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The Activity of Titanocene T Against Xenografted Caki-1 Tumors

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Abstract: The indole-substituted titanocene dichloride derivative Titanocene T, which is completely water-soluble and shows micromolar activity against the human renal cancer cells Caki-1, was tested *in vivo* an against xenografted human renal cell tumors in mice. Titanocene T was then given at 25 and 50 mg/kg, seven times every four days during three weeks to two groups (n=6) of Caki-1 tumor-bearing female NMRI:nu/nu mice, while the control group was treated with solvent only. At both doses Titanocene T induced a moderate to good tumor growth reduction with respect to the solvent-treated control group, with an optimal T/C value of 51% and 32% and showed neither mortality nor toxicity. Immunohistochemical analysis revealed that the expression of the proliferation marker Ki-67 was reduced in the high-dosage group. Furthermore, anti-angiogenic activity was identified by CD31 staining; the number of micro vessels in a defined tumor area decreased by 27% and 29% due to Titanocene T treatment.

Keywords: Anticancer drug, anti-angiogenic drug; Titanocene; Renal cell cancer; Xenograft.

INTRODUCTION

Renal cell carcinoma (RCC) is the most common malignant disease of the adult kidney, which accounts for approximately 3% of adult malignancies [1]. If not detected early, these cancers develop to an invasive adenocarcinoma, which have very limited treatment options and poor outcomes. New targeted compounds like Bevacizumab, Sunitinib, Sorafenib, Temsirolimus and others give a certain amount of hope to patients with advanced renal-cell cancer, since these compounds can block the VEGF or mTOR pathway and are therefore anti-angiogenic [2]. Nevertheless these new drugs cannot cure advanced or metastatic renal cell cancer and give the patient only a few extra months of survival. These clinical facts suggest new therapeutic regimens must be explored in the quest to develop an effective therapy for these metastatic or advanced forms of renal cell cancer.

There is significant unexplored space for titanium-based drugs targeting cancer. Titanocene dichloride reached clinical trials, but the efficacy of this compound in Phase II clinical trials in patients with metastatic renal cell carcinoma [3] or metastatic breast cancer [4] was too low to be pursued. The field got renewed interest with P. McGowan's elegant synthesis of ring-substituted cationic titanocene dichloride derivatives, which are water-soluble and show significant activity against ovarian cancer [5]. More recently, novel methods starting from fulvenes [6] allow direct access to antiproliferative titanocenes *via* reductive dimerisation with titanium dichloride, hydridolithiation or carbolithiation of the fulvene followed by transmetallation with titanium tetrachloride in the latter two cases.

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Hydridolithiation of 6-anisyl fulvene and subsequent reaction with titanium tetrachloride led to bis-[(*p*-methoxybenzyl)-cyclopentadienyl] titanium(IV) dichloride (Titanocene Y) [7], which has an IC50 value of 21 μM when tested on the LLC-PK cell line. In addition, the anti-proliferative activity of Titanocene Y and other titanocenes has been studied in 36 human tumor cell lines [8] and against explanted human tumors [9, 10]. Previous work has demonstrated that Titanocene Y induces apoptosis in a caspase-independent manner in a range of prostate cancer cells and it maintained its cytotoxic effects in Bcl-2 over expressing PC-3 cells [11]. Besides the direct apoptosis-inducing effects these titanocene derivatives give a positive immune response by up-regulating the number of natural killer (NK) cells in mice [12] and Titanocene Y proved itself as a strong anti-angiogenic compound with an IC50 value of 4.9 μM shown in a spheroid-based cellular angiogenesis assay [13]. Recently, animal studies reported the successful treatment of mice bearing xenografted A431 [14], PC-3 [15], Caki-1 [16] and MCF-7 [17] tumors with Titanocene Y and against Caki-1 [18] with the isostructural Vanadocene Y. In these xenograft experiments a good tumor growth reduction (Caki-1) or even a reduction of the tumor volume upon long-term treatment (MCF-7) and no severe side effects were observed. The body weight loss due to diarrhoea was moderate; no influence on haematological (white blood cells, platelets) and renal toxicity - associated (creatinine, blood urea nitrogen) parameters was noticed. More recently, bis-[(*p*-methoxybenzyl) cyclopentadienyl] titanium oxalate (Oxali-Titanocene Y) was synthesised [19] and showed a 13-fold cytotoxicity impro-vement on LLC-PK cells leading to an IC50 value of 1.6 mM. Furthermore, 4-diethylaminomethyl-benzyl-substituted titanocene dichloride (Titanocene Y*), which is a water-soluble titanocene derivative exhibiting nanomolar activity against Caki-1 *in vitro* [20] showed anti-tumoral activity in combination with high toxicity *in vivo* [21].

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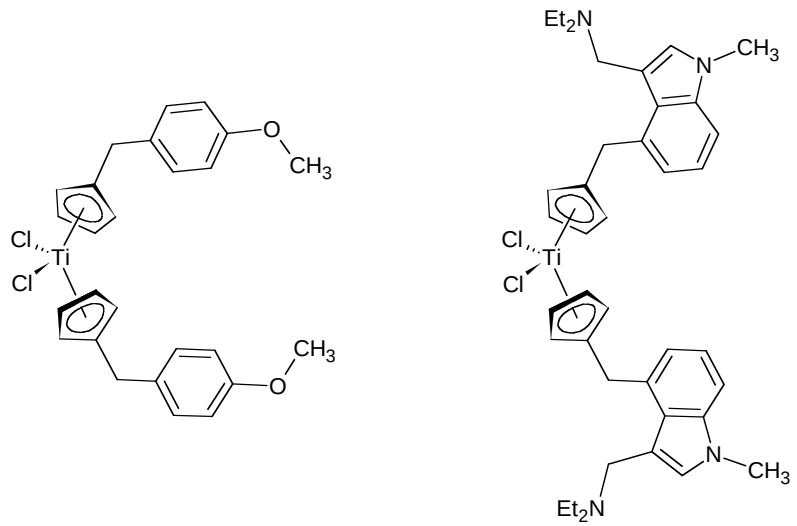

Fig. (1). Molecular structures of Titanocene Y (left) and Titanocene T (right).

This paper is now investigating the anti-proliferative effect of Titanocene T, which is a novel water-soluble analogue of Titanocene Y with micromolar cytotoxicity [22], in a Caki-1 xenograft mouse model *in vivo*.

The structures of Titanocene Y and Titanocene T are shown in Fig. (**1**).

MATERIALS AND METHODS

In Vivo Caki-1 Xenograft Model

An experiment was carried out to determine the maximum tolerable dose (MTD) of Titanocene T. The compound was dissolved in saline, which resulted in a clear and stable solution. Then male NMRI:nu/nu mice (2 mice per group) were treated with 25, 50 and 100 mg/kg/d intraperitoneally (i.p.) in single injections in order to determine the approximate MTD.

In the therapeutic in vivo experiment 10^7 Caki-1 cells (expanded in vitro in McCoy´s medium + 10% fetal bovine serum) were injected subcutaneously (s.c.) in a volume of 0.1 ml to male NMRI:nu/nu mice (6 mice per group) on day 0. When tumors were grown to a palpable size (5-6 mm diameter) mice were randomized and treatment was initiated on day 8. For groups B and C Titanocene T was injected into mice i.p. using a dose of 25 or 50 mg/kg once at days 8, 12, 16, 23, 27, 29 and 34, while the control group (A) of mice was treated with the solvent only as negative control. Tumor size was measured with a caliper instrument. Tumor growth (given as tumor volume, cm^3) was monitored during the entire experiment with the measurements of two perpendicular tumor diameters using the spheroid equation: tumor volume = [(tumor width)^2 x tumor length] x 0.5. Furthermore, the relative tumor volumes (relation to the first treatment day) and treated to control (T/C) values were calculated. Body weight and lethality of the mice were determined continuously during the experiments for an estimation of tolerability. Mice were sacrificed 24 h after their last treatment. Tumors were taken, snap frozen and used for immunohistological investigations. The animal experiments were performed according to the German Animal Protection Law and with approval from the responsible authorities. The *in vivo* procedures were consistent and in compliance with the UKCCCR guidelines.

Immunohistochemistry

The 5 μm thick sections from snap frozen Caki-1 tumors (4 per group) were fixed with 3.7% paraformaldehyde, blocked with H_2O_2 and goat serum. After incubation with the primary antibody (rat anti-mouse CD31, clone: MEC13.3, BD Pharmingen, Heidelberg, Germany), slides were incubated with an HRP-labelled goat anti-rat antibody (Jackson ImmunoResearch, Hamburg, Germany), DAB substrate (Dako, Hamburg, Germany) and counterstained with hematoxylin. Evaluation of micro vessel density was performed with AxioVision 4.5 from ZEISS (Jena, Germany). Vessels were labelled in six representative pictures of each tumor and quantified for following parameters: Micro vessel size, number and ratio (micro vessel area vs. total tumor area). For the staining of the Ki-67 protein, fixation and blocking was performed according to CD31. The slides were incubated with the primary mouse anti-human Ki-67 antibody (Dako; clone: MIB-1). A secondary antibody, the HRP-labeled anti-mouse antibody was used. For staining the DAB substrate and for couterstaining hematoxylin was used (Dako). For evaluation of Ki-67 expression five pictures of each tumor were taken and the number of positive vs. negative cells was counted in three fields of view.

Statistical Analysis

The statistical analysis was performed by the 1-way ANOVA Dunnett post test with 95% confidence intervals, using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The levels of statistical significance were defined by the p-value of $p<0.05$ (*) or $p<0.001$ (***).

RESULTS

The *in vitro* cytotoxicity of Titanocene T was determined earlier in a MTT-based Caki-1 assay, which showed an IC_{50} value of 13 μM [22]. Therefore, it was decided to proceed with the Caki-1 xenograft experiments in order to evaluate the antitumoral potential of the novel titanocene derivative Titanocene T *in vivo*.

Table 1. Overview on results obtained in the Caki-1 xenograft experiment. For tumor development, female nude mice received subcutaneous tumor cell injections on day 0. At palpable tumor size on day 6 the mice were treated with Titanocene T or solvent. Tumor size in the treated group in relation to the control group (T/C) was measured as a therapeutic marker, while body weight change (BWC) was used as a toxicity parameter. The number of Ki-67 positive cells was determined by immunohistochemistry. For the detection and semi-quantisation of micro vessels (mv) in a defined tumor area tumor sections were stained with a CD31-antibody.

Respective group marked with (*) is statistically significant compared to group A ($p<0.05$); or marked with (***) are highly statistically significant compared to group A ($p<0.001$).

Group	Number	Substance	Treatment	Dose	Deaths	BWC (%)	Opt. T/C (%)	Ki-67	CD31
	of mice		[on day]	(mg/kg/d)	[on day]	(max)	[on day]	(% pos cells)	mv nr.
A	6	Solvent	8, 12, 16, 23,		0/6	0		1.7 ± 1.2	86 ± 33
			27, 30, 34						
B	6	Titanocene T	8, 12, 16, 23,	25	0/6	0	51	1.7 ± 1.2	63 ± 15***
			27, 30, 34				[16]		
C	6	Titanocene T	8, 12, 16, 23,	50	0/6	-5	32*	1.3 ± 0.6	61 ± 21***
			27, 30, 34				[23]		

(*): $p<0.05$; (***): $p<0.001$

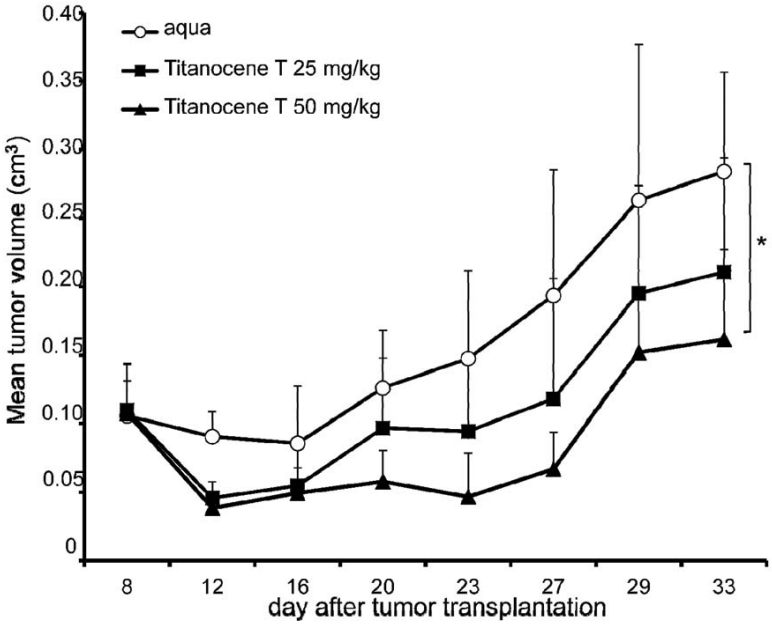

Fig. (2). Tumor growth curves of Caki-1 xenografts in NMRI nu/nu mice comparing the two Titanocene T treated groups (25 mg/kg; n=6, or 50 mg/kg Titanocene T; n=6) against the solvent (aqua) treated control group (n=6). The mean values of the respective tumor volumes are depicted. Error bars represent SD-values. Level of significance was determined by 1-way ANOVA Dunnett post test ($p<0.05$) and is indicated by (*).

IN VIVO EFFICACY

To test *in vivo* tolerability of Titanocene T, we first performed the maximum tolerated dose (MTD) test. In the MTD mouse experiment, three groups of two mice each were treated with doses of 25, 50 and 100 mg/kg/d of Titanocene T. The first group of mice showed a body weight loss (BWC) of 2%, while the value extended to 10% and 8% in the groups receiving 50 mg/kg/d or 100 mg/kg/d of Titanocene T. Only the highest dose led to toxic death of one animal after treatment. However, all other mice recovered their body weight loss within 5 days. From this MTD study doses of 25 and 50 mg/kg were chosen for the therapeutic experiment with an extended treatment (7 times every 4 days) period for the Caki-1 tumor-bearing mice.

In the Caki-1 s.c. xenograft experiment all tumors grew progressively and the tumors reached a palpable size on day 6. Therefore three groups of n=6 mice each were treated by intraperitoneal injections with solvent (controls) or Titanocene T (25 or 50 mg/kg) at days 8, 12, 16, 23, 27, 29 and 34. All observed parameters resulting from the *in vivo* experiment using Caki-1 xenografts are presented in Table **1**.

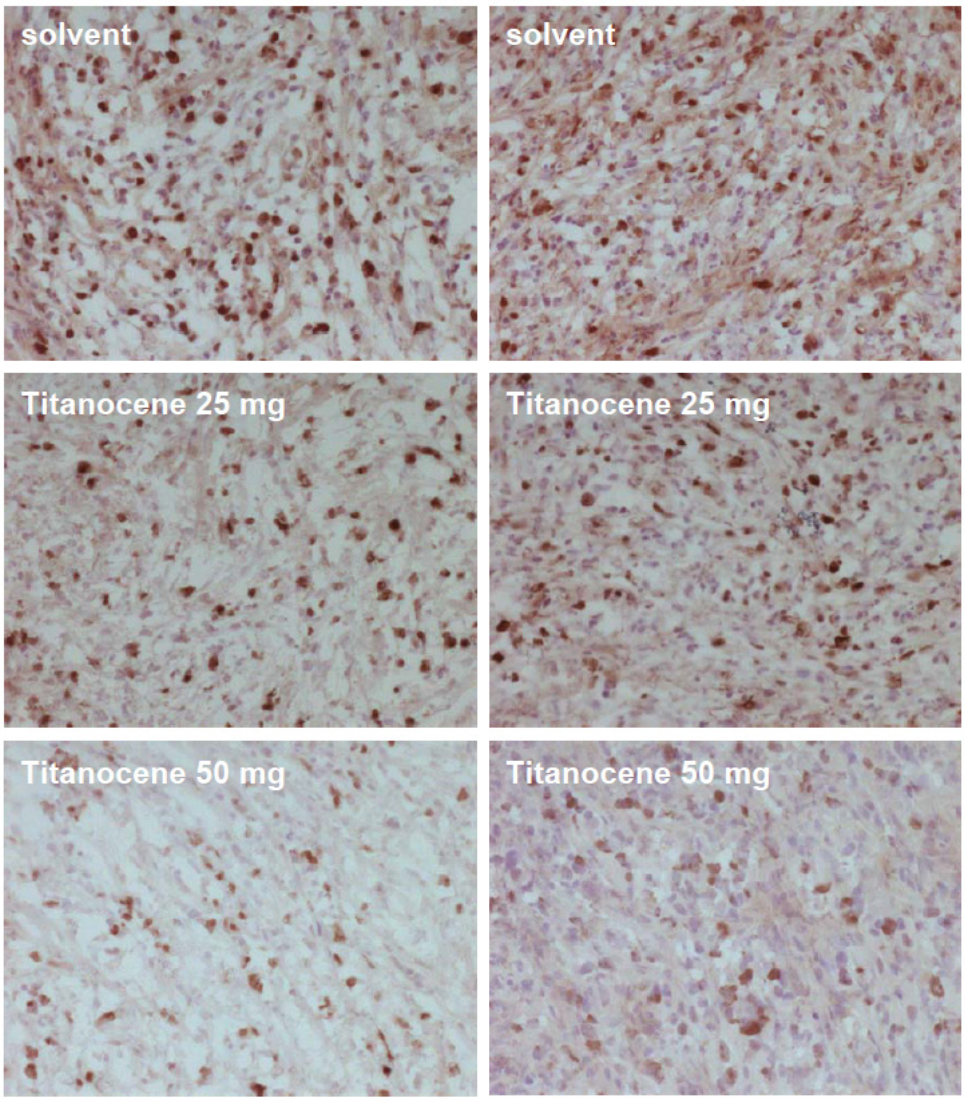

Fig. (3). Ki-67 expression in Caki-1 xenograft tumors: The immunohistochemistry images show Ki-67 positive cells within the tumor tissues as brown staining. The images were taken from two different animals, respectively. The light microscopic images were analyzed for semi-quantitative determination of Ki-67 positive cells (see table 1). Magnification: 200 x.

The two Titanocene T treated groups (25 and 50 mg/kg) of mice showed only a transient and reversible 5% body weight loss for the highest dose and no changes for the low Titanocene T dose, as well as for the control animals. However, neither of the two Titanocene T doses led to toxic death of mice treated. Therefore, both dosage groups could be evaluated in detail and 50 mg/kg is below the MTD for tumor-bearing mice, if the treatment is given during the reported time schedule.

As shown in Fig. (**2**), in the control group A tumors grew from 0.106 ± 0.025 cm^3 on day 8 to 0.284 ± 0.072 mm^3 on day 34. However, in the treated group B (25 mg/kg Titanocene T) tumors grew from 0.110 ± 0.034 to 0.210 ± 0.084 cm^3 and in the group C (50 mg/kg Titanocene T) tumors grew from 0.108 ± 0.036 to 0.162 ± 0.065 cm^3 on day 34 after seven treatments. On day 16 the optimal treated to control (T/C) value of 51% was reached for the low-dose Titanocene T group B, while the high-dose group C reached a T/C value of 32% on day 23. Statistical analysis revealed, that this value is statistically significant ($p<0.05$) with respect to the control group A. Particularly interesting is the fact that the tumor volume in group C remained almost constant (stable disease) under treatment with Titanocene T up to day 27, while there is strong or moderate tumor growth in groups A and B. The fact, that mice in groups A and B did not suffer from any body weight change (BWC) and mice in group C experienced only a moderate BWC underlines the low toxicity of Titanocene T even in the high-dose group C.

Effect of Titanocene T on Ki-67 Expression

To evaluate potential correlation of the proliferation marker Ki-67 and Titanocene T action, we performed quantitative analyses. Ki-67 expression was quantified in sextuple in all tumors (n=6) of each group by evaluation of the respective immunohistochemistry; representative light microscopic images for Ki-67 staining are shown in Fig. (**3**). As shown in Table **1** the ratio of Ki-67 positive cells in the solvent-treated tumors was 1.7 ± 1.2%. Due to treatment with

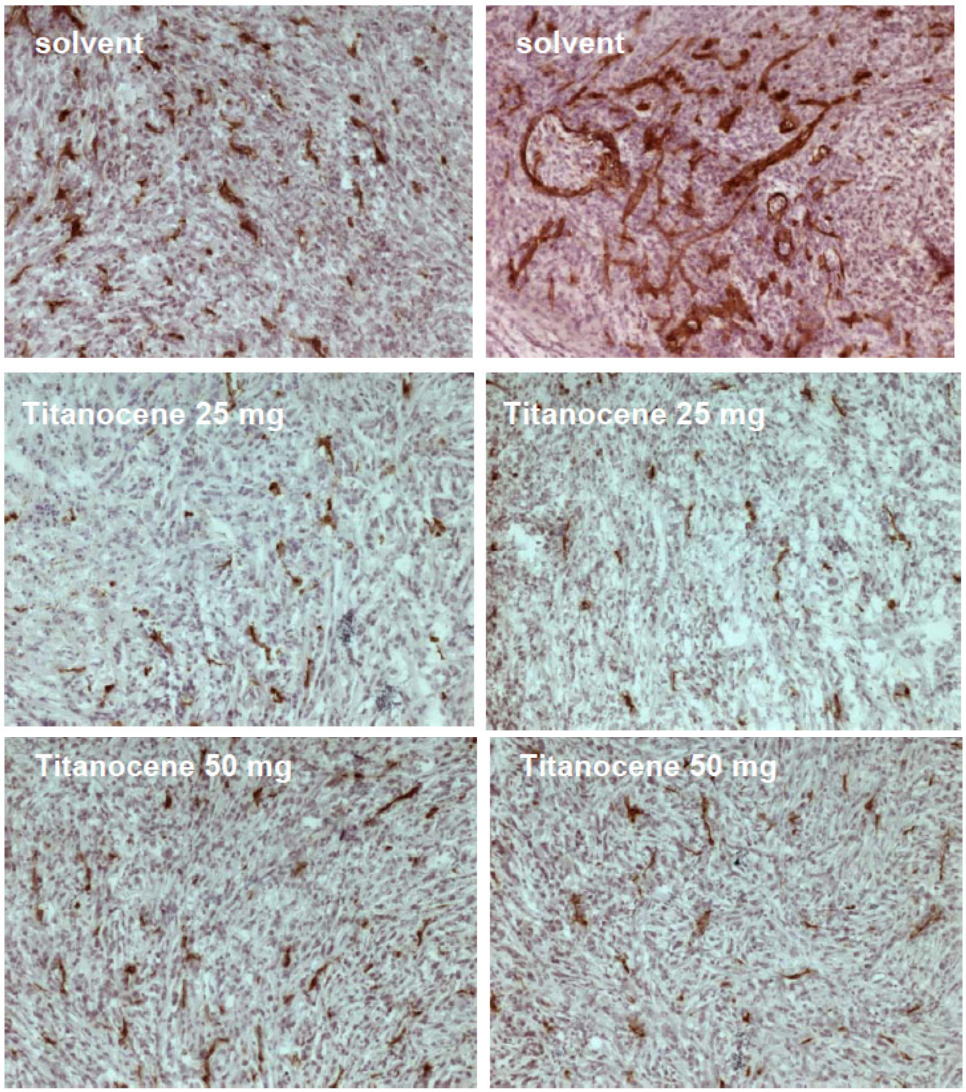

Fig. (4). Immunohistochemistry for evaluation of microvessel density in Caki-1 xenograft tumors: Light-microscopy images of representative samples per treatment group show CD31-specific brown vessel staining and blue cell nuclei. The light microscopic images were analyzed for semi-quantitative determination of CD31-positive areas (see Table **1**). The images were taken from two different animals, respectively. Magnification: 100 x.

Titanocene T the fraction of Ki-67 expressing cells was identical in group B with a value of 1.7 ± 1.2 and was decreased to only 1.3 ± 0.6% in group C. This reduction was found to be statistically insignificant (1-way ANOVA test; $p>0.05$), in part due to the high standard deviation of associated with this method. Therefore, Titanocene T treatment did not lead to alterations in the Ki-67 proliferation marker.

Effect of Titanocene T on Vascularisation

To determine, if Titanocene has anti-angiogenic activity, we analyzed the influence of the treatment on microvessel (mv) density in the tumors. Microvessels within a tumor area were detected by specific CD31 staining (Fig. **4**). The brown coloured vessels were quantified and counted by computer-based analysis in sextuple in all 6 tumors of each group. A distinct occurrence of vascularisation was determined in tumors of the control cohort, with a mean microvessel number of 86 ± 33. The treatment of mice in groups B and C with Titanocene T induced a decrease in the number of microvessels to 63 ± 15 and 61 ± 21. These reductions in groups B and C by about 27% and 29% are both highly statistically significant ($p<0.001$) with respect to group A, as determined by the 1-way ANOVA test, indicating the strong anti-angiogenic activity of this compound. This effect correlates well with the reduction in tumor growth.

DISCUSSION

This study was performed to determine the in vivo activity of Titanocene T in the Caki-1 xenograft tumor model *in vivo*, to extend the *in vitro* knowledge on the activity of this compound. Testing this new Titanocene T compound for *in vivo* activity was of particular interest, since the antitumoral activity of Titanocene Y and other Titanocenes has been studied in various human *in vitro* tumor models demonstrating their superiority over the established cisplatin [8, 9, 10, 21]. Titanocene Y has been shown to induce the effector

caspase-3 and -7 dependent apoptosis in human epidermoid carcinoma cells, but also caspase-independent apoptosis in different prostate cancer cells and, apart from the apoptotic effects, was able to up-regulate natural killer (NK) cell number in treated mice [11, 12, 14]. We therefore were interested to evaluate, if Titanocene T also has antitumoral *in vivo* activity associated with no or only minor potential side effects in the treated animals at doses comparable to those used for e.g. Titanocene Y.

Based on our MTD study, Titanocene T doses of 25 and 50 mg/kg were chosen for the therapeutic Caki-1 xenograft experiment, in which both dosage groups B and C could be evaluated, due to the good tolerability of the compound. Compared to MTD of 40 mg/kg for Titanocene Y, Titanocene T showed slightly better tolerability in the animals, reflected by the higher tolerated dose of >50 mg/kg, which is important for further development and *in vivo* testing of this compound [21].

The *in vivo* experiment of this study showed that Titanocene T induced a dose-dependent tumor growth inhibition, reflected by the moderate inhibition of optimal 51% T/C value achieved with 25 mg/kg and a stronger, significant 32% T/C value reached with 50 mg/kg. This relatively high T/C value combined with the low toxicity of Titanocene T demonstrated that the compound is active in the Caki-1 renal carcinoma xenograft model and that its therapeutic index is reasonable. Earlier studies showed similar tumor growth inhibition for Titanocene Y in the same *in vivo* tumor model at 40 mg/kg [16].

Titanocene Y proved itself as a strong anti-angiogenic compound [13]. We therefore determined this effect also for Titanocene T. Here, Titanocene T shows a highly significant diminishing effect for both Titanocene T doses on the number of microvessels in the human renal Caki-1 xenograft tumors. This evidenced its capability to interrupt neovascularisation *in vivo*. This might serve as one possible mechanism for the observed antitumoral activity of Titanocene T in the Caki-1 tumor model, paralleling the known effects of Titanocene Y [13].

However, correlation of the proliferation marker Ki-67 with the observed Titanocene T mediated tumor growth inhibition was not possible. In this regard, no significant changes were seen for Ki-67 in the two treatment groups compared to the control. In fact, this is in line with findings by Kolberg *et al.*, where also no correlation was seen between this proliferation marker and the growth inhibitory effects of Titanocene dichloride or cisplatin [23]. Further analyses will reveal, which particular mechanism (e.g. apoptosis) is associated with the in vivo tumor growth inhibition by Titanocene T.

CONCLUSION

Generally, one can conclude that Titanocene T is a water-soluble potent cytotoxic anticancer agent, which exerted a significant tumor growth inhibitory *in vivo* effect in a dose dependent way in association with anti-angiogenic activity. The treatment with Titanocene T was with no side effects at any of the two concentrations used. This makes Titanocene T the leading derivative for further detailed therapeutic *in vivo* studies and potentially an up-coming clinical trial.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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