) compounds.
- The novel compounds affect the adhesion and migration patterns of PC-3 cancer cells.
- **2b** and **2c** are more cytotoxic against Caki-1 cancer cells than cisplatin or titanocene Y.
- **2b** and specially **2c** preferentially induce apoptosis in Caki-1 cancer cells.
- **2b** and **2c** show selectivity against Caki-1 compared to non-tumorigenic HEK-293T cells.

Fig. 1.
Selected titanium compounds with relevant antitumor properties

Fig. 2.
Enantiomerically pure amino-oxime ligands and related Ti(IV) compounds

Fig. 3.
Synthesis of monocyclopentadienyl oximato titanium compounds

Fig. 4. ORTEP drawing of compound **1a** with 30% probability ellipsoids. Hydrogen bonded to carbon atoms have been omitted for clarity. Representative bond lengths (Å) and angles (deg): Ti(1)–O(1) 1.8860(19); Ti(1)–N(1) 2.102(2); N(1)–O(1) 1.401(3); N(1)–C(1) 1.281(3); O(1)–Ti(1)–N(1) 40.71(9); N(1)–O(1)–Ti(1) 77.91(14); O(1)–N(1)–Ti(1) 61.34(11); C(1)–N(1)–Ti(1) 178.9(2); C(6)–N(2)–C(21) 124.3(3).

Fig. 5.
Synthesis of titanocene amino-oximato compounds

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Fig. 7.

Compounds **2b**, **2c**, cisplatin and Titanocene Y impaired the viability of Caki-1 cells by inducing apoptosis. Following a 72 h incubation period, the compounds (5 μ M) **2b** or **2c** or (30 μ M) Cisplatin or Titanocene Y reduced Caki-1 viability by 61% (cisplatin), 48% (Titanocene Y), 56% (**2b**) and 84% (**2c**); induced necrosis in 9% (cisplatin), 5% (Titanocene Y), 17% (**2b**) and 6% (**2c**), and induced apoptosis in 38% (cisplatin), 33% (Titanocene Y), 38% (**2b**) and 76% (**2c**) of treated cells. The effect of cisplatin and Titanocene Y, **2b** and **2c** on viability, necrosis and apoptosis was assessed by measuring protease activity using the non-cell-permeable substrates, cell-permeable substrates and by measuring the total caspase-3 and -7 activities with the ApoTox-Glo Triplex Assay. The effect of each treatment was determined by comparing treated and untreated cells. From the Apotox assay a cell population that was neither necrotic nor apoptotic was detected and their mode of death could not be assigned. Those cells were not included in the graph.

Fig. 8.

Effect of [$(\eta^5$ -C₅H₅)TiCl₃], [$(\eta^5$ -C₅H₅)₂TiCl₂], **c-HCl**, **1c-HCl**, **2b** and **2c** derivatives on adhesion of PC-3 cells to type-I collagen was studied after treatment with organic and organometallic compounds for 40 min. Data are the mean $\pm$ S.E.M of at least four experiments. *, $P < 0.05$; ***, $P < 0.001$ versus Control.

Fig. 9.

Effect of $[(\eta^5\text{-C}_5\text{H}_5)\text{TiCl}_3]$, $[(\eta^5\text{-C}_5\text{H}_5)_2\text{TiCl}_2]$ **c-HCl**, **1c-HCl**, **2b** and **2c** on cell migration was studied in human androgen-independent prostate cancer PC-3 cell line. Microscopic analysis of the cell-free area was carried out at the indicated time (6 h) after the addition of the complexes and the width of the area invaded by prostate cells was estimated.

Fig. 10.

Electrophoresis mobility shift assays for cisplatin and compounds [(η⁵-C₅H₅)TiCl₃], [(η⁵-C₅H₅)₂TiCl₂], **a·HCl**-**c·HCl**, **1a·HCl**-**1c·HCl** and **2a–2c** (see Experimental Section for details). DNA refers to untreated plasmid pBR322. Lanes *a*, *b*, *c* and *d* correspond to metal/DNA bp ratios of 0.25, 0.5, 1.0 and 2.0 respectively. *OC* open circular or relaxed form (Form II), *CCC* covalently closed or supercoiled form, (Form I).

Table 1

IC₅₀ (μM) ± S.E.M.^a for compounds **1a·HCl**-**1c·HCl**, **2a–2c** and **a·HCl**-**c·HCl** in the androgen-independent prostate cancer PC-3 cells after 72 h of incubation.

Compound	IC ₅₀ values
1a·HCl	121.3 ± 5.7
1b·HCl	113 ± 6.5
1c·HCl	25.7 ± 8.6
2a	139.2 ± 8.4
2b	59.3 ± 6.8
2c	17.3 ± 5.2
a·HCl	201.7 ± 9.4
b·HCl	177 ± 5.5
c·HCl	204.7 ± 15.6
[(η⁵-C₅H₅)/TiCl₃]	>300

^aEach value represents the mean ± S.E.M. of at least three independent experiments.

Table 2

IC₅₀ values (μM) ± S.E.M.^a in human cell lines were determined with **2b** and **2c**. Reported values for cisplatin, [(η⁵-C₅H₅)₂TiCl₂], and Titanocene Y are indicated for some cell lines.

Compound	Caki-1	DLD-1	MDA-MB-231	HEK-293T
2b	8.55 ± 2.77	59.66 ± 4.97	> 100	50.99 ± 7.15
2c	4.51 ± 1.17	44.09 ± 3.10	> 100	70.22 ± 11.22
[(η ⁵ -C ₅ H ₅) ₂ TiCl ₂] ^b	> 200	----	---	> 200
Titanocene Y ^b	29.42 ± 4.18	---	---	> 200
Cisplatin ^b	29 ± 4.11	---	---	3.27 ± 0.13

^aEach value represents the mean ± S.E.M. of at least two independent experiments.

^bValues obtained with the same technique, cell lines and incubation times [27,30].