Titanium Salan Complexes Displays Strong Antitumor Properties In Vitro and In Vivo in Mice

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Abstract

The anticancer activity of titanium complexes has been known since the groundbreaking studies of Köpf and Köpf-Maier on titanocene dichloride. Unfortunately, possibly due to their fast hydrolysis, derivatives of titanocene dichloride failed in clinical studies. Recently, the new family of titanium salan complexes containing tetradeutate ONNO ligands with anti-cancer properties has been discovered. These salan complexes are much more stable in aqueous media. In this study we describe the biological activity of two titanium salan complexes in a mouse model of cervical cancer. High efficiency of this promising complex family was demonstrated for the first time in vivo. From these data we conclude that titanium salan complexes display very strong antitumor properties exhibiting only minor side effects. Our results may influence the chemotherapy with metallotherapeutics in the future.

Introduction

The discovery of the anticancer effects of cisplatin and its clinical introduction in the 1970s represents an important step in the history of the development of cancer drugs. Today, this metal based complex and its next generation analogues, carboplatin and oxaliplatin, play an important role in tumor therapy, but the existing complexes are efficient in only a limited range of cancers and can induce acquired resistance upon treatment [1]. They often cause severe side-effects like nausea, bone marrow suppression and kidney toxicity [1,2]. Current research on metal complexes is therefore directed to the investigation of other platinum-based compounds as well as non-platinum metal-based systems with little cross resistance to cisplatin and less side effects [3–7]. Among these, titanium[TIV]complexes showed encouraging antitumor activity in various cell lines [8–10].

Till today, three classes of cytoxic titanium complexes are known. Most of the complexes investigated so far are derivatives of either titanocene dichloride (Cp₂TiCl₂) [11] or diketonato complexes like budotitane [Ti(bzac)₂(OEt)₂; Hbzac = Phenylbutane-1,3-dione] [12]. Representatives of these two classes of complexes were tested in clinical trials, but unfortunately, no objective clinical responses were observed [13,14]. The main disadvantages of these titanium complexes are their fast hydrolysis under physiological conditions: in an aqueous environment, the loss of the labile groups occurs within seconds [15,16].

With hydrolytic half lives in the range of hours, salan complexes of titanium are much more stable [17]. Exploring this new, third class of titanium complexes with antitumor activity, we could demonstrate that their biological properties can be fine-tuned by varying the substitution pattern of the complexes. The most promising subgroup contains salan complexes bearing halogen residues at the aromatic rings. The IC₅₀ values of these halo-salan complexes are comparable to cisplatin and in contrast to their alkyl-substituted counterparts, they almost exclusively induce apoptotic cell death [18]. Moreover, we could demonstrate that these complexes are effective in a cisplatin-resistant cell line [17].

Up to now, few is known about the mechanism of action and cellular uptake of the titanium salan complexes on the one and selectivity and in vivo applicability on the other hand. Therefore, we have selected cervical cancer for first preclinical studies. Worldwide approximately 370,000 cases of cervical cancer are being diagnosed each year and almost 200,000 deaths are attributed to this disease [19]. In developed areas the treatment bases mainly on surgery and chemotherapy but nevertheless even when optimal treatment is available about 40% of all cervical cancer patients die of this disease [20]. Therefore, the development of effective and save new anticancer drugs is necessary.

In the current study we analyzed the potency of two titanium salan complexes to induce cell death in C3 cells in vitro and tumor regression in C57BL/6 mice.

Results

As we reported previously, the halogen substituted complexes are highly active against various human cancer cells and nearly exclusively induce apoptotic cell death. Moreover, they display an
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Table 1. Physicochemical and biological properties of complexes 1 and 2.

| properties of complexes 1, 2 | complex 1 | complex 2 |
|----------------------------|-----------|-----------|
| \( IC_{50} \) value in Hela S3 | 1.6 ± 0.1 \( \mu M \) | 5.3 ± 0.2 \( \mu M \) |
| \( IC_{50} \) value in Hep G2 | 2.2 ± 0.2 \( \mu M \) | 4.0 ± 0.2 \( \mu M \) |
| hydrolytic half life | 6 h | 108 h |
| apoptotic/dead cells in Hela S3 | 90.1 ± 4.4% | 95.5 ± 2.0036% |

\( IC_{50} \) values were determined using an AlamarBlue Assay after 48 h of incubation with different concentrations of the complexes. The hydrolytic half life was determined using time resolved \( ^{1}H \)-NMR. These hydrolysis experiments were conducted in a mixture of 95% [D8]-THF and 4.8% D2O at 37°C. As an internal standard, 0.2% DMSO was added. Cell death was followed via double staining with propidium iodide and fluorescein isothiocyanate-labeled annexin V.

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astounding hydrolytic stability. Especially complex 2 with a half life of more than 4 days in aqueous media is therefore a promising substance (for an overview of their physicochemical and biological properties see table 1) [17,18]. The aim of this study was to characterize the activity of two promising titanium salan complexes in more detail and to evaluate their potential clinical value. For this purpose we investigated the biological activity using a broad variety of cancer cell lines in vitro and, importantly, the well established C3 mouse model in vivo.

Titanium salan complexes were synthesized in high yields

Complexes 1 and 2 were accessible by refluxing the appropriate phenol, \( N,N' \)-diacetylbenzylamine, and formaldehyde in methanol and metallating the resulting ligands with titanium tetraisopropoxide (Ti(O\( \left( \text{OPr}\right)\)4) [18]. To remove trace impurities, the complexes were recrystallized at least once from \( \alpha \)-hexane or toluene and purities were proven by combustion analysis (for details see materials and methods). This elegant two step synthesis allowed for the preparation of the complexes in high yields (more than 10 g, respectively) and purity (Figure 1).

Complexes 1 and 2 are able to suppress cell proliferation in the murine C3 model of cervical cancer but also in various human cancer cells in vitro

To test efficacy of complexes 1 and 2 in C3 cells, both compounds were tested using the well-established AlamarBlue assay. This assay commonly used to determine cell proliferation is known to be highly reproducible and more sensitive than the MTT assay. The obtained dose-activity curves for complexes 1 and 2 in C3 cells are shown in Figure 2. With an IC\( _{50} \) value of \( 2.2 \pm 0.1 \mu M \) complex 1 was highly active in C3 cells. In fact, it represents one of the most active titanium salan complexes found so far. Complex 2 was slightly less toxic, the IC\( _{50} \) value was \( 0.6 \pm 1.3 \mu M \) (Figure 2). Both IC\( _{50} \) values are given as mean values from at least three independent experiments each done in four replicates. These IC\( _{50} \)-values are comparable to those found for these complexes in the human cancer cell lines HeLa S3 and Hep G2.

To further show the broad applicability of these halogen substituted titan salan complexes, complex 1, representing the most active complex, was sent to the US National Cancer Institute (NCI) for testing its activity in 57 human tumor cell lines (NCI60 anticancer drug screen). In 17 cell lines the complex was only slightly active with an IC\( _{50} \) value of more than 100 \( \mu M \). In particular, it was not toxic to every of the leukemia cell lines tested so far. In further 20 cell lines, the complex showed a mediocre activity between 10 and 100 \( \mu M \). In 19 tumor cell lines, the complex was highly active with IC\( _{50} \) values below 10 \( \mu M \). Especial melanoma cells seem to be extremely sensitive to titanium salan complexes. Together, we conclude that complex 1 suppresses tumor cell proliferation in vitro in more than two thirds of all cell lines investigated (Table 2, 3, and 4). The complete data are shown in the supporting information (Figure S1).

Both titanium salan complexes are well tolerated in animals up to single doses of 25 mg/kg

In a first experiment we investigated the tolerance of titanium salan complexes 1 and 2 in direct comparison to conventional cisplatin in animals. Thereby, we hypothesized that the complexes are much more tolerated in direct comparison to cisplatin and decided to compare a well established dose of cisplatin in the murine model with different doses of the compounds. Mice (n = 6/group) received 2 mg/kg cisplatin [21] or once 50, 25, 12.5 or 5 mg/kg of the complexes 1 and 2, respectively. Noteworthy, after 30 days all mice of the cisplatin group and all animals of the 50 and 25 mg/kg groups (complex 1 and 2, respectively) displayed clear signs of side effects like hair loss, apathy up to death (data not shown). Interestingly, all animals of the other complex-treated groups (25, 12, 5 mg/kg, respectively) survived and no fatal side effects were observed by eye. From this outcome we decided to test first a 30 mg/kg dose given at multiple applications (m.a.) (5 mg/kg on days 0, 2, 4, 7, 9, 11, respectively [30 mg/kg m.a. group]) versus a 50 mg/kg dose also given at m.a. (30 mg/kg on day 0 and 20 mg/kg on day 9 [50 mg/kg m.a. group]) treatment regime in the next set of experiments.

Figure 1. Synthesis of the salan ligands and titanium salan complexes. The halo-salan ligands L1 and L2 were synthesised via a Mannich-condensation of the respective phenol, formaldehyde and \( N,N' \)-ethylenediamine. The ligands were then metallated with Ti(O\( \left( \text{OPr}\right)\)4 in toluene to give complexes 1 and 2.

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Titanium salan complexes mediate tumor regression in a transplantable mouse tumor model for cervical cancer

We were interested to investigate the therapeutic in vitro effects of the two titanium salan complexes and took advantage of the well established C3 tumor model of cervical cancer [22]. After animals (n = 10/group) had developed palpable tumors (2–4 mm² in average) treatment was started (day 0).

Two groups received 30 mg/kg at m.a. (days 0, 2, 4, 7, 9, 11) and control animals received DMSO/PBS/Tween in the same time scale. We observed a significantly reduced tumor growth in the 30 mg/kg m.a. group that received complex 1 (122±23 mm² vs 240±32 mm² in the control group, p-value: 0.008) at day 31 whereas the effect was dramatically enhanced within the 30 mg/kg m.a. dose group treated with complex 2 (34±12 mm², p-value to the control group: 0.0001, p-value to the 30 mg/kg m.a. complex 1 group: 0.03) (table 5).

In order to determine the effects of a 50 mg/kg dose regime combined with a lower application frequency on the tumor growth, two further groups were treated with 30 mg/kg (day 0) followed by an injection of 20 mg/kg (day 9) with the respective titanium salan complex (50 mg/kg m.a.). Compared to the control group (DMSO/PBS/Tween) both compounds mediate significant tumor regression (p-values for both complexes: 0.0001).

Interestingly, no total tumor regressors were found within the 30 mg/kg m.a. groups. In contrast, 2 total tumor regressors (no tumor palpable) were documented in the 50 mg/kg complex 1 group.

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**Table 2.** IC₅₀ values of titanium salan complex 1 in Breast-, Colon-, and CNS human cancer cell lines.

| Breast Cancer | IC₅₀ | Colon Cancer | IC₅₀ | CNS Cancer | IC₅₀ |
|---------------|------|--------------|------|------------|------|
| MCF7          | >100 μM | COLO 463 μM  | 2.4 μM | SF-268 7.4 μM |
| MDA-MB-231/ATCC | >100 μM | HCT-116 29.2 μM | 2.9 μM | SF-295 6.0 μM |
| HS 578T       | >100 μM | HCT-15 12.6 μM | 2.9 μM | SF-539 >100 μM |
| BT-549        | 32.5 μM | HT29 8.7 μM  | 2.9 μM | SNB-19 >100 μM |
| T-47D         | 60.1 μM | KM12 17.6 μM | 2.9 μM | SNB-75 8.8 μM |
| MDA-MB-468    | 9.9 μM | SW-620 9.4 μM | 2.9 μM | U251 4.6 μM |

Cells were cultivated in 96 well plates and incubated with a dilution series containing five different concentrations of complex 1. The cells were fixed, stained with sulforhodamine B and compared to a control. From the dose-response curves (see supplements, Figure S1), the IC₅₀ values were calculated. The full test protocol is available under http://dtp.nci.nih.gov/branches/btb/ivclsp.html.

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**Table 3.** IC₅₀ values of titanium salan complex 1 in Melanoma-, Lung-, and Renal human cancer cell lines.

| Melanoma | Lung Cancer | IC₅₀ | Renal Cancer | IC₅₀ |
|----------|-------------|------|--------------|------|
| LOX IMVI | A549/ATCC   | 6.5 μM | 39.8 μM | 786-0 14.8 μM |
| MALME-3M | EKVX        | 4.3 μM | 59.5 μM | A498 6.8 μM |
| M14      | HOP-92      | 6.7 μM | 3.6 μM | ACHN >100 μM |
| MDA-MB-435 | NCI-H226 | 5.7 μM | >100 μM | CAK0-1 44.9 μM |
| SK-MEL-2 | NCI-H23     | 37.6 μM | 6.6 μM | RXF 4.4 μM |
| SK-MEL-28 | NCI-H322M   | 5.4 μM | 20.3 μM | SN12C 43.3 μM |
| SK-MEL-5 | NCI-H460    | 4.3 μM | 30.9 μM | TK-10 >100 μM |
| UACC-257 | NCI-H522    | 12.6 μM | 5.3 μM | UO-31 24.2 μM |
| UACC-62 | >100 μM     |      |      |      |

Conditions of treatment, see table 2.

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group (tumor size: 12 ± 8 mm²) and 3 total regressors were observed within the complex 2 treated animals (tumor size: 11 ± 6 mm²). In direct comparison of the 30 mg/kg vs the 50 mg/kg groups, both complexes are more effective at higher doses (p-values: 0.0003 for complex 1 and 0.1 for complex 2) (table 5).

But in contrast to the 30 mg/kg m.a. application regime side effects like alopecia and apathy were observed in all animals treated with 50 mg/kg, especially in the complex 1 group (data not shown). From this set of experiments we conclude that titanium salan complex 2 is well tolerated and highly active in vitro at 30 mg/kg dose given at m.a.

Next, we wanted to know if the observed therapeutic tumor effect of the well tolerated complex 2 (30 mg/kg at m.a.) could be enhanced without strengthening any side effects. Therefore, tumor-bearing animals (n = 10/group) were treated either with 30 mg/kg at m.a. (day 0: 30 mg/kg, day 7: 20 mg/kg) or with a single dose of 30 mg/kg (day 0). Again, control animals received DMSO/PBS/Tween (days 0, 7) and reached at day 29 a tumor size of 268 ± 24 mm². A comparable therapeutic effect as in the first set of in vivo experiments was observed in the 50 mg/kg m.a. group (tumor size: 12 ± 3 mm², p-value to the control: <0.0001) (see table 6). As mentioned above, side effects like alopecia and apathy were observed. In contrast, animals of the 30 mg/kg s.a. group developed significant larger tumors (64 ± 9 mm², p-value to the 50 mg/kg m.a. group: <0.0001); and no alopecia but mild apathy were observed. A comparison of the 30 mg/kg m.a. (table 5) with the 30 mg/kg s.a. (table 6) reveals higher efficacy if the drug is applied distributed over 11 days (p-value: 0.06). The single application (s.a.) of 30 mg/kg of complex 2 is therefore less effective than the application of the same complex amount at multiple days.

Together, these experiments imply that the titanium salan complex 2 combines strong antitumor effects in vitro and in vivo and is highly tolerated when given at low doses distributed over multiple injections.

**Discussion**

In this study we were able to confirm the cytotoxicity of two titanium salan complexes in various cell lines and showed for the first time the activity of this class of complexes in vivo. We were able to demonstrate that one out of two complexes investigated in this study combines high antitumoral effects with a low adverse reaction profile.

Titanium salan complexes represent a promising group of chemotherapeutic agents, belonging to the class of metallo drugs. Cisplatin as the first metallo therapeutic agent used in chemotherapy was first synthesized in the nineteenth century but clinical application did not commence until the 1970s. After this major landmark in the history of successful drugs for the treatment of cancer, carboplatin as a safer second generation analogue was introduced approximately ten years later. Next, the development of improved analogues slowed for many years but recently the interest in well tolerated and effective metallo therapeutic compounds has increased. Aiming for more effective and less toxic substances, titanium complexes attracted attention. Herein, we could show that such titanium complexes are effective in a variety of cancer cells. In tests on 57 human cancer cell lines, titanium salan complexes showed mediocre activity in 20 cell lines and high activity with IC₅₀ values below 10 μM in 19 tumor cell lines. These results encouraged us to further investigate the applicability of these complexes as anti cancer drugs. As we decided to use the well established mouse C3 model for cervical cancer, we tested the activity of our complexes against C3 cells in vivo and, more importantly, in vitro.

Indeed, cervical cancer is one potential field of application for the titanium salan complexes due to the fact that survival in women with recurrent or metastatic cervical cancer remains poor and therefore, more effective and less toxic regimes are needed. The superiority of the complex 2 in contrast to the complex 1 counterpart is especially interesting because showed a nearly four fold higher activity against C3 cells in vitro. This observation could be explained by the much higher stability of complex 2 in aqueous media. With a half life of 106 h, this complex is nearly 20 times as stable as its fluoro counterpart complex 1. It seems that a hydrolytic detoxification of complex 1 leads to a lower effective dose of the active substance on the one hand. On the other hand,

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**Table 4.** IC₅₀ values of titanium salan complex 1 in Ovarian-, Leukemia-, and Prostate human cancer cell lines.

| Ovarian Cancer | IC₅₀ | Leukemia | IC₅₀ | Prostate Cancer | IC₅₀ |
|---------------|-----|----------|-----|----------------|-----|
| IGROV1        | 6.2 μM | CCRF-CEM | >100 μM | PC-3 | 28.6 μM |
| OVCAR-3       | 4.6 μM | HLR-60(TB) | >100 μM | DU-145 | 21.0 μM |
| OVCAR-4       | 78.7 μM | K-562 | >100 μM |     |     |
| OVCAR-5       | 57.2 μM | MOLT-4 | >100 μM |     |     |
| OVCAR-8       | >100 μM | SR | >100 μM |     |     |
| NCI/ADR-RES   | >100 μM |     |     |     |     |
| SK-OV-3       | 59.1 μM |     |     |     |     |

Conditions of treatment, see table 2. doi:10.1371/journal.pone.0017869.t004

**Table 5.** Growth of established C3 tumors in C57BL/6 mice after treatment with titanium salan complexes 1, 2.

| Complex | Average tumor size at day 0 (mm²) ± S.E.M | Average tumor size at day 31 (mm²) ± S.E.M | Number of total regressors at day 31 |
|---------|-------------------------------------------|-------------------------------------------|-------------------------------------|
| Complex 1* (30 mg/kg m.a.) | 3 ± 2 | 122 ± 23 | 0 |
| Complex 2* (30 mg/kg m.a.) | 3 ± 1 | 34 ± 12 | 0 |
| Complex 1* (50 mg/kg m.a.) | 2 ± 1 | 12 ± 8 | 2 |
| Complex 2* (50 mg/kg m.a.) | 4 ± 2 | 11 ± 6 | 3 |
| DMSO/PBS/0.5% Tween 80 | 2 ± 1 | 240 ± 32 | 0 |

Mice received tumor cells s.c. into the flank and were treated either with 30 mg/kg at multiple applications (m.a.) (days 0, 2, 4, 7, 9, 11 with 5 mg/kg, respectively) or with 50 mg/kg at m.a. (day 0: 30 mg/kg and day 9: 20 mg/kg). Data gives the mean ± SEM at days 0 and 31 and the number of completely tumor free animals. Because of the size of tumors in the control group the experiment was terminated at day 31. One representative of two experiments is shown. doi:10.1371/journal.pone.0017869.t005
Table 6. Growth of established C3 tumors in C57BL/6 mice after treatment with titanium salan complexes 1, 2.

| Complex | Average Tumor Size at Day 0 (mm²) ± S.E.M | Average Tumor Size at Day 29 (mm²) ± S.E.M | Number of Total Regressors at Day 29 |
|---------|------------------------------------------|------------------------------------------|-------------------------------------|
| 2 (30 mg/kg s.a.) | 2 ± 1 | 64 ± 9 | 0 |
| 2 (50 mg/kg m.a.) | 2 ± 1 | 12 ± 3 | 2 |
| DMSO/PBS/0.5% Tween 80 | 3 ± 2 | 268 ± 24 | 0 |

Mice received tumor cells s.c. into the flank and were treated either with 50 mg/kg at multiple applications (m.a.) (day 0: 30 mg/kg and day 7: 20 mg/kg) or with a single application (s.a.) of 30 mg/kg on day 0 (6). Data gives the mean ± SEM at days 0 and 29 and the number of completely tumor free animals. Because of the size of tumors in the control group the experiment was terminated at day 29. One representative of two experiments is shown.

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Materials and Methods

Synthesis of the ligands

Synthesis of the complexes was conducted using standard Schlenk technique, all solvents were purified according to standard procedures. NMR spectra were recorded on a JEOL Eclipse 400 or a Bruker Avance DRX 600 spectrometer. Structure assignments were done based on 2D NMR experiments (COSY, HMBC, HSQC). Elemental analyses were performed at the microanalytical laboratory of the University of Constance.

The respective (20 mmol) 2,4-disubstituted phenol was suspended in methanol (10 ml) and formaldehyde solution (7.5 ml, 36%) and N,N'-dimethylethylene diamine (10 mmol) were added. The mixture was refluxed for 24 h and allowed to cool to room temperature. Upon cooling to 6°C, the products crystallized as colorless solids.

1: 1H-NMR (600 MHz, CDCl3): δ = 6.76–6.72 (m, 2H, H(2)), 6.52–6.48 (m, 2H, H(6)), 5.09 (sept., J = 6.1 Hz, 2H, Ti(OCH)2), 4.62 (d, J = 13.7 Hz, 2H, NCH2Car), 3.15 (d, J = 13.7 Hz, 2H, NCH2Car), 2.97 (d, J = 9.4 Hz, 2H, NCH2CH2N), 2.46 (s, 6H, 2x NCH3), 1.90 (d, J = 9.4 Hz, 2H, NCH2CH2N), 1.28 (d, J = 6.2 Hz, 6H, CH(CH2)2), 1.24 ppm (s, J = 6.2 Hz, 6H, CH(CH2)2). 13C-NMR (101 MHz, CDCl3): δ = 153.3 (dd, J = 237.1 Hz, 11.2 Hz, FCu), 150.6 (dd, J = 246.3 Hz, 12.4 Hz, FCu), 146.5 (dd, J = 12.4 Hz, 2.8 Hz, CHCu), 126.0 (dd, J = 8.5 Hz, 4.1 Hz, OCa), 110.4 (dd, J = 22.5 Hz, 3.3 Hz, HCa), 103.7 (dd, 26.1 Hz, 22.5 Hz, HCa), 78.9 (TiOCH), 63.7 (dd, J = 2.8 Hz, 2.2 Hz, NCH2Car), 51.8 (NCH2CH2N), 46.9 (NCH3), 25.8 ppm CH(CH3)2). Elemental analysis calc’d (%) for C18H20Cl4N2O2: C: 49.34; H: 4.60; N: 6.39, found: C: 49.38; H: 4.51; N: 6.33.

Synthesis of the complexes

The ligands (3.9 mmol) were dissolved in toluene (20 ml) and Ti(OPr)4 (3.9 mmol, 420 mg, 860 μl) was added under nitrogen atmosphere. The yellow reaction mixture was allowed to stir over night at room temperature. The solvent was removed under reduced pressure and the obtained yellow solid was recrystallized to give complexes 1, 2.

1: 1H-NMR (600 MHz, CDCl3): δ = 6.78–6.72 (m, 2H, H(2)), 6.52–6.48 (m, 2H, H(6)), 5.09 (sept., J = 6.1 Hz, 2H, Ti(OCH)2), 4.62 (d, J = 13.7 Hz, 2H, NCH2Car), 3.15 (d, J = 13.7 Hz, 2H, NCH2Car), 2.97 (d, J = 9.4 Hz, 2H, NCH2CH2N), 2.46 (s, 6H, 2x NCH3), 1.90 (d, J = 9.4 Hz, 2H, NCH2CH2N), 1.28 (d, J = 6.2 Hz, 6H, CH(CH2)2), 1.24 ppm (s, J = 6.2 Hz, 6H, CH(CH2)2). 13C-NMR (101 MHz, CDCl3): δ = 153.3 (dd, J = 237.1 Hz, 11.2 Hz, FCu), 150.6 (dd, J = 246.3 Hz, 12.4 Hz, FCu), 146.5 (dd, J = 12.4 Hz, 2.8 Hz, CHCu), 126.0 (dd, J = 8.5 Hz, 4.1 Hz, OCa), 110.4 (dd, J = 22.5 Hz, 3.3 Hz, HCa), 103.7 (dd, 26.1 Hz, 22.5 Hz, HCa), 78.9 (TiOCH), 63.7 (dd, J = 2.8 Hz, 2.2 Hz, NCH2Car), 51.8 (NCH2CH2N), 46.9 (NCH3), 25.8 ppm CH(CH3)2). Elemental analysis calc’d (%) for C18H20Cl4N2O2: C: 49.34; H: 4.60; N: 6.39, found: C: 49.38; H: 4.51; N: 6.33.

Cell culture

C3 tumor cells (C57BL/6 origin) derived from embryonic mouse cells transfected with the complete HPV-16 genome [22] were cultured in RPMI 1640 supplemented with heat-inactivated 5% (v/v) fetal calf serum (FCS, Gibco, Egggenstein, Germany), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml)
The cells were then incubated for 48 h with 100 μl DMSO/PBS/0.5% Tween 80 and control animals received DMSO/PBS/0.5% Tween 80 only. The treatment was repeated as mentioned up to five times. Tumor sizes were measured with a caliper and were determined every 2–4 days until mice had to be sacrificed (tumor size of 400 mm² or when tumors were bleeding). Tumor sizes of the mice within a group were calculated as arithmetic means with standard error of the means (SEMs). All operations on live animals were performed under Isoflurane anesthesia (CuraMed Pharma, Karlsruhe, Germany).

Statistical analysis
Differences of means between experimental and control group were considered statistically significant when p was <0.05 by unpaired Students t-test.

Supporting Information
Figure S1 IC₅₀values of titanium salan complex 1 in different human cancer cell lines. Dose response curves of complex 1 in 57 different human cancer cell lines. The data show the broad spectrum of activity of titanium salan complexes. (TIF)

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Author Contributions
Conceived and designed the experiments: TAI UG TH PO¨ . Performed the animal experiments.

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