SUPPLEMENTARY INFORMATION

Enantiopure titanocene complexes – direct evidence for paraptosis in cancer cells.

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1. MTT assays: representative dose-response curves.



Figure S1. Inhibition of Mia PaCa-2, HCT 116, MDA-MB-468 carcinoma and MRC5 fibroblast growth by (*R*,*R*)-4, (*S*,*S*)-4 and cisplatin following 72 h exposure. Mean \pm SD values within single representative MTT experiments (n=8); \geq 3 independent trials. T₀, timoe of test agent addition; GI₅₀, concentration of test agent estimated to inhibit cell growth by 50%.

2. Characterisation studies

2.1 TEM Instrumentation/cell culture

Bright field TEM images of fixed and microtomed sections are shown in figure S2 (control sample) and figure S3 (agent and concentration 6 h exposure to the titanocene 4). As highly evident in figure S3, extensive formation of vacuoles was present in titanocene treated cells, which is indicative of paraptosis, a characteristic feature of type III cell death - a primitive form of programmed cell death. Furthermore, features characteristic of apoptosis – chromatin condensation, nuclear fragmentation, cytoplasmic shrinkage, membrane blebbing, formation of apoptotic bodies were absent.

Titanocene dichloride 1 derived Ti in cells detectable *via* TEM analysis has been reported previously^{S1,S2}. In the first example, EAT cells of mice were harvested on day 6 after transplantation, fixed with buffer, post-fixed with osmium tetroxide and embedded in Mikropal (resin). Ultra-thin sections were then mounted on copper mesh, stained with uranyl acetate and lead citrate and then finally dipped for 15-30 min in 8000 μ M titanocene dichloride 1. Ti levels of a comparable intensity to the N levels were located in structures within the cell using EELS. The biological relevance of exposing dead sectioned cells to such vast excesses of strongly Lewis acidic 1 has never been questioned as far as we can tell. In the 1983 work, EAT cells were cultured *in vitro* as a suspension culture cell line. Groups of 4 subcultures each containing about 5 x 10⁶ EAT cells per 20

mL were exposed for 40 min to 1 in final concentrations of 1000 μ M and 10,000 μ M. (GI₅₀ of 1 was reported as 500 μ M under these conditions). 'Semi-quantitative' EELS analysis again showed Ti levels of a comparable intensity to the N signal in certain intracellular structures. Although not discussed therein, the potential for hydrolysis of 1 to insoluble titanium species that would not have been removed by the saline wash used is significant. In our work, the more modern Gatan Tridiem detector for EELS and the state-of-the-art Oxford Instruments detector for EDX are expected to show a significantly greater sensitivity to Ti, and would both be sufficient to detect Ti significantly below the levels reported^{S1,S2}. However, in our work, even a 40 minute exposure at a very high concentration (1000 μ M) did not reproducibly result in a detectable level of Ti in the microtomed section by either EDX or EELS, despite an expected sensitivity of at least 0.1 at% and 1 at%, respectively given the section thickness and electron doses used in this work.

STEM-EDX (Section 2.3) mapping of the cells was performed to investigate any variance in Ti content across the cell. The work of Köpf-Maier and Krahl reported no Ti in mitochondria or lipids, but a remarkable level in other structures^{S1,S2} – comparable to the signal from N - maps in our work found the Ti signal remained at the level of background noise across the cell, while the N and O signals are clearly visible (SI 2.4). Similar results were obtained using EELS, once potential artefacts are subtracted. Identical cells analysed by ICP-MS revealed only very low levels of titanium (*ca.* 3 femtomol per cell). Assuming hydrolysis to TiO₂, its known density (4.23 g cm⁻³) and a homogenous distribution of the Ti throughout the cell, there would have to be >10⁷ aggregates of less than 1-2 nm dimensions to be below our detector's threshold.

2.2 Bright field TEM images

i) MDA-MB-468 cells - Control samples







Figure S2. Bright field TEM images MDA-MB-468 cells - Control samples

ii) *MDA-MB-468 cells - Treated 6 h (meso/rac)-4 - 50 \muM.* Extensive formation of vacuoles, as observed herein, is characteristic of type III cell death – paraptosis, a primitive form of programmed cell death







Figure S3. Bright field TEM images MDA-MB-468 cells - Treated 6 h (*rac/meso*)-4 - 50 μ M. Extensive formation of vacuoles, as observed herein, is characteristic of type III cell death – paraptosis, a primitive form of programmed cell death



0.33 h

0.66 h

Figure S4. Bright field confocal live cell microscopy 0.33-0.66 h after exposure to **4**. Appearance of membrane invaginations curved into crater- like cups on the cell surface membrane indicating an endocytic-like activity as in literature^{S16}.



Figure S5. Dark Field Z-contrast montage after 3 h exposure to 4 50 μ M showing extensive vacuolization.

2.3 STEM-EDX - mapping of the cells





Figure S6. Annular dark field scanning TEM image and associated EDX elemental maps. N and O levels can be seen clearly; however the Ti signal is consistent with volume sensitive background noise only.

2.4 EDX quantification



Figure S7. EDX quantification. 3 h (*meso-rac*)-4 50 μ M exposure - spectrum map sum XMAX-TLE EDX spectra and quantification. Ti is not detected. Os, Pb and U are due to staining, Au is from the TEM holder. The Cu signal from the supporting TEM grid has been de-convoluted.



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Element	Wt%	Wt% Sigma
С	79.85	0.12
N	2.27	0.09
Ο	13.43	0.07
Si	0.19	0.01
Р	0.30	0.02
S	0.15	0.01
Cl	1.03	0.01
Ti	0.00	0.00
Os	2.54	0.05
Au	0.23	0.02
Total:	100.00	

Figure S8. EDX quantification. 6 h (*meso-rac*)-4 50 μ M exposure - spectrum map sum XMAX-TLE EDX spectra and quantification unstained sample. Ti is not detected. Au is from the TEM holder. The Cu signal from the supporting TEM grid has been deconvoluted.

2.5 EELS analysis

EELS analysis of low levels of Ti against a strong C background signal is compromised by a signal due to the carbon appearing in a range that overlaps the default Ti detection window used by the standard Gatan software (Fig. S8). The contribution of this artefact can be significantly increased by acquiring the signal in Diffraction mode, which could lead to an incorrect identification of Ti (Fig. S9). Careful comparison of the signal observed in this region in this work indicates any deviation from the power law background as being likeliest to be due to the carbon contribution by comparison with a signal from an amorphous carbon film of similar thickness to the microtomed section.



Figure S9. EELS artefact producing a deviation from the power law background in the Ti region from a signal acquired in Bright Field mode from an amorphous carbon film - C and O content only.



Figure S10. EELS artefact producing a significant deviation in the Ti sensitive region from a signal acquired in Diffraction mode from an amorphous Carbon film - C and O content only.



Figure S11. EELS signal from a sample treated with (rac/meso)-4 (50 μ M, 6 h) - Oxygen can be detected, no unambiguous Ti signal can be confirmed.

2.6 ICP-MS

Analyses conducted on a Thermo Scientific iCAP QC indicated that treated cells contained 3.3 ± 0.3 femtomol Ti per cell. Cells untreated with (*S*,*S*)-4 showed only background levels (0.3-0.35 femtomol Ti per cell). If complete hydrolysis to TiO₂ of standard density (4.23 g cm⁻³) was attained at 3.3 femtomol/cell the titanium would have to be present in the cell as >10⁷ aggregates of maximum 1-2 nm dimensions to have avoided detection in our TEM studies. Analyses of the wash samples from (*S*,*S*)-4 (30 μ M) 4 h treated MDA-MB-468 cells indicates that the majority (>99.9%) of the applied titanium dose is recovered.

3. Spectroscopy

3.1: Mass spectrometry



Figure S12: ESMS (in 1% DMSO/99% D₂O) and LCMS studies (in 1% DMSO/99% D₂O or RPMI 1640 medium alone) point to a single major homogeneous species whose daughter ions are consistent with the presence of $[(Cp^R)_2Ti(OH)(OH_2)]Cl$ (I) being the major soluble intermediate attained upon dissolution of (*S*,*S*)-4.



Figure S13: ESMS spectrum of (*S*,*S*)-4 in 1% DMSO / 99% D₂O. The molecular ion peak $[(Cp^R)_2Ti(OMe)]^+$ is due to the presence of methanol from the 'sipper' robot in the MS system.

Intens.								+1	MS, 1.0-1.2	min #(73-87)	
x10 ⁵	Expected ions not found!										
0.8-											
0.6-											
0.4-											
0.4											
0.21											
0.0-	600	800	1000	1200	1400	1600	1800	2000	2200	2400m/	
#	m/z	1%									
1	503.2265	8.5									
2	504.2271	10.8									
3	505.2219	100.0									
4	506.2254	36.6									
5	507.2233	13.2									
6	508.2248	3.0									
7	509.2197	0.6									
8	519.2027	1.4									
9	520.2053	0.6									
10	531.2007	0.8									
11	545.2171	1.1									
12	546.2186	0.5									
13	548.5061	0.5									
14	553.4606	1.3									
15	554.4655	0.5									
16	647.4605	1.4									
17	648.4648	0.6									
18	769.4874	0.9									
19	770.4879	0.5									
20	981 4082	0.4									

Figure S14: GC-MS spectrum of (S,S)-4 in 1% DMSO / 99% D₂O after 20 h (presence of dimer).



Figure S15: LC-MS spectrum of RPMI-1640 medium only.



Figure S16: LC-MS spectrum of RPMI-1640 medium + (S,S)-4.

3.2: Hydrolysis NMR spectroscopy





Figure S19: NMR spectrum of (rac/meso)-4 at 0 h (blue) and 48 h (red) from dissolution of sample in D₆-DMSO.



Figure S20: Ar region NMR spectrum of (*rac/meso*)-4 at 0 h (blue) and 48 h (red), from dissolution of sample in D_6 -DMSO.



Figure S21: NMR spectrum of (R,R)-4 in 1% D₆-DMSO / 99% D₂O at [I]_{max}





Figure S23: NMR spectra stack of (R,R)-4 aromatic region in 1% D₆-DMSO / 99% D₂O at [A] to [I]_{max}.



Figure S24: NMR spectra stack of (*R*,*R*)-4 aromatic region in 1% D_6 -DMSO / 99% D_2O at [**A**] to [**I**]_{max}.



Figure S25: NMR spectra stack of (*R*,*R*)-4 cyclopentadienyl region in 1% D_6 -DMSO / 99% D_2O at [I]_{max} to [P].



Figure S26: NMR spectra stack (R,R)-4 aromatic region 20% D₆-DMSO / 80% D₂O [A] to [I]_{max}



Figure S27: NMR spectra stack (R,R)-4 aromatic region 20% D₆-DMSO / 80% D₂O [I]_{max} to [P].



Figure S28: NMR spectrum stack (*rac/meso*)-4 (blue line) at $[I]_{max}$ vs free cyclopentadienyl tautomers (red line) in 20% D₆-DMSO / 80% D₂O.



Figure S29: NMR spectrum stack 0 ppm – 4.5 ppm (*rac/meso*)-4 (blue line) at $[I]_{max}$ vs free cyclopentadienyl tautomers (red line) in 20% D₆-DMSO / 80% D₂O.



Figure S30: NMR spectrum stack 5 ppm – 8 ppm (*rac/meso*)-4 (blue line) at $[I]_{max}$ vs free Cp tautomers (red line) in 20% D₆-DMSO / 80% D₂O.



Figure S31: NMR spectra (*rac/meso*)-4 (blue line) at $[I]_{max}$ vs (*R/R*)-4 at $[I]_{max}$ (red line) in 20% D₆-DMSO / 80% D₂O.



Figure S32: NMR spectrum stack (*rac/meso*)-4 (blue line) at $[I]_{max}$ vs (*R/R*)-4 at $[I]_{max}$ (red line) in 20% D₆-DMSO / 80% D₂O.



Figure S33: NMR spectra aromatic region (*rac/meso*)-4 (blue line) at $[I]_{max}$ vs (*R/R*)-4 at $[I]_{max}$ (red line) in 20% D₆-DMSO / 80% D₂O.



Figure S34: NMR spectra stack aromatic region (*rac/meso*)-4 (blue line) at $[I]_{max}$ vs (*R/R*)-4 at $[I]_{max}$ (red line) in 20% D₆-DMSO / 80% D₂O.



Figure S35: NMR spectra stack aromatic region (*R*,*R*)-4 (blue line) at $[I]_{max}$ *vs* (*R*/*R*)-4 at $[I]_p$ (red line) *vs* free cyclopentadienyl tautomers (green line) in 20% D₆-DMSO / 80% D₂O.

References

- S1. Köpf-Maier, P., Krahl, D., Naturwissenschaften 1981, 68, 273-274.
- S2. Köpf-Maier, P., Krahl, D., Chem. Biol. Interactions 1983, 44, 317-328.