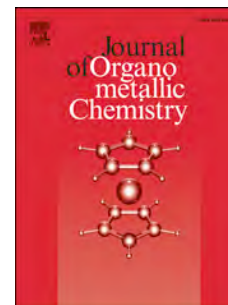


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1 Study of the Anticancer Properties of Optically 2 Active Titanocene Oximato Compounds

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5

6 *Dedicated to the memory of Prof. Dr. Pascual Royo, who loved aquo titanium chemistry*

7

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13 **Keywords**

14 titanium, chiral, enantiomer, DNA, cytotoxic

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17 ABSTRACT

1 New water soluble and optically active cyclopentadienyl titanium derivatives $[(\eta^5-$
2 $C_5H_5)_2Ti\{(1R,4S)-\kappa ON,(R)NH\}Cl]$ (R = Bn (Benzyl) **1a'**, 2-pic (2-picolylamine) **1b'**)
3 have been synthesized. The novel compounds along with those previously described
4 $[(\eta^5-C_5H_5)_2Ti\{(1S,4R)-\kappa ON,(R)NH\}Cl]$ (R = Bn **1a**, 2-pic **1b**) were evaluated by
5 polarimetry, ultra-violet and circular dichroism spectroscopy. The structure of **1b** was
6 determined by single crystal X-ray crystallography and showed a unique terminal
7 monohapto Ti-O disposition of the oximato ligand. All enantiomers have been tested
8 against several cancer cell lines *in vitro*: prostate PC-3 and DU-145, lung A-549,
9 pancreas MiaPaca-2, colorectal HCT-116, leukemia Jurkat and cervical HeLa. In
10 addition, **1a**, **1b** and **1b'** were tested against non-tumorigenic prostate RWPE-1 cell line.
11 After 24 h of incubation, **1b** and **1b'** were moderately active against Jurkat and A-549
12 cells. The anti-proliferative effect of titanium compounds on prostate PC-3, DU-145 and
13 RWPE-1 cell lines was also assessed after 72 h of drug exposure. The cytotoxic profile
14 of the enantiomers was similar, exception made for the PC-3 cells, with *S,R*-isomers
15 exhibiting cytotoxicities 2 to 3 times higher than *R,S*-compounds. Under these
16 conditions, derivative **1b** showed calculated IC_{50} values better than those of Tacke's
17 Titanocene-Y (bis-[(*p*-methoxybenzyl)cyclopentadienyl]titanium(IV) dichloride) on
18 both the prostate PC-3 and DU-145 cells. **1a** and **1b** cytotoxic behaviour shows certain
19 selectiveness, with activities 2-4 times lower on normal prostate RWPE-1 than on
20 cancer PC-3 cells. Furthermore, **1b** produces higher cytotoxicity on prostate PC-3, DU-
21 145 and RWPE-1 cells than the additive dose of titanocene dichloride and pro-ligand
22 **b·HCl**. Additionally, compound-DNA interactions have been investigated by
23 equilibrium dialysis, Fluorescence Resonance Energy Transfer (FRET) melting assays
24 and viscometric titrations, which suggest that these metal complexes and/or their
25 hydrolysis products bind DNA either in the minor groove or externally.

1 1. Introduction

2 Since the successful introduction of cisplatin (*cis*-[PtCl₂(NH₃)₂]) as an anticancer
3 drug, much effort has been devoted to investigation of the anticancer activity of other
4 coordination and/or organometallic transition metal compounds [1-7]. The titanium
5 derivatives titanocene dichloride ([η^5 -C₅H₅)₂TiCl₂], TDC) [8,9] and budotitane (*cis*-
6 diethoxybis(1-phenylbutane-1,3-dionato)titanium(IV)) [10,11] were the first metal
7 compounds to enter clinical trials after platinum complexes. Although these derivatives
8 showed promising properties in preliminary studies, they failed advanced clinical trials
9 due to low antitumor efficacy *in vivo*, rapid hydrolysis and limited solubility in
10 biological media [12-19]. Since then, a plethora of modified titanium based compounds
11 have been synthesized and studied as potential antitumor agents [17-29].

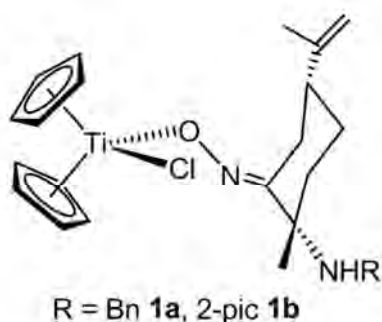
12 The effect of stereochemistry on biological activity is of great importance in medicinal
13 chemistry, as many of the biological targets are chiral [30,31]. The anticancer properties
14 of chiral metal derivatives have been largely studied [32-46], but the role of the
15 stereochemistry in the biological activity of non-platinum based compounds has been
16 less investigated [22,47-61]. Effect of the absolute configuration on the anticancer
17 efficiency of titanium compounds was firstly explored by Tshuva in 2010 [50]. The
18 enantiomers of C₂-symmetrical Ti(IV) compounds with chiral diamine bis(phenolato)
19 ligands showed different antitumor activities by factors of 2-4 on human colorectal
20 (HT-29) and ovarian (OVCAR-1) carcinoma cells [50,51,56,60]. According to these
21 results, the authors proposed that stereochemistry should be considered in the design,
22 modification, and improvement of active compounds [60]. The same year, Baird
23 published a family of enantiomerically pure titanocene derivatives bearing chiral
24 alkylammonium groups, but a relationship between the anticancer activity and chirality
25 could not be established due to the low cytotoxicity showed on the cancer cell lines

1 evaluated [62]. Enantiomer-dependent activity was found in chiral substituted
2 titanocene compounds by Cini et al [22,58], with the (*S,S*) enantiomer of $\text{Cp}^{\text{R}}_2\text{TiCl}_2$
3 ($\text{Cp}^{\text{R}} = \eta^5\text{-C}_5\text{H}_4\text{CH}(\text{CH}_2\text{CH}_3)\text{C}_6\text{H}_5\text{OMe}$) being twice as active as the (*R,R*) isomer
4 towards pancreatic, breast and colon cancer cell lines, after 24 h of treatment.
5 Interestingly, lack of enantiomer recognition was observed at 72 h when screening the
6 compounds in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
7 assays.

8 Within this context, enantiomerically pure, naturally occurring terpenes are useful
9 building blocks for asymmetric synthesis [63,64]. They are inexpensive and
10 commercially available reagents in optically pure form, and easily tailored by
11 stereoselective functionalization [65]. On the other hand, oxime groups are presented as
12 excellent chemical modifiers, with a wide versatility of coordination modes going from
13 mono κNO , κON , to dihapto $\kappa^2\text{N,O}$; either with *side on* or bridging coordination, which
14 could offer an increased stability of the final compounds when bonded to Ti(IV) acid
15 centres [66-68].

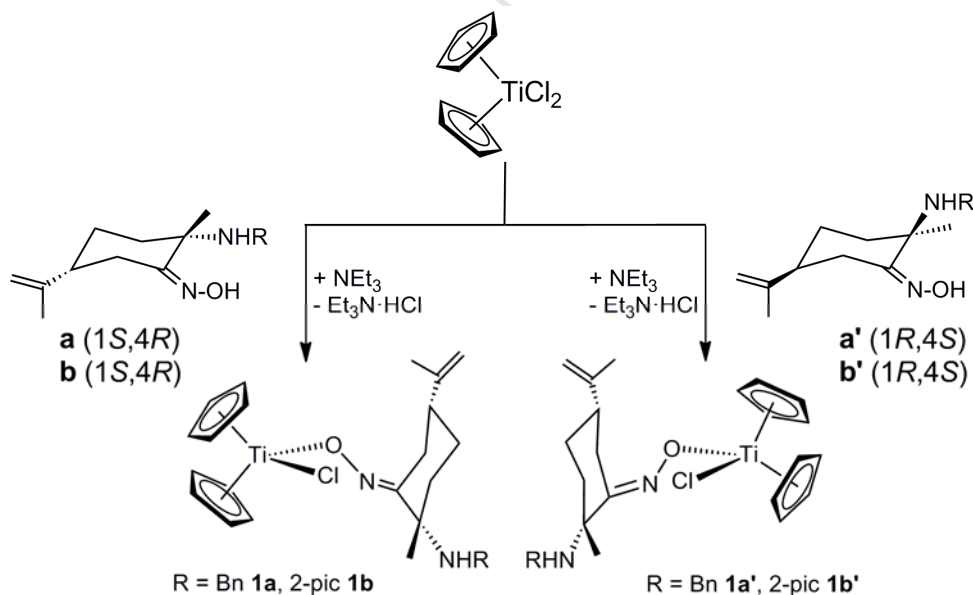
16 We have recently reported a new family of enantiopure cyclopentadienyl titanium(IV)
17 compounds with amino-oximato ligands derived from *R*-limonene, of formula $[(\eta^5\text{-}$
18 $\text{C}_5\text{H}_5)_2\text{Ti}\{(1*S*,4*R*)-\kappa\text{ON},(\text{R})\text{NH}\}\text{Cl}]$ (R = Bn **1a**, 2-pic **1b**) (Fig. 1), with relevant
19 antitumor properties. Our compounds show significant effects on cytotoxicity, cell
20 adhesion to collagen and migration of androgen-independent prostate cancer cells while
21 they do not seem to exhibit strong interactions with plasmid DNA by electrophoretic
22 mobility shift assays. Compounds **1a** or **1b** suffered hydrolysis in water or phosphate
23 buffered saline (PBS) solutions. However, the additive doses of TDC and **a**•HCl or
24 **b**•HCl produced lower antiproliferative effects on prostate cancer PC3 cells than those
25 observed after treatment with oximato titanocenes **1a** or **1b**, respectively. This fact led

1 us to the conclusion that the active operating titanium species was positively influenced
 2 by the presence of the oximato ligand [69].



3
 4 **Fig. 1** Optically active titanocene compounds containing ligands derived from *R*-
 5 limonene

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



9
 10
 11 **Fig. 2** Synthesis of optically active titanocene oximato compounds

12 We report here on the synthesis and characterization of corresponding
 13 cyclopentadienyl Ti(IV) enantiomers $[(\eta^5\text{-C}_5\text{H}_5)_2\text{Ti}\{(1*R*,4*S*)\text{-}\kappa\text{ON},(\text{R})\text{NH}\}\text{Cl}]$ (R = Bn

1 **1a'**, 2-pic **1b'**). Their hydrolytic behaviour has been studied by ¹H NMR, Ultraviolet-
2 visible (UV-Vis) spectroscopy and circular dichroism (DC). These novel compounds
3 along with those previously described have been evaluated against several cancer cell
4 lines *in vitro*: prostate PC-3 and DU-145, lung A-549, pancreas MIA PaCa-2, colorectal
5 HCT-116, leukemia Jurkat and cervical HeLa. In addition, the compounds were tested
6 against the non-tumorigenic human prostate RWPE-1 cell line. DNA interactions of the
7 metal derivatives and/or their hydrolysis products have been further investigated by
8 FRET melting assays, equilibrium dialysis and viscometric titrations experiments.

10 2. Experimental Section

11 2.1. Chemicals and synthesis

12 Manipulations involving the synthesis of titanium compounds **1a**, **1a'**, **1b** and **1b'** and
13 Titanocene-Y (bis-[(*p*-methoxybenzyl)cyclopentadienyl]titanium(IV) dichloride) were
14 performed at an argon/vacuum manifold using standard Schlenk techniques or in a
15 MBraun MOD System glove-box. Solvents were dried by known procedures and used
16 freshly distilled. Titanocene-Y [72], (1*S*,4*R*)-{NH(R),NOH}, (R = Bn **a** [70], 2-pic **b**);
17 (1*R*,4*S*)-{NH(R),NOH} (R = Bn **a'**; 2-pic **b'**); corresponding adducts (1*S*,4*R*)-
18 {NH(R)·HCl,NOH}, (R = Bn **a**·HCl, 2-pic **b**·HCl); (1*R*,4*S*)-{NH(R)·HCl,NOH} (R =
19 Bn **a'**·HCl, 2-pic **b'**·HCl) [63,73] and metal compounds [(η⁵-C₅H₅)₂Ti{(1*S*,4*R*)-
20 κON,(R)NH}Cl] (R = Bn **1a**, 2-pic **1b**) [69] were prepared according to previous
21 reports. *R*- or *S*-limonene and isopentyl nitrite were reacted following the standard
22 method described by Carman et al in 1977 [73]. *R*-limonene, *S*-limonene, TDC and
23 cisplatin were purchased from Sigma-Aldrich. Commercially available reagents were
24 used without further purification. Nuclear Magnetic Resonance (NMR) spectra were
25 recorded on a Bruker 400 Ultrashield. ¹H and ¹³C chemical shifts are reported relative to

1 tetramethylsilane. ^{15}N chemical shifts are reported relative to liquid ammonia (25 °C).
2 Coupling constants J are given in Hertz. Elemental analysis was performed on a LECO
3 CHNS 932 Analyzer at the Universidad de Alcalá or, alternatively, at the Universidad
4 Autónoma de Madrid. Fourier Transform Infrared (FT IR) spectra were recorded on IR
5 FT Perkin Elmer (Spectrum 2000) spectrophotometer on KBr pellets. The pH was
6 measured in a HANNA HI208 pHmeter in distilled water solutions. Circular Dichroism
7 (CD) spectra were recorded on a J-715 CD spectropolarimeter (Jasco, UK) at ambient
8 temperature (297 K). The spectra were determined at a concentration of 0.5 mM in
9 water using a quartz cuvette of 0.5 cm path length, scan speed of 20 nm·min⁻¹, 0.1 nm
10 band width, 0.5 nm data pitch and 0.5 s of response time. Optical rotations of all the
11 compounds solutions were recorded on a Perkin Elmer 341 polarimeter, using the
12 sodium D line (589 nm) at ambient temperature (297 K) in a quartz cell of 1 dm path
13 length. Specific optical rotation values were calculated according to the equation $[\alpha]_{\text{D}}^{24}$
14 = $100 \cdot \alpha_{\text{obs}} / l \cdot c$ [74]. Analytical balance and volumetric pipettes (2.0 mL) were used to
15 prepare CHCl_3 solutions of the compounds at concentrations within a range of 7.50-7.80
16 g·dL⁻¹. UV-Vis spectra were measured at room temperature on water solutions of the
17 compounds with a Perkin Elmer Lambda 35 spectrophotometer.

18 2.1.1. (*1R,4S*)-{NH(2-pic),NOH} (**b'**). An analogous procedure to that described
19 before for the synthesis of **b** [63] was used, starting from *S*-limonene [70,71,73]. $[\alpha]_{\text{D}}^{23}$
20 (deg·dm⁻¹·cm³·g⁻¹) -126 ± 1.3 (**b'** at $c = 0.7839$ g·dL⁻¹, $\alpha_{\text{obs}} = -0.957$ deg); +127 ± 1.3 (**b**
21 at $c = 0.7604$ g·dL⁻¹, $\alpha_{\text{obs}} = +0.954$ deg). All analytical and spectroscopic data are
22 identical to those observed for **b**. Anal. Calcd. for C₁₆H₂₃N₃O: C, 70.30; H, 8.48; N,
23 15.37; Found: C, 70.13; H, 8.07; N, 15.20. FT IR (KBr, $\lambda_{\text{max}}/\text{cm}^{-1}$): 3086-3314 (br,
24 $\nu_{\text{OH/NH}}$), 1650, 1598 ($\nu_{\text{C=N}}$). UV-Vis (0.1 mM in H₂O:DMSO 99:1): λ_{max} (ϵ): 261
25 (316), 340 (10). ^1H NMR (plus two dimensional correlation spectroscopy (COSY),

1 400.1 MHz, 293 K, chloroform-*d*₁): δ 9.80 (=NOH), 8.49, 7.60, 7.28, 7.11 (m, each 1H,
 2 NC₅H₄), 4.75 (br, 2H, =CH₂), 3.87, 3.61 (both d, each 1H, ³J_{HH} = 6, -CH₂-C₅H₄N), 3.28
 3 (d, 1H, ²J_{HH} = 12, -CH₂³), 2.60 (br, 1H, NH), 2.09 (m, 1H, -CH⁴), 2.03 (dd, 1H, ²J_{HH} =
 4 12, ³J_{HH} = 3, -CH₂³), 2.00, 1.69 (m, each 1H, -CH₂⁶ + -CH₂⁵), 1.85 (m, 1H, CH₂⁶), 1.75
 5 (s, 3H, CH₃-C=), 1.65 (m, 1H, CH₂⁵), 1.32 (s, 3H, -CH₃-Cq-N). ¹³C NMR (plus
 6 Attached Proton Test (APT), plus gradient Heteronuclear Single Quantum Coherence
 7 (gHSQC), plus Heteronuclear Multiple Bond Correlation (HMBC), 100.6 MHz, 293 K,
 8 chloroform-*d*₁): δ 162.4 (Cq=NOH, Cq is quaternary carbon), 161.1 (C_{ipso}-C₅H₄N),
 9 148.9 (C=CH₂), 149.2, 136.8, 122.7, 122.1 (C₅H₄N), 109.6 (=CH₂), 56.9 (Cq-NH), 48.1
 10 (CH₂-C₅H₄N), 45.0 (CH⁴), 40.5 (-CH₂⁶), 26.4 (-CH₂⁵), 25.6 (-CH₂³), 23.5 (-CH₃-CNH),
 11 21.0 (CH₃-C=). ¹⁵N NMR (gHMBC, 40.5 MHz, 293 K, chloroform-*d*₁): δ 346.7 (C=N-),
 12 305.3 (C₅H₄N), 51.8 (-NHpic).

13 2.1.2. [(η^5 -C₅H₅)₂Ti{(1*R*,4*S*)- κ ON,(Bn)NH}Cl] (**1a'**). An analogous procedure to that
 14 described for [(η^5 -C₅H₅)₂Ti{(1*S*,4*R*)- κ ON,(Bn)NH}Cl] [69] was followed, starting from
 15 TDC (0.20 g, 0.80 mmol), (1*R*,4*S*)-{NH(Bn),NOH} (0.22 g, 0.80 mmol) and NEt₃ (0.11
 16 mL, 0.80 mmol). Compound **1a'** was obtained as a yellow-orange solid. Yield: 0.32 g
 17 (88%). [α]_D²³ (deg·dm⁻¹·cm³·g⁻¹) -88.9 \pm 1.2 (**1a'** at c = 0.7602 g·dL⁻¹, α_{obs} = -0.676
 18 deg), +89.2 \pm 1.2 (**1a** at c = 0.7497 g·dL⁻¹, α_{obs} = +0.681 deg). Analytical and
 19 spectroscopic data of the compound are identical to those already reported [69].
 20 Solubility in H₂O at 24 °C (mM): 6.6 \pm 0.2. Value of pH ([2.0 mM]) in H₂O at 24 °C:
 21 5.54. Anal. Calcd for C₂₇H₃₃ClN₂OTi: C, 66.88; H, 6.86; N, 5.78; Found: C, 66.80; H,
 22 6.90; N, 5.76. FT IR (KBr, λ_{max} /cm⁻¹): 3370 (m, NH), 1646, 1601 (both m, C=N). ¹H
 23 NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, chloroform-*d*₁): δ
 24 7.32 (m, 5H, -C₆H₅), 6.39, 6.39 (both s, each 5H, C₅H₅), 4.76, 4.74 (both s, each 1H,

1 =CH₂), 3.76, 3.55 (both m, each 1H, -CH₂Ph), 2.92 (m, 1H, -CH₂³), 2.05 (m, 1H, -CH-
 2 C=), 1.90 (m, 1H, -CH₂⁶), 1.72 (m, 1H, -CH₂³), 1.68 (m, 1H, -CH₂⁵), 1.59 (m, 1H, -
 3 CH₂⁶), 1.56 (m, 1H, -CH₂⁵), 1.25 (br, 1H, NH), 1.47, 1.25 (both s, each 3H, NC-CH₃ +
 4 CH₃C=). ¹³C NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K,
 5 chloroform-*d*₁): δ 159.2 (Cq=N), 149.3 (=Cq-Me), 141.6 (C_{ipso}Ph), 128.7, 128.7, 127.2
 6 (C₆H₅), 117.1, 117.1 (C₅H₅), 109.4 (=CH₂), 57.1 (Cq-NH), 47.3 (-CH₂Ph), 45.6 (-CH⁴),
 7 41.2 (-CH₂⁶), 27.8 (-CH₂³), 26.2 (-CH₂⁵), 23.9, 21.3 (CH₃-CNH + CH₃-C=). ¹⁵N NMR
 8 (gHMBC, 40.5 MHz, 293 K, chloroform-*d*₁): δ 398.9 (C=N), 60.0 (NHBn).

9 2.1.3. [(η⁵-C₅H₅)₂Ti{(1*R*,4*S*)-κON,(2-pic)NH}Cl] (**1b'**). An analogous procedure to
 10 that described for [(η⁵-C₅H₅)₂Ti{(1*S*,4*R*)-κON,(2-pic)NH}Cl] [69] was followed,
 11 starting from TDC (0.30 g, 1.20 mmol), (1*R*,4*S*)-{NH(2-pic),NOH} (0.33 g, 1.20 mmol)
 12 and NEt₃ (0.11 mL, 1.20 mmol). Compound **1b'** was obtained as a yellow-orange solid.
 13 Yield: 0.35 g (60%). [α]_D²³ (deg·dm⁻¹·cm³·g⁻¹) -75.7 ± 1.2 (**1b'** at c = 0.7534 g·dL⁻¹,
 14 α_{obs} = -0.570 deg), +74.2 ± 1.2 (**1b** at c = 0.7772 g·dL⁻¹, α_{obs} = +0.570 deg). Solubility
 15 in H₂O at 24 °C (mM): 15.7 ± 1.7. Value of pH ([2.0 mM]) in H₂O at 24 °C: 5.22. Anal.
 16 Calcd for C₂₆H₃₂ClN₃OTi: C, 64.27; H, 6.64; N, 8.65; Found: C, 64.62; H, 7.25; N,
 17 8.54. FT IR (KBr, λ_{max}/cm⁻¹): ν̄ 3304 (m, NH), 1640, 1591, 1569 (all s, C=N). ¹H
 18 NMR (plus HSQC, plus HMBC, plus COSY, 400.1 MHz, 293 K, chloroform-*d*₁): δ
 19 8.50, 7.60, 7.30, 7.12 (all m, each 1H, -NC₅H₄), 6.38, 6.38 (both s, each 5H, C₅H₅),
 20 4.77, 4.74 (both s, each 1H, =CH₂), 3.91, 3.70 (both m, each 1H, CH₂-C₅H₄N), 2.84 (m,
 21 1H, -CH₂³), 2.07 (m, 1H, -CH-C=), 1.98 (m, 2H, overlapped -CH₂⁶⁺³), 1.78 (s, 3H,
 22 CH₃C=), 1.64 (m, 1H, -CH₂⁶), 1.62 (m, 1H, -CH₂⁵), 1.60 (m, 1H, -CH₂⁵), 1.48 (br, 4H,
 23 NC-CH₃ + NH). ¹³C NMR (plus APT, plus gHSQC, plus HMBC, 100.6 MHz, 293 K,
 24 chloroform-*d*₁): δ 157.6 (Cq=N), 148.1 (=Cq-Me), 160.2 (C_{ipso}C₅H₄N), 149.3, 136.7,

1 122.9, 122.9 (C₅H₄N), 117.1, 117.1 (C₅H₅), 109.6 (=CH₂), 48.5 (-CH₂-C₅H₄N), 45.3 (-
2 CH⁴), 41.1 (-CH₂⁶), 27.7 (-CH₂³), 26.2 (-CH₂⁵), 23.9, 21.3 (CH₃-CNH + CH₃-C=). ¹⁵N
3 NMR (gHMBC, 40.5 MHz, 293 K, chloroform-*d*₁): δ 402.1 (C=N), 312.5 (C₅H₄N), 52.6
4 (NHpic).

5 2.1.5. ¹H NMR experiments at physiological pH. Phosphate buffered saline solution
6 (PBS) was prepared according to Cold Spring Harbor Protocols
7 (<http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8247>) using NaCl, KCl, Na₂HPO₄
8 and K₂HPO₄ in D₂O. Adjustment of pD (pD = pH* + 0.4, where pH* = pHmeter
9 reading in D₂O) was carried out using a solution of DCl (0.01M) or NaOD (0.01M) in
10 D₂O, with the help of a HANNA HI208 pHmeter. Titanium compounds were then
11 dissolved in 2000 μL of the freshly prepared PBS, final pD measured (7.30-7.38) and
12 time-dependent ¹H NMR spectra of 500 μL aliquots of final solutions were carried out
13 at 25 °C.

14 2.2. Single-crystal X-ray structure determination

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

23 The structure was solved, using WINGX package [75], by intrinsic phasing methods
24 (SHELXT) [76], and refined by least-squares against F² (SHELXL-2014/7) [77].

1 Crystals of **1b** were refined as a two-component inversion twin, and also had two
2 independent molecules in the asymmetric unit with no significant differences. All non-
3 hydrogen atoms were anisotropically refined. Positions of the amine hydrogen atoms,
4 H(2) and H(21), were located in the difference Fourier map. H(2) was refined
5 isotropically, while U_{iso} for H(21) was fixed with a value of 0.05. The rest of the
6 hydrogen atoms were positioned and refined by using a riding model. *Crystal data for*
7 **1b**: ($\text{C}_{26}\text{H}_{32}\text{ClN}_3\text{OTi}$), FW = 485.89, Monoclinic, space group $P2_1$, crystal dimensions
8 (mm^3) 0.30 x 0.27 x 0.27, $a = 10.470(1)$, $b = 11.631(1)$, $\beta = 91.53(1)$, $c = 19.856(3)$ Å,
9 $V = 2417.2(5)$ Å³, $Z = 4$, $\rho_{\text{calcd}} = 1.335$ g cm⁻³, $\mu = 0.488$ mm⁻¹, $F(000) = 1024$, θ range
10 = 3.08 to 27.50 deg, no. of rflns collected = 42638, no. of indep rflns / $R_{\text{int}} = 10939 /$
11 0.074, no. of data / restraints / params = 10939 / 1 / 589, $R1 / wR2 (I > 2\sigma(I)) = 0.068 /$
12 0.141, $R1 / wR2$ (all data) = 0.089 / 0.151, GOF (on F^2) = 1.167, Absolute structure
13 parameter = 0.04(5). Final difference Fourier maps did not show peaks higher than
14 0.695 nor deeper than -0.329 eÅ⁻³. CCDC-1572920 contains the supplementary
15 crystallographic data for this paper. These data can be obtained free of charge from The
16 Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

17 2.3. Cell culture, cytotoxicity assays and cell death analysis

18 2.3.1. Cell culture

19 The prostate androgen-unresponsive cancer cell line PC-3 was obtained from the
20 American Type Culture Collection (Manassas, VA) and may be related to recurrent
21 prostate cancers that have achieved androgen independence. All culture media were
22 supplemented with 1% penicillin/streptomycin/amphoterycin B (Life Technologies,
23 Barcelona, Spain). The culture was performed in a humidified 5% CO₂ environment at
24 37 °C. After the cells reached 70–80% confluence, they were washed with PBS,
25 detached with 0.25% trypsin/0.2% ethylenediaminetetraacetic acid (EDTA) and seeded

1 at 30,000–40,000 cells·cm⁻². The culture medium was changed every 3 days. A549
2 (lung carcinoma) cells were maintained in high glucose DMEM (Dulbecco's Modified
3 Eagle's Medium) and RWPE-1 (non-tumorigenic prostate) cells in DMEM/F12
4 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12), supplemented with 5%
5 fetal bovine serum (FBS), 200 U·mL⁻¹ penicillin, 100 µg·mL⁻¹ streptomycin and 2 mM
6 L-glutamine. DU-145 (prostate carcinoma), MIA PaCa-2 (pancreas carcinoma), HCT-
7 116 (colorectal carcinoma), HeLa (cervical cancer) and Jurkat (leukemic cancer) cells
8 were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium
9 supplemented with 5% FBS, 200 U·mL⁻¹ penicillin, 100 µg·mL⁻¹ streptomycin and 2
10 mM L-glutamine. Cultures were maintained in a humidified atmosphere of 95% air:5%
11 CO₂ at 37 °C. Adherent cells were allowed to attach for 24 h prior to addition of
12 compounds.

13 2.3.2. MTT Toxicity Assays

14 For toxicity assays, cells (5×10^4 for Jurkat cells and 10^4 for adherent cell lines) were
15 seeded in flat-bottom 96-well plates (100 µL/well) in complete medium. Adherent cells
16 were allowed to attach for 24 h prior to addition of cisplatin or tested compounds. Stock
17 solutions of Titanocene-Y, TDC and ammonium-oxime pro-ligands were freshly
18 prepared in 1% of dimethyl sulfoxide (DMSO) in water, while cisplatin and oximato
19 titanium compounds were dissolved in culture medium. The stock solutions were then
20 diluted in complete medium and used for sequential dilutions to desired concentrations.
21 The final concentration of DMSO in the cell culture medium did not exceed 0.1%.
22 Control groups with and without DMSO (0.1%) were included in the assays.
23 Compounds were then added at different concentrations in quadruplicate. Cells were
24 incubated with compounds for 24 h or 72 h, and then cell proliferation was determined
25 by a modification of the MTT-reduction method. Briefly, 10 µL/well of [3-(4,5-

1 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) ($5 \text{ mg}\cdot\text{mL}^{-1}$ in PBS)
2 was added, and plates were incubated for 1–3 h at $37 \text{ }^\circ\text{C}$. Finally, formazan crystals
3 were dissolved by adding $100 \text{ }\mu\text{L}/\text{well}$ *i*PrOH (0.05 M HCl) and gently shaking. The
4 optical density was measured at 550 nm using a 96-well multi-scanner auto-reader
5 Enzyme-Linked Immuno Sorbent Assay (ELISA).

6 2.4. DNA interaction studies

7 2.4.1. Equilibrium Dialysis

8 Duplex DNA from calf thymus (CT DNA), (Deoxyribonucleic acid, Activated, Type
9 XV) was directly purchased from Sigma Aldrich and used as provided. Duplex-forming
10 oligonucleotides ds17-1 ($5\text{'-CCA GTT CGT AGT AAC CC-3'}$) and ds17-2 (5'-GGG
11 $\text{TTA CTA CGA ACT GG-3'}$) were acquired High Performance Liquid Chromatography
12 (HPLC) -purified and desalted from Integrated DNA Technologies (IDT). Dialysis
13 membranes (Spectra/Por® molecular porous membrane tubing MWCO: $3.5\text{--}5.0 \text{ kDa}$;
14 6.4 mm diameter) were purchased from Spectrum Laboratories Inc. Aqueous solutions
15 of surfactant sodium dodecyl sulphate (SDS) (10%) were purchased from Sigma
16 Aldrich. The buffer employed in this experiment was 10 mM phosphate buffer
17 $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, $\text{pH} = 7.2$, with either 10 mM or 100 mM NaCl . The solutions of
18 DNA were prepared in the working phosphate buffer at $75 \text{ }\mu\text{M}$ monomeric unit (mum.)
19 concentrations, in base pairs. For the preparation of the short oligonucleotide solution,
20 an annealing step was needed, with heating at $90 \text{ }^\circ\text{C}$ for 10 min and then gradually
21 cooling to $25 \text{ }^\circ\text{C}$ during 3 h . The solutions were left at $4 \text{ }^\circ\text{C}$ overnight.

22 Dialysis bags, previously washed with milli-Q water, were filled with $75 \text{ }\mu\text{M}$ (m.u.)
23 of DNA duplex ($200 \text{ }\mu\text{L}$ each bag) and placed in a beaker containing 225 mL of ca. 2
24 μM solution of the tested compound. The beaker was covered with parafilm and
25 aluminium foil and allowed to equilibrate during 24 h at room temperature. Experiments

1 were run, at least, in triplicate. Once the dialysis process had been completed, the
2 solutions from each dialysis bag were transferred to Eppendorf tubes. The content of
3 each bag was then mixed with an aqueous detergent solution (10%) to reach a 1%
4 concentration (v/v) of SDS. The concentrations of free compound in the dialysate
5 solution and compound in the dialysis bags were determined by absorbance
6 measurements using the extinction coefficients of the metal complexes (determined in
7 the presence and absence of the detergent) and apparent association constants were
8 calculated [78].

9 2.4.2. DNA FRET melting assay

10 The DNA melting assay was performed on a quantitative PCR kit ABI PRISM® 7000
11 Sequence Detection System (Applied Biosystems) in a 96-well plate format (96-Well
12 Optical MicroAmp® Reaction Plate, Applied Biosystems, Life Technologies
13 Corporation). The oligonucleotide sequence employed in this experiment, F10T (5'-
14 FAM-AGC TAT TA /sp18/ TA TA GCT ATA-TAMRA-3') was produced, HPLC-
15 purified and desalted by IDT. FAM is 6-carboxyfluorescein and TAMRA is
16 carboxytetramethylrhodamine. The buffer system used in this experiment was: 10 mM
17 sodium cacodylate, 100 mM LiCl, (pH = 7.3). First, the duplex-forming oligonucleotide
18 was dissolved in water (grade BPC) and a 50 μ M stock solution was prepared, which
19 was then diluted to 0.5 μ M. Then, the diluted DNA solution was mixed with the
20 working buffer (2x) and water Biotechnology Performance Certified (BPC) grade. The
21 DNA solution was heated at 90 °C for 10 min, cooled down slowly for 3 h and left at
22 4 °C overnight. Compounds to be tested were dissolved in water and approximately 1
23 mM stock solutions were prepared. The exact concentrations were checked by UV-Vis.
24 Stock solutions were then diluted with buffer to obtain 50 μ M solutions of each
25 compound. In a 96-well microplate, DNA solutions were mixed with solutions of tested

1 compound and buffer to reach a total volume of 50 μL with a F10T concentration of 0.2
2 μM and a compound concentration ranging between 1 and 10 μM .

3 The experimental protocol consisted of an incubation for 5 min at 24 $^{\circ}\text{C}$, followed by
4 a temperature ramp with heating rate 1 $^{\circ}\text{C}/\text{min}$. Fluorescence values corresponding to
5 the fluorophore FAM at wavelength of 516 nm (after excitation at 492 nm) were
6 collected at each degree of temperature. Afterwards, the fluorescence data were
7 normalized, plotted against temperature ($^{\circ}\text{C}$) at each compound concentration, and T_m
8 values were determined.

9 2.4.3. Viscometric titrations

10 Duplex DNA from CT (Deoxyribonucleic acid, Activated, Type XV) was purchased
11 from Sigma Aldrich and used as provided. The buffer employed in this experiment was
12 10 mM phosphate buffer $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, $\text{pH} = 7.2$. The viscosity measurements
13 were performed in a Visco System AVS 470 at 25.00 ± 0.01 $^{\circ}\text{C}$, using a
14 microUbbelohde ($K = 0.01$) capillary viscometer. 6 mL of DNA solution (0.4 mM in
15 nucleotides) in phosphate buffer were equilibrated for 20 min at 25.00 $^{\circ}\text{C}$ and then 20
16 flow times were registered. Small aliquots (30–50 μL) of solutions of metal complexes
17 (1.6–2.3 mM) were added to the same DNA solution. Before each flow time
18 registration, the solutions were equilibrated for 20 min to 25.00 $^{\circ}\text{C}$ and then 20 flow
19 times were measured. With the averaged time of the different flow time measurements
20 and the viscometer constant, the viscosities (μ) for each point were calculated. The
21 viscosity results were plotted as $(\mu/\mu_0)^{1/3}$, where μ_0 represents the DNA solution
22 viscosity in the absence of the ligand, versus (r), representing the ratio $[\text{ligand}]/[\text{DNA}]$.

23 2.5. Data analysis

24 Results were subjected to computer-assisted statistical analysis using One-Way
25 Analysis of Variance ANOVA, Bonferroni's post-test, and Student's t-test. Data are

1 shown as the means of individual experiments and presented as the mean \pm SD
2 (Standard deviation). Differences of $P < 0.05$ were considered to be significantly
3 different from the controls.

4 **3. Results and Discussion**

5 3.1. Synthesis and characterization of metal compounds

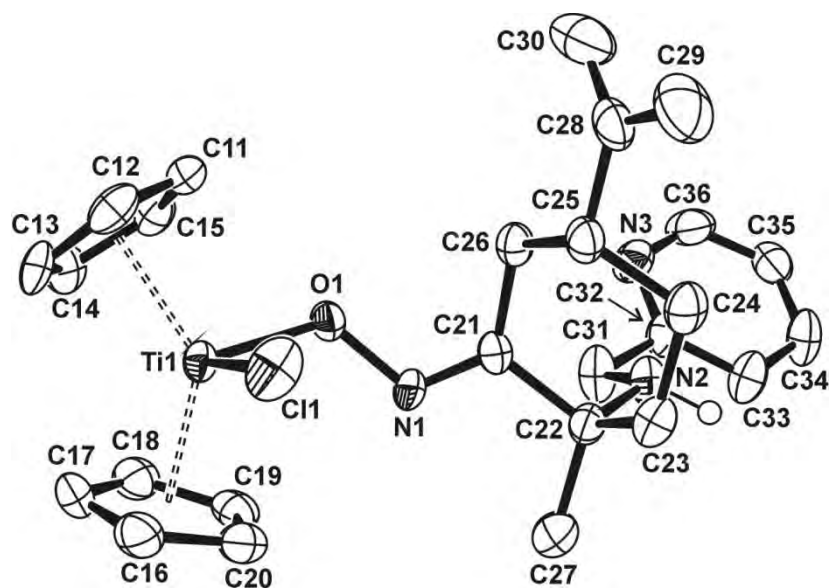
6 Synthesis of the novel Ti(IV) compounds was carried out analogously to that of
7 previously described enantiomers **1a** and **1b** [69]. Treatment of TDC and amino-oxime
8 derivatives **a'** or **b'** in the presence of NEt_3 allows isolation of novel chiral-at-ligand
9 titanium compounds **1a'** or **1b'**, respectively (Fig. 2), which are formed together with
10 $\text{Et}_3\text{N}\cdot\text{HCl}$.

11 Analytical and spectroscopic data of the novel compounds **1a'** and **1b'** are identical to
12 those reported before for **1a** and **1b**, respectively (see ref [69], Experimental Section
13 and Online Resource, Fig. S3-S9).

14 Calculated data of specific optical rotation in chloroform solution for the ligands and
15 novel metal derivatives ($[\alpha]_{\text{D}}^{23}$ ($\text{deg}\cdot\text{dm}^{-1}\cdot\text{dL}\cdot\text{g}^{-1}$) = -127 ± 1.3 **a'**, $+130 \pm 1.3$ **a**, $-126 \pm$
16 1.3 **b'**, $+127 \pm 1.3$ **b**, -88.9 ± 1.2 **1a'**, $+89.2 \pm 1.2$ **1a**, -75.7 ± 1.2 **1b'**, $+74.2 \pm 1.2$ **1b**)
17 evidence the enantiomeric relationship of the stereoisomers. Furthermore, absolute
18 configuration of the compound **1b** has been confirmed through X-ray structure
19 determination (Fig. 3, and Online Resource Table S1, S2 and Fig. S16).

20

21



1

2 **Fig. 3** ORTEP drawing of compound **1b** with 50% probability ellipsoids. Hydrogen
 3 bonded to carbon atoms have been omitted for clarity. Representative bond lengths (Å)
 4 and angles (deg): Ti(1)-Ct(1) 2.073; Ti(1)-Ct(2) 2.065; Ti(1)-Cl(1) 2.380(2); Ti(1)-O(1)
 5 1.899(4); Ti(1)···N(1) 2.866(5); N(1)-O(1) 1.403(6); N(1)-C(21) 1.273(8); Cl(1)-Ti(1)-
 6 O(1) 92.4(2); Ti(1)-O(1)-N(1) 119.6(3); O(1)-N(1)-C(21) 114.1(5); Ct(1)-Ti(1)-Ct(2)
 7 130.3; (Ct(1) is the centroid of the C(11)-C(15) ring, Ct(2) is the centroid of the C(16)-
 8 C(20) ring)

9

10 The X-ray crystal structure determination of **1b** shows the presence of two
 11 independent molecules in the asymmetric unit, with the same absolute configuration of
 12 the two chiral centers; an ORTEP diagram of one of them is presented in Fig. 3. The
 13 crystallographic study confirms a monohapto coordination of the oximate unit to the
 14 titanium atom. The compound shows a pseudotetrahedral environment around the metal
 15 centre, with Ti-O bond distances and O-N-C angles slightly shorter and closer (Online
 16 Resource Fig. S16), respectively, than those found in analogous biscyclopentadienyl

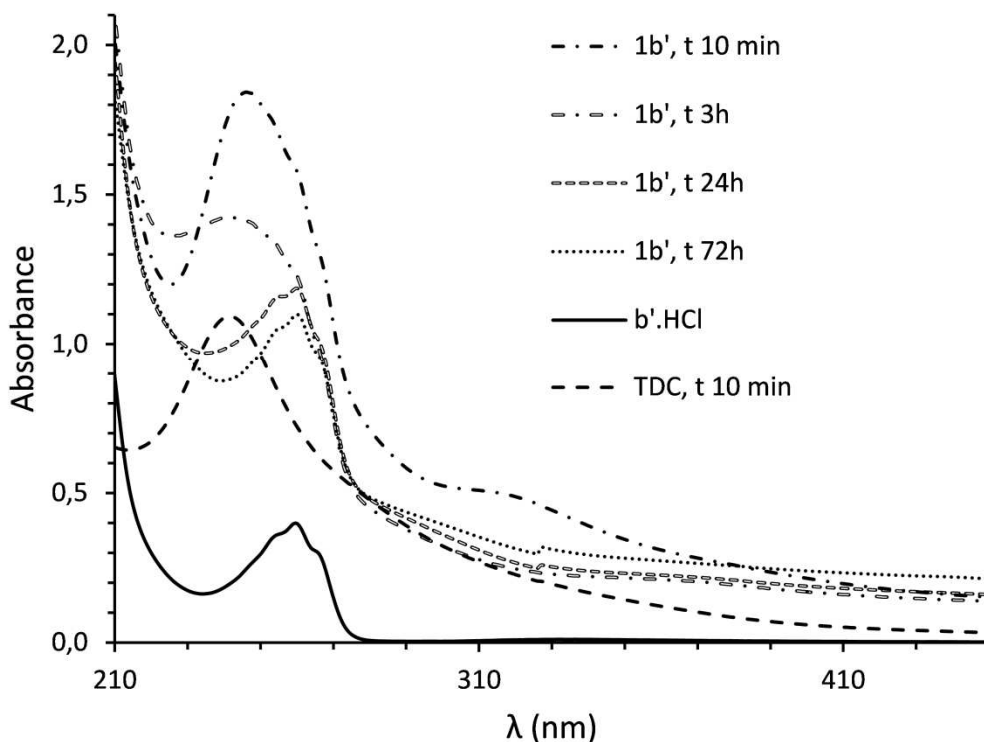
1 oximato titanium(IV) derivatives [66,68] or alcoxoximato titanium(IV) compounds
2 [79-82] with a dihapto $\kappa^2\text{NO}$ coordination of the oximato unit to the titanium centre.

3 To the best of our knowledge, this is the first example found of an oximato titanium
4 derivative with a terminal monohapto Ti-ON= coordination, where this coordination
5 mode is probably caused by the large steric requirements of the functionalized
6 cyclohexane residue. This terminal coordination may account for the hydrolysis
7 suffered for the compounds in aqueous media. In contrast, dihapto titanocene oximato
8 compounds $[(\eta^5\text{-C}_5\text{H}_5)_2\text{Ti}(\text{H}_2\text{O})(\kappa^2\text{O}=\text{NR})]^+$ ($\text{R} = \text{CMe}_2; \text{C}_6\text{H}_{10}$), reported by Thewalt et
9 al [66], were described as surprisingly stable against air and water.

10 The reactions in water or PBS solutions of **1a** or **1b** were elucidated in a previous report
11 and afford soluble ammonium-oxime pro-ligands (1*S*,4*R*)-{NH(R)·HCl,NOH} ($\text{R} = \text{Bn}$
12 **a·HCl** or 2-pic **b·HCl**, respectively), together with aqua-oxo or -hydroxo
13 biscyclopentadienyltitanium(IV) species [69,83,84] which are detected at least during
14 the first three hours after dilution. The same behavior as that described before has now
15 been observed for novel compounds **1a'** and **1b'** when their solutions in water-*d*₂ or
16 PBS were studied by ¹H NMR spectroscopy (see Online Resource, Fig. S10).

17 We decided to further investigate the existence of an amino-oxime ligand containing
18 Ti(IV) species, which could account for the observed stereoisomer-dependent cytotoxic
19 behaviour of the compounds on the prostate cancer PC-3 cell line. Since UV-Vis
20 spectroscopy is considered a more sensitive technique than NMR, we recorded time-
21 dependent UV-Vis spectra for compounds **1a'** and **1b'** in PBS solution. Right after
22 dilution, UV-Vis spectrum of **1a'** (Online Resource Fig. S13) and **1b'** (Fig. 4) shows
23 two very broad absorption bands centered at 240 and 325, and 246 and 322 nm,
24 respectively, ascribed to overlapping of LMCT bands due to cyclopentadienyltitanium
25 aquo cations and the absorption bands corresponding to proligands **a'·HCl** and **b'·HCl**.

1 After 24 h, only the absorption bands assigned to $\mathbf{a}'\cdot\mathbf{HCl}$ or $\mathbf{b}'\cdot\mathbf{HCl}$, at 250 and 332,
 2 and 260 and 332 nm, respectively, are detected. Similar UV-Vis spectra are obtained
 3 after 72 h. Analogous results were obtained when the compounds are diluted in pure
 4 water.



5
 6 **Fig. 4** Comparison of time-dependent UV-Vis spectra of $\mathbf{1b}'$ with $\mathbf{b}'\cdot\mathbf{HCl}$ and TDC
 7 spectra in PBS solution

8 CD spectra were also recorded for each pair of enantiomers. However, the spectra of
 9 derivatives $\mathbf{1a}$, $\mathbf{1b}$ and $\mathbf{1a}'$, $\mathbf{1b}'$ are identical to those obtained for ammonium-oxime
 10 compounds $\mathbf{a}\cdot\mathbf{HCl}$, $\mathbf{b}\cdot\mathbf{HCl}$, $\mathbf{a}'\cdot\mathbf{HCl}$, $\mathbf{b}'\cdot\mathbf{HCl}$ (see Online Resource Fig. S14, S15), 15
 11 min after dilution or after 72 h, leading to the assumption that those are the only
 12 detectable optically active, soluble in water products of the hydrolysis of titanium
 13 oximate compounds.

1 3.2. *In vitro* cell studies

2 3.2.1. Anti-proliferative studies

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

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

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

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

5 **Table 1.** IC₅₀ values (μM) of cisplatin, Titanocene-Y and enantiomers **1a**, **1a'**, **1b** and
 6 **1b'** in prostate cancer PC-3, DU-145 and non-tumorigenic RWPE-1 cell lines,^a (n.m.
 7 not measured)

Compound	PC-3	DU-145	RWPE-1
1a	> 150 (24 h) 48.7 ± 3.2 (72 h)	> 150 (72 h)	> 200 (72 h)
1a'	> 150 (24 h) > 150 (72 h)	> 150 (72 h)	n.m.
1b	> 150 (24 h) 14.5 ± 3.1 (72 h)	27.1 ± 1.1 (72 h)	30.8 ± 0.57 (72 h)
1b'	> 150 (24 h) 49.9 ± 7.0 (72 h)	23.9 ± 8.6 (72 h)	43.8 ± 7.2 (72 h)
1b + 1b'	37.5 ± 5.1 (72 h)	n.m.	n.m.
Titanocene-Y	> 200 (24 h) 58.1 ± 11.2 (72 h)	> 150 (72 h)	42.9 ± 0.73 (72 h)
cisplatin	104.2 ± 8.1 (24 h) 14.5 ± 2.5 (72 h)	3.7 ± 0.6 (72 h)	19.9 ± 1.1 (72 h)

8 ^a Each value represents the mean ± S.D. (n = 3)

9
 10 Under these conditions, the enantiomer **1b** shows IC₅₀ values on the prostate PC-3
 11 and DU-145 cell lines 2-5 times lower than Tacke's compound, Titanocene-Y. The
 12 cytotoxicity on PC-3 cells of the titanium enantiomers **1a** and **1b**, with the absolute
 13 configuration *S,R*-, is higher than that of the *R,S*-stereoisomers by a factor of ca. 2-3,
 14 while the racemic mixture of **1b** and **1b'** afford IC₅₀ values average between the two
 15 enantiomers. In contrast, no enantiomer recognition is observed on the prostate DU-145
 16 cells for derivatives **1b**, **1b'**, while **1a**, **1a'** resulted not to be efficient in this non-
 17 hormone dependent cancer cell line.

1 Titanocene-Y has already shown an encouraging activity in PC-3 tumour-bearing
2 mice [85]. Other titanium compounds which have proved their *in vitro* antitumor
3 activity in prostate cancer cell lines under similar time exposure conditions are Schiff-
4 base titanium (IV) derivatives [87] (IC₅₀ values within the range 5-18 μM, in PC-3) or
5 heterometallic titanocene-gold compounds (IC₅₀ values ranged from 27-40 μM in PC-3
6 [88,89], and 11.8-27.6 μM in DU-145 [24,90]).

7 In order to analyse the cytotoxic selectiveness to healthy cells, the isomers **1a**, **1b** and
8 **1b'** were also tested in the non-tumorigenic human prostate (RWPE-1) cells. Regarding
9 selectivity, **1a** and **1b** are less toxic to the non-tumorigenic RWPE-1 than to the cancer
10 PC-3 cells (from 2 to 4 times less toxic), while **1b'** shows a similar behaviour relative to
11 the cancer DU-145 cells.

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

22

23

24

25

1 **Table 2.** Comparison of IC₅₀ values (μM) of **1b**, **b·HCl**, TDC and TDC+**b·HCl** in
 2 prostate cancer PC-3, DU-145 and non-tumorigenic RWPE-1 cell lines^a (after 72 h of
 3 exposure to the drug).

Compound	PC-3	DU-145	RWPE-1
1b	14.5 ± 3.1	27.1 ± 1.1	30.8 ± 0.57
b·HCl	> 100	106.1 ± 10.4	140.5 ± 23.0
TDC+ b·HCl	39.5 ± 2.1	54.9 ± 13.5	> 150
TDC	> 150	> 150	> 150

4 ^a Each value represents the mean ± S.D. (n = 3)

5

6 3.3. DNA binding

7 To date, various distinct mechanisms have been proposed for titanium-based
 8 therapeutics. DNA binding is still thought to be one important potential mode of action
 9 for titanocene compounds, although interactions with DNA have been found to be
 10 generally very weak at physiological pH conditions [16,17]. The study of DNA
 11 interactions for these particular metal complexes does often represent an experimental
 12 challenge, since the compounds can easily hydrolyse in water solutions. That said,
 13 investigation in this area may be used to shed some light about the nature of the
 14 interactions that may partially account for the biological activity observed in
 15 physiologically relevant aqueous environments, albeit the results obtained should be
 16 interpreted cautiously. Our previous results showed that titanocenes **1a** or **1b** did not
 17 exhibit strong interactions with plasmid DNA by electrophoretic mobility shift assays,
 18 but the absence of a shift in the electrophoretic bands did not allow us to rule out DNA
 19 binding. Having established the interesting antitumor properties of metal compounds
 20 **1a**, **1a'**, **1b** and **1b'**, our aim with the study presented now was to further analyse and

1 compare the kind of potential interactions of the enantiomers with DNA, by using other
2 techniques to complement previous studies.

3 Dialysis experiments, based on the fundamental thermodynamic principle of
4 equilibrium dialysis [78,91], were performed to determine apparent binding constants
5 between DNA and the metal compounds, following the protocol described by Chaires
6 [78] with some modifications. As the DNA targets, we selected CT DNA and a short
7 oligonucleotide duplex of known sequence (ds17, 17 bp).

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

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

23 In these experiments, a 10-bp oligonucleotide (F10T) labelled with two fluorophores,
24 FAM at its 5' end and TAMRA at the 3' end, was selected [93]. If the metal complex
25 binds to DNA affecting the stability of the helix, changes in the value of DNA T_m

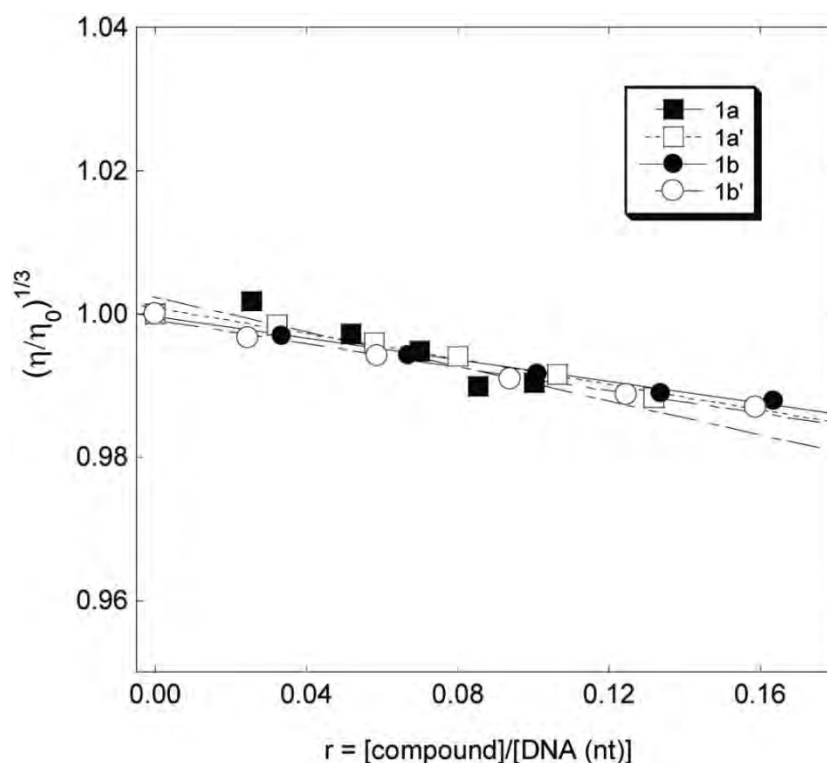
1 should be expected. Stabilization of duplex DNA usually results in increased values of
2 T_m .

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

10 Finally, DNA viscometric titrations were carried out because viscosity measurements
11 can provide a simple way to discriminate between the different binding modes of
12 potential DNA ligands (such as intercalation *versus* groove or external binding) [94].
13 According to the theory of Cohen and Eisenberg [95], from gradual titration of DNA
14 solutions with the compounds of interest, linear plots of the cubed root of the relative
15 DNA viscosity $(\eta/\eta_0)^{1/3}$ versus the molar ratio of bound ligand to DNA nucleotide (r)
16 can be obtained. The slope values in these plots correlate well with the DNA-ligand
17 binding modes. Groove binding compounds normally display a slope close to 0.0,
18 whereas classical mono-intercalants result in a slope close to 1.0 [94,95].

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

23



1
2 **Fig. 5** Viscometric titrations of CT DNA and metal complexes **1a**, **1a'**, **1b** and **1b'** at 25
3 °C (10 mM sodium phosphate buffer, pH 7.2)

4
5 These results are in good agreement with the FRET DNA melting assays and point
6 towards an external or groove interaction of the titanium metal complexes and/or their
7 hydrolysis products that does not result in overall changes of contour length or thermal
8 stabilization of the DNA double helix structure.

9 10 **4. Conclusions**

11 Optically active amino-oxime ligands derived from natural products are useful and
12 inexpensive starting materials for the design of enantiopure titanocene compounds. In
13 contrast with the resistance to hydrolysis of other $\kappa^2\text{N,O}$ oximato-Ti biscyclopentadiene
14 compounds described before, our systems suffer hydrolysis in water at physiological
15 conditions, most likely due to the monohapto κON coordination mode of the highly

1 sterically demanding limonene residue of the oximato ligand. Regarding their cytotoxic
2 behaviour, the oxime-containing Ti(IV) compound **1b** has shown potent anticancer
3 activities against both prostate cancer PC-3 and DU-145 cell lines after 72 h of
4 incubation time. The cytotoxicity of the enantiomers **1a**, **1a'** and **1b'**, **1b'** towards all
5 the cancer cell lines tested showed no significant differences, exception made for the
6 PC-3 cells. In addition, isomers **1a** and **1b** showed certain selectivity in their toxicity
7 against prostate cancer PC-3 versus non-tumorigenic RWPE-1 cells. Furthermore,
8 compound **1b** shows higher activity than the additive dose of TDC and proligand **b·HCl**
9 on the prostate PC-3, DU-145 and RWPE-1 cell lines. These results point towards the
10 existence of an influence of the oximato-Ti unit on the hydrolysis process and/or the
11 cytotoxicity mechanism. Compound-DNA interactions have been investigated by
12 equilibrium dialysis, FRET melting assays and viscometric titrations. The experimental
13 results suggest that these metal complexes and/or their hydrolysis products can bind
14 DNA either in the minor groove or externally, irrespective of the ligand
15 stereochemistry.

1

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8 **Online Resource.** Supplementary data associated with this article can be found in the
9 online version, at <http://>. These data include: Representative NMR, UV-Vis and CD
10 spectra of compounds **a**, **a'**, **b**, **b'**, **1a**, **1a'**, **1b**, **1b'**. Selected biological data. Selected
11 crystallographic data and bond lengths and angles for X-ray molecular structures of **1b**.

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7

ACCEPTED MANUSCRIPT

Highlights (85 characters)

- Synthesis of novel enantiopure titanocene amino-oximato compounds is reported.
- The X-ray crystal structure of one of the compounds shows a unique monohapto Ti-ON coordination.
- One enantiomer shows IC₅₀ values lower than Titanocene-Y on both PC-3 and DU-145 cells.
- One enantiomer is more active than additive doses of Ti(η^5 -C₅H₅)Cl₂ and oxime pro-ligand.