Study of the anticancer properties of optically active titanocene oximato compounds

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Abstract

New water soluble and optically active cyclopentadienyl titanium derivatives [(η5-C5H5)2Ti(1R,4S)-\(\text{ON}-(R)\text{NH})\text{Cl}] (R = Bn (Benzyl) 1a, 2-pic (2-picolylamine) 1b) have been synthesized. The novel compounds along with those previously described [(η5-C5H5)2Ti(1S,4R)-\(\text{ON}-(R)\text{NH})\text{Cl}] (R = Bn 1a, 2-pic 1b) were evaluated by polarimetry, ultra-violet and circular dichroism spectroscopy. The structure of 1b was determined by single crystal X-ray crystallography and showed a unique terminal monohapto Ti-O disposition of the oximato ligand. All enantiomers have been tested against several cancer cell lines in vitro: prostate PC-3 and DU-145, lung A-549, pancreas MiaPaca-2, colorectal HCT-116, leukemia Jurkat and cervical HeLa. In addition, 1a, 1b and 1b' were tested against non-tumorigenic prostate RWPE-1 cell line. After 24 h of incubation, 1b and 1b' were moderately active against Jurkat and A-549 cells. The anti-proliferative effect of titanium compounds on prostate PC-3, DU-145 and RWPE-1 cell lines was also assessed after 72 h of drug exposure. The cytotoxic profile of the enantiomers was similar, except for the PC-3 cells, with S,R-isomers exhibiting cytotoxicities 2 to 3 times higher than R,S-compounds. Under these conditions, derivative 1b showed calculated IC50 values better than those of Tacke’s Titanium-Y (bis-[\(\text{p-methoxybenzyl}\)]cyclopentadienyl)[titanium(IV) dichloride) on both the prostate PC-3 and DU-145 cells. 1a and 1b cytotoxic behaviour shows certain selectiveness, with activities 2–4 times lower on normal prostate RWPE-1 than on cancer PC-3 cells. Furthermore, 1b produces higher cytotoxicity on prostate PC-3, DU-145 and RWPE-1 cells than the additive dose of titanocene dichloride and pro-ligand b-HCl. Additionally, compound-DNA interactions have been investigated by equilibrium dialysis, Fluorescence Resonance Energy Transfer (FRET) melting assays and viscometric titrations, which suggest that these metal complexes and/or their hydrolysis products bind DNA either in the minor groove or externally.

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1. Introduction

Since the successful introduction of cisplatin (cis-[PtCl2(NH3)2]) as an anticancer drug, much effort has been devoted to investigation of the anticancer activity of other coordination and/or organometallic transition metal compounds [1–7]. The titanium derivatives titanocene dichloride [(η5-C5H5)2TiCl2, TDC] [8,9] and budotitane [(cis-dithioxybis(1-phenylbutane-1,3-dionato)tinanium(IV)) [10,11] were the first metal compounds to enter clinical trials after platinum complexes. Although these derivatives showed promising properties in preliminary studies, they failed advanced clinical trials due to low antitumor efficacy in vivo, rapid hydrolysis and limited solubility in biological media [12–19]. Since then, a plethora of modified titanium based compounds have been synthesized and studied as potential antitumor agents [17–29].

The effect of stereochemistry on biological activity is of great importance in medicinal chemistry, as many of the biological targets are chiral [30,31]. The anticancer properties of chiral metal derivatives have been largely studied [32–46], but the role of the stereochemistry in the biological activity of non-platinum based compounds has been less investigated [22,47–61]. Effect of the absolute configuration on the anticancer efficiency of titanium...
Compounds was firstly explored by Tshuva in 2010 [50]. The enantiomers of C2-symmetrical Ti(IV) compounds with chiral diamine bis(phenolato) ligands showed different antitumor activities by factors of 2–4 on human colorectal (HT-29) and ovarian (OVCAR-1) carcinoma cells [50,51,56,60]. According to these results, the authors proposed that stereochemistry should be considered in the design, modification, and improvement of active compounds [60]. The same year, Baird published a family of enantiomerically pure titanocene derivatives bearing chiral alkylammonium groups, but a relationship between the anticancer activity and chirality could not be established due to the low cytotoxicity showed on the cancer cell lines evaluated [62]. Enantiomer-dependent activity was found in chiral substituted titanocene compounds by Cini et al. [22,28], with the (SS) enantiomer of Cp5TiCl2 (Cp5 = η5-C5H4CH(CH2CH3) C6H5OMe) being twice as active as the (RR) isomer towards pancreatic, breast and colon cancer cell lines, after 24 h of treatment. Interestingly, lack of enantiomer recognition was observed at 72 h when screening the compounds in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays.

Within this context, enantiomerically pure, naturally occurring terpenes are useful building blocks for asymmetric synthesis [63,64]. They are inexpensive and commercially available reagents in optically pure form, and easily tailored by stereoselective functionalization [65]. On the other hand, oxime groups are presented as excellent chemical modifiers, with a wide versatility of coordination modes going from mono- to di-NO, α-ON, to dihapto αα,N,O; either with side on or bridging coordination, which could offer an increased stability of the final compounds when bonded to Ti(IV) acid centres [66–68].

We have recently reported a new family of enantiopure cyclopentadienyl titanium(IV) compounds with amino-oximato ligands derived from R-limonene, of formula [(η5-C5H4CH(CH2CH3) C6H5OMe]Ti{(1R,4S)-ON,(R)NH}Cl (R = Bn a, 2-pic b) (Fig. 1), with relevant antitumor properties. Our compounds show significant effects on cytotoxicity, cell adhesion to collagen and migration of androgen-independent prostate cancer cells while they do not seem to exhibit strong interactions with plasmid DNA by electrophoretic mobility shift assays. Compounds 1a or 1b suffered hydrolysis in water or phosphate buffered saline (PBS) solutions. However, the additive doses of TDC and a-HCl or b-HCl produced lower antiproliferative effects on prostate cancer PC3 cells than those observed after treatment with oximato titanocenes 1a or 1b, respectively. This fact led us to the conclusion that the active operating titanium species was positively influenced by the presence of the oximato ligand [69].

Encouraged by these previous results, we decided to explore the reactions of TDC with the already described amino-oxime chiral compounds (1R,4S)-(NH(R),NOH) (R = Bn a, 2-pic b) [65,70,71], derived from S-limonene.

We report here on the synthesis and characterization of corresponding cyclopentadienyl Ti(IV) enantiomers [(η5-C5H4CH(CH2CH3) C6H5OMe]Ti{(1R,4S)-α-ON,(R)NH}Cl (R = Bn 1a, 2-pic 1b). Their hydrolytic behaviour has been studied by 1H NMR, Ultraviolet-visible (UV–Vis) spectroscopy and circular dichroism (DC). These novel compounds along with those previously described have been evaluated against several cancer cell lines in vitro: prostate PC-3 and DU-145, lung A-549, pancreas MIA PaCa-2, colorectal HCT-116, leukemia Jurkat and cervical Hela. In addition, the compounds were tested against the non-tumorigenic human prostate RWPE-1 cell line. DNA interactions of the metal derivatives and/or their hydrolysis products have been further investigated by FRET melting assays, equilibrium dialysis and viscometric titrations experiments.

2. Experimental Section

2.1. Chemicals and synthesis

Manipulations involving the synthesis of titanium compounds 1a, 1a’, 1b and 1b’ and Titanocene-Y (bis-{(p-methoxybenzyl) cyclopentadienyl}titanium(IV) dichloride) were performed at an argon/vacuum manifold using standard Schlenk techniques or in a MBraun MOD System glove-box. Solvents were dried by known procedures and used freshly distilled. Titanocene-Y [72], (154R)-(NH(R),NOH) (R = Bn a, 2-pic b), (154R)-(NH(R),NOH) (R = Bn a, 2-pic b); corresponding adducts (154R)-(NH(R) HCl,NOH), (R = Bn a·HCl, 2-pic b·HCl); (1R,4S)-(NH(R) HCl,NOH) (R = Bn a’·HCl, 2-pic b’·HCl) [63,73] and metal compounds [(η5-C5H4CH3)2Ti{(1R,4S)-(α-ON,(R)NH}Cl (R = Bn 1a, 2-pic 1b) [69] were prepared according to previous reports. R- or S-limonene and isopentyl nitrite were reacted following the standard method described by Carman et al., in 1977 [73]. R-limonene, S-limonene, TDC and cisplatin were purchased from Sigma-Aldrich. Commercially available reagents were used without further purification. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker 400 Ultraschild. 1H and 13C chemical shifts are reported relative to tetramethylsilane. 15N chemical shifts are reported relative to liquid ammonia (25 °C). Coupling constants J are given in Hertz. Elemental analysis was performed on a LECO CHNS932 Analyser at the Universidad de Alcalá or, alternatively, at the Universidad Autónoma de Madrid. Fourier Transform Infrared (FT IR) spectra were recorded on IR FT Perkin Elmer (Spectrum 2000) spectrophotometer on KBr pellets. The pH was measured on a HANNA HI208 pHmeter in distilled water solutions. Circular Dichroism (CD) spectra were recorded on a J-715 CD spectropolarimeter (Jasco, UK) at ambient temperature (297 K). The spectra were determined at a concentration of 0.5 mM in water using a quartz cuvette of 0.5 cm path length, scan speed of 20 nm min−1, 0.1 nm band width, 0.5 nm data pitch and 0.5 s of response time. Optical rotations of all the compounds solutions were recorded on a Perkin Elmer 341 polarimeter, using the sodium D line (589 nm) at ambient temperature (297 K) in a quartz cell of 1 dm path length. Specific optical rotation values were calculated according to the equation [α]D25°C = 100 c [D]Cu1 cm-1 [74]. Analytical balance and volumetric pipettes (2.0 mL) were used to prepare CHCl3 solutions of the compounds at concentrations within a range of 7.50–7.80 g dL−1. UV–Vis spectra were measured at room temperature on water solutions of the compounds with a Perkin Elmer Lambda 35 spectrophotometer.

2.1.1. (1R,4S)-(NH(2-pic),NOH) (b’)

An analogous procedure to that described before for the synthesis of b [53] was used, starting from S-limonene [70,73], [x]D25°C = (deg dm3 g−1 cm−1) = 128 ± 1.3 (b’ at c = 0.7839 g dL−1), [α]D25°C = 25°C = 0.957 deg); +127 ± 1.3 (b at c = 0.7604 g dL−1), [α]D25°C = 25°C = 0.954 deg). All analytical and spectroscopic data are identical to those observed for b. Anal. Calc. for C50H52N2O3: C, 70.30; H, 8.48; N, 15.37; Found: C, 70.13; H, 8.07; N, 15.20. FT IR (KBr, λmax/cm−1):
1.20 mmol) and NEt3 (0.11 mL, 1.20 mmol). Compound 45.0 (CH4), 40.5 (C

3086–3314 (br, vOH/NH), 1650, 1598 (vC = N). UV–Vis (0.1 mM in H2O:DMSO 99:1); λmax (ε) 261 (316), 340 (10). 1H NMR (plus two dimensional correlation spectroscopy (COSY), 400.1 MHz, 293 K, chloroform-d1): δ 9.80 (=NOH), 8.49, 7.60, 7.28, 7.11 (m, each 1H, NC6H5), 4.75 (br, 2H, =CH2), 3.87, 3.61 (both d, each 1H, JHH = 6, −CH2−C≡CH-N), 3.28 (d, 1H, JHH = 12, −CH2=), 2.60 (br, 1H, NH), 2.09 (m, 1H, −CH3), 2.03 (dd, 1H, JHH = 12, =CH2), 1.69 (m, each 1H, −CH2−CH3 =), 1.85 (m, 1H, CH2), 1.75 (s, 3H, CH3−C =), 1.65 (m, 1H, CH3), 1.32 (s, 3H, −CH3−C=N−). 13C NMR (plus Attached Proton Test (APT), plus gradient Heteronuclear Single Quantum Coherence (gHSQC), plus Heteronuclear Multiple Bond Correlation (HMBC), 100.6 MHz, 293 K, chloroform-d1): δ 52.0 (CH3−C), 40.5 (C=N), 39.8 (C=CH2), 35.3 (C≡CH), 33.1 (CH), 28.4 (m, each 1H, −CH2−CH2−CH3 =), 21.0 (CH3−C =), 19.9 (CH3−C=N−). FT IR (KBr, νC=N): 1520, 1492, 1451, 1387, 1350, 1289, 1276, 1164, 1174, 1092 (C≡CH), 1053, 982, 970 (C=CH2), 873, 753, 721, 703, 695 (vO/NH). Anal. calc. for C86H80Cl3N10O7Ti: C, 48.5, H, 4.79, N, 5.34, Ti, 8.91; Found: C, 48.5, H, 4.76, N, 5.37. 15N NMR (gHMBC, 40.5 MHz, 293 K, chloroform-d1): δ 434.7 (C=N−C=N−C=N−), 308.6 (C=N−C=N−C=N−), 253.0 (C=N−C=N−C=N−), 200.0 (C=N−C=N−C=N−), 151.2 (C=N−C=N−C=N−), 108.0 (C≡C−), 102.0 (C≡C−). Anal. calc. for C86H80Cl3N10O7Ti: C, 48.5, H, 4.79, N, 5.34, Ti, 8.91; Found: C, 48.5, H, 4.76, N, 5.37. 15N NMR (gHMBC, 40.5 MHz, 293 K, chloroform-d1): δ 434.7 (C=N−C=N−C=N−), 308.6 (C=N−C=N−C=N−), 253.0 (C=N−C=N−C=N−), 200.0 (C≡C−), 108.0 (C≡C−). 1H NMR experiments at physiological pH

Phosphate buffered saline solution (PBS) was prepared according to Cold Spring Harbor Protocols (http://chpprotocols.cshlp.org/content/2006/1/pdb.rect8247) using NaCl, KCl, Na2HPO4 and K2HPO4 in D2O. Adjustment of pH (pH = pH + 0.4, where pH = pMter reading in D2O) was carried out using a solution of DCl (0.01M) or NaOD (0.01M) in D2O, with the help of a HANNA HI208 pHmeter. Titanium compounds were then dissolved in 200 μL of the freshly prepared PBS, final pH measured (7.30–7.38) and time-dependent 1H NMR spectra of 500 μL aliquots of final solutions were carried out at 25°C.

2.2. Single-crystal X-ray structure determination

Yellow crystals of pure enantiomer 1b were grown from a hexane-toluene solution. The crystals were removed from the vial and covered with a layer of a viscous perfluoropolyether. A suitable crystal was selected with the aid of a microscope, mounted on a cryo-loop, and placed in the low-temperature nitrogen stream of the diffractometer. The intensity data sets were collected at 200 K on a Bruker-Nionus Kaiser CCD diffractometer equipped with an Oxford Cryostream 700 unit. The molybdenum radiation (λ = 0.71073) was used in both cases, graphite monochromated, and enhanced with an MIRACOL collimator.

The structure was solved, using WINGX package [75], by intrinsic phases method [76], and refined by least-squares against F2 (SHELXL-2014/7) [77]. Crystals of 1b were refined as two-component inversion twin, and also had two different molecules in the asymmetric unit with no significant differences. All non-hydrogen atoms were anisotropically refined. Positions of the amine hydrogen atoms, H(2) and H(21), were located in the difference Fourier map. H(2) was refined isotropically, while Uiso(H21) was fixed with a value of 0.05. The rest of the hydrogen atoms were positioned and refined by using a riding model. Crystal data for 1b: (C26H32ClN3OTi), FW = 485.89, Monoclinic, space group P21/c, crystal dimensions (mm3) 0.30 × 0.27 × 0.12, a = 10.470(1), b = 11.631(3), c = 19.856(3) Å, β = 94.27(2)°, Z = 4, V = 1417.25 Å3, μ = 0.488 mm−1, F(000) = 1024, θ = 3.08–27.50 deg, 0.0081 < Fobs < 0.0084, 10939/15053, 1.67, Absolute structure parameter = 0.04 (5). Final difference Fourier maps did not show peaks higher than 0.695 nor deeper than −0.329 e Å−3. CCD-1579290 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

2.3. Cell culture, cytotoxicity assays and cell death analysis

2.3.1. Cell culture

The prostate androgen-unresponsive cancer 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. All culture media were supplemented with 1% penicillin/streptomycin/amphotericin 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 PBS, detached with 0.25% trypsin/0.2% ethylenediaminetetraacetic acid (EDTA) and
seeded at 30,000–40,000 cells cm⁻². The culture medium was changed every 3 days. A549 (lung carcinoma) cells were maintained in high glucose DMEM (Dulbecco’s Modified Eagle’s Medium) and RWPE-1 (non-tumorigenic prostate) cells in DMEM/F12 (Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12), supplemented with 5% fetal bovine serum (FBS), 200 U mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin and 2 mM l-glutamine. DU-145 (prostate carcinoma), MIA PaCa-2 (pancreas carcinoma), HCT-116 (colorectal carcinoma), HeLa (cervical cancer) and Jurkat (leukemic cancer) cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 5% FBS, 200 U mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin and 2 mM l-glutamine. Cultures were maintained in a humidified atmosphere of 95% air:5% CO₂ at 37 °C. Adherent cells were allowed to attach for 24 h prior to addition of compounds.

2.3.2. MTI toxicity assays

For toxicity assays, cells (5 × 10⁴ for Jurkat cells and 10⁴ for adherent cell lines) were seeded in flat-bottom 96-well plates (100 μL/well) in complete medium. Adherent cells were allowed to attach for 24 h prior to addition of cisplatin or tested compounds. Stock solutions of Titanocene-Y, TDC and ammonium-oxime proligands were freshly prepared in 1% of dimethyl sulfoxide (DMSO) in water, while cisplatin and oximato titanium compounds were dissolved in culture medium. The stock solutions were then dilute 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. Compounds were then added at different concentrations in quadruplicate. Cells were incubated with compounds for 24 h or 72 h, and then cell proliferation was determined by a modification of the MTT-reduction method. Briefly, 10 μL/well of [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] (MTT) (5 mg mL⁻¹ in PBS) was added, and plates were incubated for 1–3 h at 37 °C. Finally, formazan crystals were dissolved by adding 100 μL/well IPrOH (0.05 M HCl) and gently shaking. The optical density was measured at 550 nm using a 96-well multi-scanner auto-reader Enzyme-Linked Immuno Sorbent Assay (ELISA).

2.4. DNA interaction studies

2.4.1. Equilibrium dialysis

Duplex DNA from calf thymus (CT DNA), (Deoxyribonucleic acid, Activated, Type XV) was directly purchased from Sigma Aldrich and used as provided. Duplex-forming oligonucleotides ds17-1 (5’-CCA GTT CTT AGT AAC CC-3’) and ds17-2 (5’-GGG TTA CTA CGA ACT GC-3’) were acquired High Performance Liquid Chromatography (HPLC) -purified and desalted from Integrated DNA Technologies (IDT). Dialysis membranes (Spectra/Por™ molecular porous porous membrane tubing MWC0: 3.5–50 kDa: 6.4 mm diameter) were purchased from Spectrum Laboratories Inc. Aqueous solutions of surfactant sodium dodecyl sulphate (SDS) (10%) were purchased from Sigma Aldrich. The buffer employed in this experiment was 10 mM phosphate buffer Na₂HPO₄/Na₂HPO₄, pH = 7.2. The viscosity measurements were performed in a Visco System AVS 470 at 25.00±0.01 °C, using a microUbbelohde (K = 0.01) capillary viscometer. 6 mL of DNA solution (0.4 mM in nucleotides) in phosphate buffer were equilibrated for 20 min at 25.00 °C and then 20 flow times were registered. Small aliquots (30–50 μL) of solutions of metal complexes (1.6–2.3 mM) were added to the same DNA solution. Before each flow time registration, the solutions were equilibrated for 20 min to 25.00 °C and then 20 flow times were measured. With the averaged time of the different flow time measurements and the viscometer constant, the viscosities (μ) for each point were calculated as: 10 mM sodium cacodylate 100 mM LiCl, (pH = 7.3). First, the duplex-forming oligonucleotide was dissolved in water (grade BPC) and a 50 μM stock solution was prepared, which was then diluted to 0.5 μM. Then, the diluted DNA solution was mixed with the working buffer (2x) and water Biotechnology Performance Certified (BPC) grade. The DNA solution was heated at 90 °C for 10 min, cooled down slowly for 3 h and left at 4 °C overnight. Compounds to be tested were dissolved in water and approximately 1 mM stock solutions were prepared. The exact concentrations were checked by UV–Vis. Stock solutions were then diluted with buffer to obtain 50 μM solutions of each compound. In a 96-well microplate, DNA solutions were mixed with solutions of tested compound and buffer to reach a total volume of 50 μL with a FIOT concentration of 0.2 μM and a compound concentration ranging between 1 and 10 μM.

The experimental protocol consisted of an incubation for 5 min at 24 °C, followed by a temperature ramp with heating rate 1 °C/min. Fluorescence values corresponding to the fluorophore FAM at wavelength of 516 nm (after excitation at 492 nm) were collected at each degree of temperature. Afterwards, the fluorescence data were normalized, plotted against temperature (°C) at each compound concentration, and Tm values were determined.

2.4.3. Viscometric titrations

Duplex DNA from CT (Deoxyribonucleic acid, Activated, Type XV) was purchased from Sigma Aldrich and used as provided. The buffer employed in this experiment was 10 mM phosphate buffer Na₂HPO₄/Na₂HPO₄, pH = 7.2. The viscosity measurements were performed in a Visco System AVS 470 at 25.00±0.01 °C, using a microUbbelohde (K = 0.01) capillary viscometer. 6 mL of DNA solution (0.4 mM in nucleotides) in phosphate buffer were equilibrated for 20 min at 25.00 °C and then 20 flow times were registered. Small aliquots (30–50 μL) of solutions of metal complexes (1.6–2.3 mM) were added to the same DNA solution. Before each flow time registration, the solutions were equilibrated for 20 min to 25.00 °C and then 20 flow times were measured. With the averaged time of the different flow time measurements and the viscometer constant, the viscosities (μ) for each point were calculated as: 10 mM sodium cacodylate 100 mM LiCl, (pH = 7.3). Finally, for mazan crystals were dissolved by adding 100 μL/well IPrOH (0.05 M HCl) and gently shaking. The optical density was measured at 550 nm using a 96-well multi-scanner auto-reader Enzyme-Linked Immuno Sorbent Assay (ELISA).
2.5. Data analysis

Results were subjected to computer-assisted statistical analysis using One-Way Analysis of Variance ANOVA, Bonferroni’s post-test, and Student’s t-test. Data are shown as the means of individual experiments and presented as the mean ± SD (Standard deviation). Differences of P < 0.05 were considered to be significantly different from the controls.

3. Results and discussion

3.1. Synthesis and characterization of metal compounds

Synthesis of the novel Ti(IV) compounds was carried out analogously to that of previously described enantiomers 1a and 1b [69]. Treatment of TDC and amino-oxime derivatives a’ or b’ in the presence of NET3 allows isolation of novel chloro- and ligand titanium compounds 1a’ or 1b’, respectively (Fig. 2), which are formed together with Et3N·HCl.

Analytical and spectroscopic data of the novel compounds 1a’ and 1b’ are identical to those reported before for 1a and 1b, respectively (see Ref. [69], Experimental Section and Online Resource, Figs. S3–S9).

Calculated data of specific optical rotation in chloroform solution for the ligands and novel metal derivatives ([x]D0 (deg dm−1·dL·g−1) = −127 ± 1.3 a’, +130 ± 1.3 a, −126 ± 1.3 b’, +127 ± 1.3 b, −88.9 ± 1.2 1a’, +89.2 ± 1.2 1a, −75.7 ± 1.2 1b’, +74.2 ± 1.2 1b) evidence the enantiomeric relationship of the stereoisomers. Furthermore, absolute configuration of the compound 1b has been confirmed through X-ray structure determination (Fig. 3, and Online Resource Tables S1 and S2 and Fig. S16).

The X-ray crystal structure determination of 1b shows the presence of two independent molecules in the asymmetric unit, with the same absolute configuration of the two chiral centres; an ORTEP diagram of one of them is presented in Fig. 3. The crystallographic study confirms a monohapto coordination of the oximate unit to the titanium atom. The compound shows a pseudotetrahedral environment around the metal centre, with Ti–O bond distances and O–N–C angles slightly shorter and closer (Online Resource Fig. S16), respectively, than those found in analogous bis(cyclopentadienyl)oximate(IV) derivatives [66,68] or alcoxo oximate titanium(IV) compounds [79–82] with a dihapto a2-NO coordination of the oximate unit to the titanium centre.

To the best of our knowledge, this is the first example found of an oximate titanium derivative with a terminal monohapto Ti–ON coordination, where this coordination mode is probably caused by the large steric requirements of the functionalized cyclohexane residue. This terminal coordination may account for the hydrolysis suffered for the compounds in aqueous media. In contrast, dihapto titanocene oximato compounds [(n3-C5H5)2Ti(κ2-O)=NR]+ (R = CMe2; C6H4), reported by Thewalt et al. [69], were described as surprisingly stable against air and water.

The reactions in water or PBS solutions of 1a or 1b were elucidated in a previous report and afford soluble ammonium-oxime pro-ligands (1S,4R)-[NH(R)-HCl]NO (R = Bn, a-HCl or 2-pic b-HCl, respectively), together with aqua-oxo or -hydroxo bis(cyclopentadienyl)titanium(IV) species [69,83,84] which are detected at least during the first 3 h after dilution. The same behaviour as that described before has now been observed for novel compounds 1a’ and 1b’ when their solutions in water-d2 or PBS were studied by 1H NMR spectroscopy (see Online Resource, Fig. S10).

We decided to further investigate the existence of an aminooxime ligand containing Ti(IV) species, which could account for the observed stereoisomer-dependent cytotoxic behaviour of the compounds on the prostate cancer PC-3 cell line. Since UV–Vis spectroscopy is considered a more sensitive technique than NMR, we recorded time-dependent UV–Vis spectra for compounds 1a’ and 1b’ in PBS solution. Right after dilution, UV–Vis spectrum of 1a’ (Online Resource Fig. S13) and 1b’ (Fig. 4) shows two very broad absorption bands centered at 240 and 325, and 246 and 322 nm, respectively, ascribed to overlapping of LMCT bands due to cyclopentadienyltitanium aquo cations and the absorption bands corresponding to proligands a’-HCl and b’-HCl. After 24 h, only the absorption bands assigned to a’-HCl or b’-HCl at 250 and 332, and 260 and 332 nm, respectively, are detected. Similar UV–Vis spectra are obtained after 72 h. Analogous results were obtained when the compounds are diluted in pure water.

CD spectra were also recorded for each pair of enantiomers. However, the spectra of derivatives 1a, 1b and 1a’, 1b’ are identical to those obtained for ammonium-oxime compounds a-HCl, b-HCl, a’-HCl, b’-HCl (see Online Resource Figs. S14, S15), 15 min after dilution or after 72 h, leading to the assumption that these are the only detectable optically active, soluble in water products of the hydrolysis of titanium oximato compounds.

Fig. 2. Synthesis of optically active titanocene oximate compounds.

Fig. 3. ORTEP drawing of compound 1b with 50% probability ellipsoids. Hydrogenbonded to carbon atoms have been omitted for clarity. Representative bond lengths (Å) and angles (deg): Ti(1)-Ct(1) 2.073; Ti(1)-Ct(2) 2.065; Ti(1)-O(1) 1.899(4); Ti(1)-N(1) 2.866(5); N(1)-O(1) 1.403(6); N(1)-C(21) 1.273(8); C(1)-Ti(1)-O(1) 127.4(2); Ti(1)-O(1)-N(1) 119.6(3); C(1)-N(1)-C(21) 114.3(3); Ct(1)-Ti(1)-Ct(2) 130.3; Ct(1) is the centroid of the C(11)-C(15) ring, Ct(2) is the centroid of the C(16)-C(20) ring.)
3.2. In vitro cell studies

3.2.1. Anti-proliferative studies

Chiral compounds 1a and 1b have already shown their promising anticancer properties on the human prostate and renal cancer cell lines PC-3 and Caki-1. Both titanocenes, especially 1b, are considerably less toxic to the non-tumorigenic human embryonic kidney cell line HEK-293T than to Caki-1 renal cells (7–15-fold less toxic) [69].

In order to compare and evaluate the versatility of the different enantiomers, the cytotoxic activity of pro-ligands HCl, a’HCl, b’HCl and metal compounds TDC, Tacke’s Titanocene-Y [72,85,86], 1a, 1a’ , 1b and 1b’ was assessed after 24 h of incubation time on a wide variety of human cancer cell lines, i.e. prostate PC-3 and DU-145, lung A-549, pancreas MIA PaCa-2, colorectal HT-116, leukemia Jurkat and cervical Hela. The in vitro effect of the compounds on cytotoxicity was firstly evaluated by monitoring their ability to inhibit cell growth using the MTT assay.

Under these conditions, pro-ligands a’HCl, a’HCl, b’HCl, b’HCl and metal compounds 1a and 1a’, TDC, and Titanocene-Y are poorly cytotoxic in all tested cell lines (IC50 > 150 μM under these experimental conditions). Enantiomers 1b and 1b’ are also not effective, after 24 h of exposure, in prostate PC-3, pancreatic MIA PaCa-2 or colon HT-116 human carcinoma cell lines, but show inhibitory activities of 40–50% and 20–25% at concentrations of 50 μM against human lung carcinoma A-549 (Online Resource Fig. S17) and leukemia Jurkat-T cell lines respectively. Cell morphology evaluation of A-549 cells indicated that titanium derivatives 1b and 1b’ did not induced apoptotic cell death, since no apoptotic cells, characterized by condensed nuclei and membrane blebbing, were detected. Cisplatin was included in the experiment as a positive control.

Since compounds 1a and 1b had shown to be efficiently cytotoxic on the PC-3 cell line after 72 h of incubation with the cells [69], we decided to assess the anti-proliferative effect of titanium compounds on prostate PC-3 and DU-145 cell lines as the IC50 value after 72 h of drug exposure. The results are summarized in Table 1.

Under these conditions, the enantiomer 1b shows IC50 values on the prostate PC-3 and DU-145 cell lines 2–5 times lower than Tacke’s compound, Titanocene-Y. The cytotoxicity on PC-3 cells of the titanium enantiomers 1a and 1b, with the absolute configuration S,R-, is higher than that of the R,S-stereoisomers by a factor of ca. 2–3, while the racemic mixture of 1b and 1b’ aﬀord IC50 values average between the two enantiomers. In contrast, no enantiomer recognition is observed on the prostate DU-145 cells for derivatives 1b, 1b’ while 1a, 1a’ resulted not to be efficient in this non-hormone dependent cancer cell line.

Titanocene-Y has already shown an encouraging activity in PC-3 tumour-bearing mice [85]. Other titanium compounds which have proved their in vitro antitumor activity in prostate cancer cell lines under similar time exposure conditions are Schiff-base titanium (IV) derivatives [87] (IC50 values within the range 5–18 μM, in PC-3) or heterometallic titanocene-gold compounds (IC50 values ranged from 27 to 40 μM in PC-3E) [88,89], and 11.8–27.6 μM in DU-145 [24,90].

In order to analyse the cytotoxic selectiveness to healthy cells, the isomers 1a, 1b, and 1b’ were also tested in the non-tumorigenic human prostate (RWPE-1) cells. Regarding selectivity, 1a and 1b are less toxic to the non-tumorigenic RWPE-1 than to the cancer PC-3 cells (from 2 to 4 times less toxic), while 1b showed a similar behaviour relative to the cancer DU-145 cells.

Titanium compound 1b was selected for a further study in vitro. We evaluated a combination of TDC and pro-ligand HCl on the cellular viability after 72 h of exposure to the drug. As already described in the PC-3 cell line [69], the additive dose of both starting materials also produced lower anti-proliferative effects than those observed after treatment with only 1b (Table 2) in the prostate DU-145 and RWPE-1 cell lines. These results are consistent with the involvement of metal oxime containing species in the cytotoxicity mechanism. While water soluble hydrolysis species detected in our studies are the same as those formed from a mixture of TDC and amino-oxime proligand, the existence of polyclinical ligand influenced species formed in a colloidal phase of hydrolysis cannot be ruled out.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>PC-3 IC50 (M)</th>
<th>DU-145 IC50 (M)</th>
<th>RWPE-1 IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>&gt;150 (24 h)</td>
<td>&gt;150 (72 h)</td>
<td>&gt;200 (72 h)</td>
</tr>
<tr>
<td>1a’</td>
<td>&gt;150 (24 h)</td>
<td>&gt;150 (72 h)</td>
<td>n.m.</td>
</tr>
<tr>
<td>1b</td>
<td>&gt;150 (24 h)</td>
<td>27.1 ± 1.1 (72 h)</td>
<td>30.8 ± 0.57 (72 h)</td>
</tr>
<tr>
<td>1b’</td>
<td>&gt;150 (24 h)</td>
<td>23.9 ± 8.6 (72 h)</td>
<td>43.8 ± 7.2 (72 h)</td>
</tr>
<tr>
<td>1b + 1b’</td>
<td>37.5 ± 5.1 (72 h)</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>Titanocene-Y</td>
<td>&gt;200 (24 h)</td>
<td>&gt;150 (72 h)</td>
<td>42.9 ± 0.73 (72 h)</td>
</tr>
<tr>
<td>cisplatin</td>
<td>104.2 ± 8.1 (24 h)</td>
<td>3.7 ± 0.6 (72 h)</td>
<td>19.9 ± 1.1 (72 h)</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± S.D. (n = 3).

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>PC-3 IC50 (M)</th>
<th>DU-145 IC50 (M)</th>
<th>RWPE-1 IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>14.5 ± 3.1</td>
<td>27.1 ± 1.1</td>
<td>30.8 ± 0.57</td>
</tr>
<tr>
<td>bHCl</td>
<td>&gt;100</td>
<td>106.1 ± 10.4</td>
<td>140.5 ± 23.0</td>
</tr>
<tr>
<td>TDC + bHCl</td>
<td>39.5 ± 2.1</td>
<td>54.9 ± 13.5</td>
<td>&gt;150</td>
</tr>
<tr>
<td>TDC</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± S.D. (n = 3).
titanium-based therapeutics. DNA binding is still thought to be one important potential mode of action for titanocene compounds, although interactions with DNA have been found to be generally very weak at physiological pH conditions [16,17]. The study of DNA interactions for these particular metal complexes does often represent an experimental challenge, since the compounds can easily hydrolyse in water solutions. That said, investigation in this area may be used to shed some light about the nature of the interactions that may partially account for the biological activity observed in physiologically relevant aqueous environments, albeit the results obtained should be interpreted cautiously. Our previous results showed that titanocenes 1a or 1b did not exhibit strong interactions with plasmid DNA by electrophoretic mobility shift assays, but the absence of a shift in the electrophoretic bands did not allow us to rule out DNA binding. Having established the interesting antituumor properties of metal compounds 1a, 1a’, 1b and 1b’, our aim with the study presented now was to further analyse and compare the kind of potential interactions of the enantiomers with DNA, by using other techniques to complement previous studies.

Dialysis experiments, based on the fundamental thermodynamic principle of equilibrium dialysis [78,91], were performed to determine apparent binding constants between DNA and the metal compounds, following the protocol described by Chaires [78] with some modifications. As the DNA targets, we selected CT DNA and a short oligonucleotide duplex of known sequence (ds17, 17 bp).

Unfortunately, under the conditions employed, large dispersion data sets were obtained, which prevented the precise determination of association constants between the titanium(IV) compounds and DNA. This is likely to be a consequence of the hydrolysis of these complexes in aqueous media. However, even if the results should be interpreted with caution, a significant increase in compound concentration was invariably observed in the dialysis bags of replicate experiments, suggesting effective DNA binding by metal complexes 1a, 1a’, 1b, 1b’ and/or their hydrolysis products.

With the purpose of determining the effect that these compounds may exert on the DNA denaturing temperature, Tm, we used a variable-temperature (FRET-melting) assay. This experiment requires little DNA consumption, allows the assessment of a wide range of compound concentrations, can be adapted to a high-throughput fashion, and it has been extensively used to determine the degree of thermal stabilization of different DNA structures in the presence of potential ligands [92]. Thus, FRET experiments were used to establish whether metal complexes 1a, 1a’, 1b and 1b’ were able to thermally stabilize duplex DNA structures.

In these experiments, a 10-bp oligonucleotide (F10T) labelled with two fluorophores, FAM at its 5’ end and TAMRA at the 3’ end, was selected [93]. If the metal complex binds to DNA affecting the stability of the helix, changes in the value of DNA Tm should be expected. Stabilization of duplex DNA usually results in increased values of Tm.

Compounds 1a, 1a’, 1b and 1b’ were analysed for their ability to affect duplex DNA melting within the 1–10 µM concentration range. However, under these conditions, the titanium(IV) derivatives were not able to produce a significant change in the DNA melting temperature. Furthermore, none of the enantiomers of the precursor ligand, a-HCl, a’-HCl, showed DNA stabilization (see Online Resource Fig. S18). These results suggest that the compounds may interact with DNA in an external, mainly electrostatic fashion or through partial recognition of the DNA grooves.

Finally, DNA viscometric titrations were carried out because viscosity measurements can provide a simple way to discriminate between the different binding modes of potential DNA ligands (such as intercalation versus groove or external binding) [94]. According to the theory of Cohen and Eisenberg [95], from gradual titration of DNA solutions with the compounds of interest, linear plots of the cubed root of the relative DNA viscosity (\(\sqrt[3]{\eta/\eta_0}\)) versus the molar ratio of bound ligand to DNA nucleotide (r) can be obtained. The slope values in these plots correlate well with the DNA-ligand binding modes. Groove binding compounds normally display a slope close to 0.0, whereas classical mono-intercalants result in a slope close to 1.0 [94,95].

The tested compounds showed a linear (\(\sqrt[3]{\eta/\eta_0}\)) versus r correlation in the typical r range used in these experiments (Fig. 5). Complexes 1a, 1a’, 1b and 1b’, irrespective of the amino-bound ligand and the stereochemistry of the metal complex, gave rise to slope values practically equal to zero.

These results are in good agreement with the FRET DNA melting assays and point towards an external or groove interaction of the titanium metal complexes and/or their hydrolysis products that does not result in overall changes of contour length or thermal stabilization of the DNA double helix structure.

4. Conclusions

Optically active amino-oxime ligands derived from natural products are useful and inexpensive starting materials for the design of enantiopure titanocene compounds. In contrast with the resistance to hydrolysis of other \(\alpha^2\text{N,O} \) oximato-Ti biscalopenta-diene compounds described before, our systems suffer hydrolysis in water at physiological conditions, most likely due to the monohapto \(\alpha\)ON coordination mode of the highly sterically demanding limonene residue of the oximate ligand. Regarding their cytotoxic behaviour, the oxime-containing Ti(IV) compound 1b has shown potent anticancer activities against both prostate cancer PC-3 and DU-145 cell lines after 72 h of incubation time. The cytotoxicity of the enantiomers 1a, 1a’ and 1b, 1b’ towards all the cancer cell lines tested showed no significant differences, exception made for the PC-3 cells. In addition, isomers 1a and 1b showed certain selectivity in their toxicity against prostate cancer PC-3 versus non-tumorigenic RWPE-1 cells. Furthermore, compound 1b shows higher activity than the additive dose of TDC and proligand b-HCl on the prostate PC-3, DU-145 and RWPE-1 cell lines. These results point towards the existence of an influence of the oximato-Ti unit on the hydrolysis process and/or the cytotoxicity mechanism.

Fig. 5. Viscometric titrations of CT DNA and metal complexes 1a, 1a’, 1b and 1b’ at 25 °C (10 mM sodium phosphate buffer, pH 7.2).
Compound-DNA interactions have been investigated by equilibrium dialysis, FRET melting assays and viscometric titrations. The experimental results suggest that these metal complexes and/or their hydrolysis products can bind DNA either in the minor groove or externally, irrespective of the ligand stereochemistry.

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