Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

1. Experimental Section

General. Chemicals and reagents were purchased from Sigma Aldrich or other commercial suppliers. MCF-7 breast adenocarcinoma and HT-29 colon carcinoma were maintained in DMEM High Glucose (PAA laboratories GmbH), supplemented with 50 mg/L gentamycin (USBiological) and 10 % (v/v) fetal calf serum (FCS, Biochrom AG) prior to use. Titanocene dichloride was from Arcos Organics. Titanocene Y[1] and Ti-Salan[2] were synthesized as previously described.[1,2] PBS: phosphate buffered saline pH 7.4; cell culture medium: minimum essential medium eagle; 30 % Danieau medium: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$ and 5 mM HEPES (pH 7.2); Egg water: 0.06 g Sea salt in 1 L destilled water

HR-CS AAS measurements. A contrAA© 700 high-resolution continuum source atomic absorption spectrometer (Analytik Jena AG) was used for the Ti measurements. Titanium was measured at a wavelength of 364.2675 nm. Samples were injected at a volume of 25 µl into coated standard graphite tubes ("AAS IC-Standardrohr, beschichtet", AnalytikJena AG). The mean integrated absorbances (AUC = area under the curve) of triple injections were used throughout the study. The limit of Ti detection in biological material was 66 µg/L.

Cell culture and preparation of cell pellets. MCF-7 breast adenocarcinoma and HT-29 human colon carcinoma cells were maintained in cell culture medium (see above) at 37.8 °C / 5 % CO_2 and passaged twice a week according to standard procedures. Cell pellets were prepared and isolated as follows: cells were grown until at least 70% confluency in 75 cm^2 cell culture flasks. The culture medium was removed, the cell layer washed with 10 mL PBS, treated with 2 mL trypsin solution (0.05 % trypsin in PBS) and incubated for 2 min at 37.8 °C / 5 % CO_2 after removal of the trypsin solution. Cells were resuspended in 10 mL PBS and cell pellets were isolated by centrifugation (RT, 3323 g, 5 min).

Antiproliferative activity in MCF-7 and HT-29. The antiproliferative effects in HT-29 colon carcinoma and MCF-7 breast adenocarcinoma cells after 72 h or 96 h exposure to the titanium complexes were evaluated according to an established procedure.[3] For the experiments, the compounds were prepared freshly as stock solutions in DMF and diluted with the cell culture medium to the final assay concentrations (0.1 % v/v DMF). The IC_{50} value was described as that concentration reducing proliferation of untreated control cells by 50 %.

Zebrafish embryo collection. Embryos were obtained by natural pair-wise mating as described.[4] Eggs were collected in the morning and maintained in Petri dishes in embryo water at 27 °C for 7 h, when the medium was displaced by 30% Danieau medium containing 0.2 mM 1-phenyl-2-thiourea (PTU) to inhibit pigment formation. Unfertilized or dead embryos were sorted out to prevent contamination of the surviving embryos. 24 hours post fertilization (hpf) zebrafish embryos were dechorionated by enzymatic digestion with 1 mg/ml protease for 20 min at room temperature and washed triple with Danieau medium containing 0.2 mM PTU.

Zebrafish embryo toxicity. Substances were administered once to dechorionated 24 hpf zebrafish embryos. For this purpose, the embryos were distributed into 6-well-plates with 50 embryos per well. Titanium complexes were dissolved in DMF and the stock solutions were diluted (1:1000) in 30 % Danieau medium/0.2 mM PTU exposing the embryos to the final concentrations between 0.2 and 100 µM. The control solution contained the same concentration of DMF only (0.1 %). The development of embryo was monitored at 0, 6, 24, 48, 72 and 96 hours after compound addition, that means at 24, 30, 48, 72, 96 and 120 hpf embryos were screened for toxic effects such as malformation, heart beat failure or extensive cell death. At the time of observation dead embryos were removed from the well to prevent contamination of the surviving embryos and only healthy embryos were counted. The IC_{50}

values were calculated as the concentration of 50 percent surviving embryos. Each experiment was performed in duplicate.

Binding to Albumin. The experiments were performed according to an ethanol precipitation method with some modifications.[5] A 50 mM compound stock solution in DMF was freshly prepared and a 10 µl aliquot was mixed with 10 ml cell culture media containing 400 mg BSA (bovine serum albumin, Sigma Aldrich). The mixture was incubated in a water bath at 37 °C under gentle shaking. After 1, 2, 4, 6, 24 and 30 h an 250 µl aliquot of the solution was taken and treated with 500 µl cold (-25 °C) ethanol, and stored at -25 °C for 2 h. Afterwards, the solution was centrifuged at 2867 g for 5 min at 4 °C. A 400 µl aliquot of the supernatant was removed and stabilized with 40 µl HNO_3 (13 %) for determination of the titanium content by high-resolution continuum source atomic absorption spectrometry (HR-CS AAS, see above). The titanium amount not bound to proteins was determined as the means of four experiments and calculated as percentage of the initial compound amount. The protein binding was obtained by subtracting the unbound percentage from the total.

Binding to DNA. Precipitation of compound DNA adducts was performed according to a described method[6] with some modifications: Salmon testes DNA (Sigma Aldrich, Batch number 047K7304) was dissolved in phosphate-buffered saline pH 7.4 and compounds were added as stock solutions in DMF. The final solutions contained 50 µM drug, 250 µg/mL salmon testes DNA and 0.1% (v/v) DMF. After vortexing, the solutions were incubated at 37 °C in a water bath for 4 h. Aliquots of 200 µL were mixed with 100 µL of 0.9 M sodium acetate and 900 µL ice cold ethanol. Samples were stored at -25 °C for 30 min. The pellets were isolated by centrifugation (7167 g, 10 min, 4 °C) and resuspended in 300 µL of 0.3 M sodium acetate. 900 µL of ice cold ethanol were added, and the precipitate was collected after centrifugation (7167 g, 10 min, 4 °C). Samples were washed twice with ice cold ethanol and were stored at -25 °C. The pellets were dissolved in 500 µL of bidistilled water. The DNA content was determined by measuring the absorbance at 260 nm in a microplate reader (Perkin-Elmer Victor X4). Salmon testes DNA dissolved in bidistilled water were used for calibration purposes. An Aliquot of 200 µl was removed for titanium determination by high-resolution continuum source atomic absorption spectrometry (HR-CS AAS, see above). All samples were stabilized by addition of 20 µL of Triton X100 (1 %). The amount of drugs bound to DNA was expressed as pmol of drug per µg of DNA and nucleotide compound ratio. Results were calculated as means of three independent experiments, which were performed with two replicates.

Uptake into Cells. For cellular uptake studies cells were grown until at least 70% confluency in 75 cm^2 cell culture flasks. Stock solutions of the titanium compounds in DMF were freshly prepared and diluted with cell culture medium to the desired concentration of 10 µM for time dependent experiments. The cell culture medium of the cell culture flasks was replaced with 10 mL of the cell culture medium containing the titanium compound and the flasks were incubated at 37 °C / 5 % CO_2 for 0, 1, 4, 6, 24, 27 h. Subsequent to the incubation period the culture medium was removed, the cell layer washed with 10 mL PBS, treated with 2 mL trypsin solution (0.05 % trypsin in PBS) and incubated for 2 min at 37 °C / 5 % CO_2 after removal of the trypsin solution. Cells were resuspended in 10 mL PBS and cell pellets were isolated by centrifugation (RT, 3323 g, 5 min). Cellular lysates were prepared by resuspending an isolated cell pellet in 1 mL demineralized water followed by ultrasonication. An aliquot was removed for the purpose of protein quantification by the method of Bradford.[7] The metal content of the samples was determined by high-resolution continuum source atomic absorption spectrometry (HR-CS AAS, see above). To avoid matrix effects matrix-matched calibration was used for titanium quantification in the samples. To 200 µL of all probes and standards each 20 µL Triton X100 (1 %) and 20 µL nitric acid (13 %) were added. Results were calculated from the data of three independent experiments and are expressed as nmol of the titanium compound per milligram of cellular protein. Based on the knowledge of the mean cellular diameter and mean protein content of HT-29 cells, the values were also estimated as intracellular molar metal concentrations.[8]

Uptake into Mitochondria. For mitochondria uptake studies HT-29 cells were grown until 90-100% confluency in 175 cm^2 cell culture flasks. The cell culture medium of the cell culture flasks was replaced with 20 mL of the cell culture medium containing the titanium compound at a concentration of 25 µM and the flasks were incubated at 37 °C / 5 % CO_2 for 1, 4, 6 and 24 h. After that incubation period cell pellets were isolated as described above and resuspended in 10 mL PBS. An 2 mL aliquot was removed to determine the total cellular titanium concentration (see section uptake into cells) of the samples. Mitochondria were isolated from the isolated cell pellet using the Sigma Aldrich Mitochondria Isolation Kit for profiling cultured cells (MITOISO2, batch number 050M4042) according to the manufacturer's procedure. The obtained fractions were dissolved in 150 µL Extraction Buffer A (50 mM HEPES, pH 7.5, containing 1 M mannitol, 350 mM sucrose, and 5 mM EGTA) and an aliquot of 10 µL was removed for mitochondria protein quantification by Bradford method. For Ti quantification by high-resolution continuum source atomic absorption spectrometry (HR-CS AAS, see above) all samples were adjusted to the same protein

concentration by diluting with Storage Buffer at a final volume of 100 µL and 10 µL Triton X100 (1%) was added to each sample. Results were expressed as nmol titanium per mg mitochondrial protein as means and errors of three to five independent experiments.

Uptake into Nuclei. The nuclei of HT-29 cells were isolated according to previously described procedure[9,10] with some modifications: Cells were grown in 175 cm^2 cell culture flasks until at least 70% confluency. The cell culture medium was removed and replaced with 20 mL cell culture medium containing 25 µM titanium complex. The flasks were incubated at 37 °C / 5 % CO_2 for 1, 4, 6 and 24 h and cell pellets were isolated as described above. After centrifugation (923 g, 5 min) the pellet was resuspended in 1.0 mL of 0.9 % NaCl solution and an aliquot of 200 µL was removed before nuclei isolation to determine the total cellular titanium uptake (see above). For nuclei isolation the cell suspension was centrifuged at 923 g for 5 min and resuspended in 300 µL RSB-1 (0.01 M Tris-HCl, 0.01 M NaCl, 1.5 mM $MgCl_2$, pH 7.4) and left for 10 min in an ice-bath. Swollen cells were centrifuged (1231 g, 5 min), resuspended in 300 µL of RSB-2 (RSB-1 containing each 0.3% v/v Nonidet-P40 and sodium desoxycholate) and homogenized by 10–15 up/down-pushes using a dounce homogenizer. The homogenisate was centrifuged at 3583 g for 5 min and the resulting crude nuclei were taken up in 150 µL of 0.25 M sucrose containing 3 mM $CaCl_2$. The suspension was underlayed with 150 µL of 0.88 M sucrose and centrifuged for 10 min at 3583 g. The nuclei pellets were stored at -20 °C or immediately dissolved in 200 µL of 10 g/L EDTA-solution and disrupted by ultrasonification. The titanium content of the samples was determined by high-resolution continuum source atomic absorption spectrometry (HR-CS AAS, see above) and the nucleus protein content by the Bradford method. For HR-CS AAS measurements matrix-matched calibration was used. Results are expressed as nmol titanium per mg nuclear protein as means and errors of four independent experiments.

2. Results of Zebrafish Embryo Assays

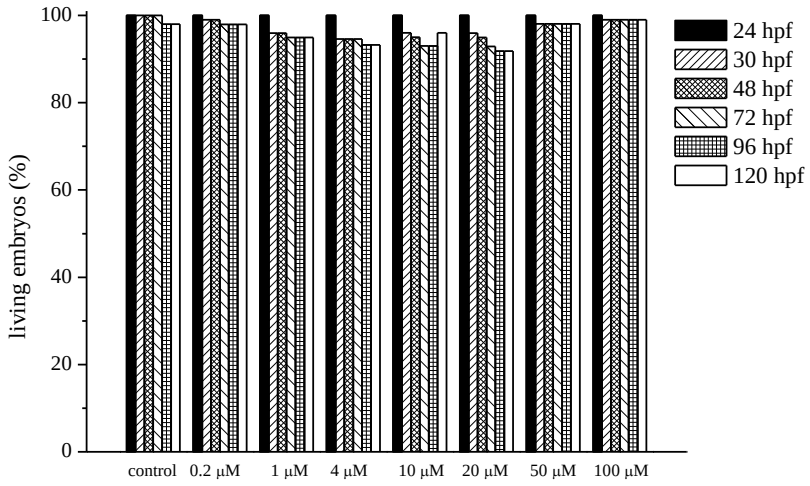

Fig. S1: *In vivo* toxicity of 0.2-100 μM Ti-Salan in 24-120 hpf zebrafish embryos. Values are expressed as means of two independent experiments.

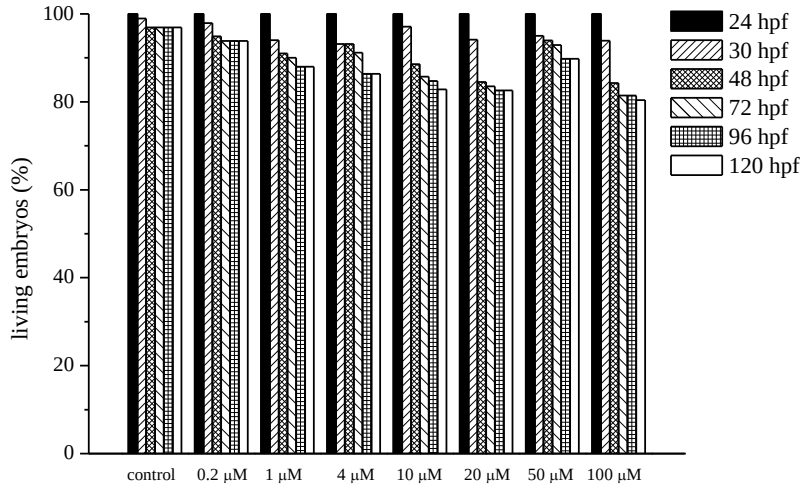

Fig. S2: *In vivo* toxicity of 0.2-100 μM Ti-Y in 24-120 hpf zebrafish embryos. Values are expressed as means of two independent experiments.

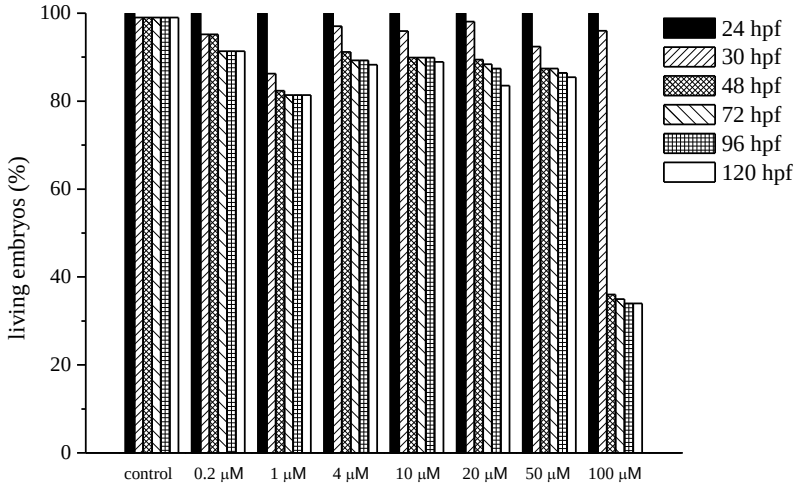

Fig. S3: *In vivo* toxicity of 0.2-100 μM $TiCp_2Cl_2$ in 24-120 hpf zebrafish embryos. Values are expressed as means of two independent experiments.

3. Binding to Albumin and Cellular Uptake Studies

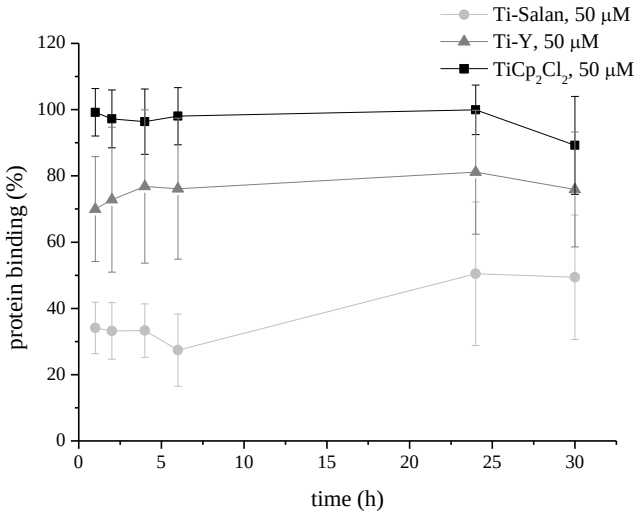

Fig. S4: Binding of titanium(IV) complexes to bovine serum albumin (BSA) in cell culture medium at 37°C determined by ethanol precipitation. The results are expressed as means ± standard deviations of four independent experiments.

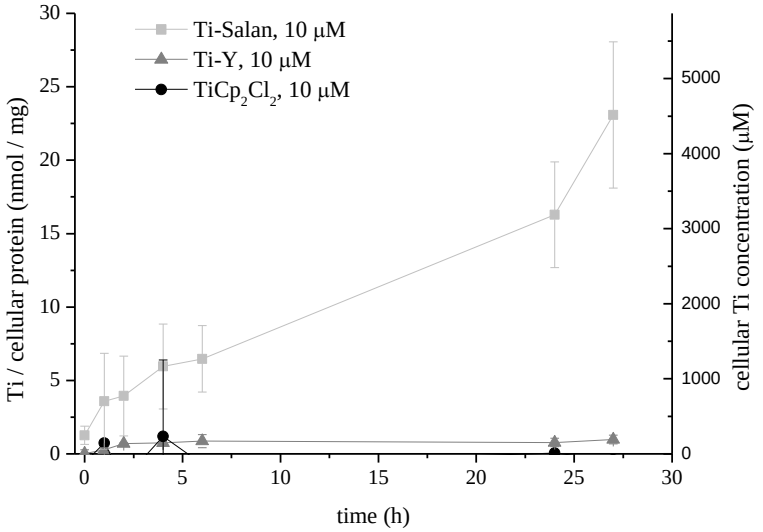

Fig. S5: Time dependent cellular uptake into HT-29 cells after exposure to titanium(IV) complexes in serum containing cell culture medium. Values are expressed as means ± standard deviations of three independent experiments.

4. References

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