Antitumor activity of Titanocene Y against freshly explanted human breast tumor cells and in xenografted MCF-7 tumors in mice

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Bis-[(*p*-methoxybenzyl)cyclopentadienyl] titanium dichloride, better known as Titanocene Y, is a newly synthesized transition metal-based anticancer drug. We studied the antitumor activity of Titanocene Y with concentrations of 2.1, 21 and 210 μmol/l against a freshly explanted human breast cancer, using an in-vitro soft agar cloning system. The sensitivity against Titanocene Y was highly remarkable in the breast cancer tumor in the full concentration range. Titanocene Y showed cell death induction at 2.1 μmol/l, well comparable to cisplatin, given at a concentration of 1.0 μmol/l. A further preclinical development of Titanocene Y was warranted and therefore an MCF-7 human breast cancer xenograft nonobese diabetic/severe combined immunodeficient mouse model was used. Titanocene Y was given for 21 days at 30 mg/kg/day (75% of the maximum tolerable dose of Titanocene Y), which resulted in the reduction of the tumor volume to around one-third, whereas no mouse was lost because of the surprisingly low toxicity of Titanocene Y. *Anti-Cancer Drugs* 18:311–315 © 2007 Lippincott Williams & Wilkins.

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Introduction

Despite the resounding success of cisplatin and closely related platinum antitumor agents, the movement of other transition-metal anticancer drugs towards the clinic has been exceptionally slow [1–3]. Metallocene dichlorides (Cp_2MCl_2) with M = Ti, V, Nb and Mo show remarkable antitumor activity [4–6]. Unfortunately, the efficacy of Cp_2TiCl_2 in phase II clinical trials in patients with metastatic renal cell carcinoma [7] or metastatic breast cancer [8] was too low to be pursued. A novel method starting from titanium dichloride and fulvenes [9–12] allows direct access to highly substituted *ansa*-titanocenes [13–20], titanocenes containing a carbon–carbon bridge. By using this method, we have synthesized [1,2-di(cyclopentadienyl)-1,2-di-(4-*N*,*N*-dimethylaminophenyl)-ethanediyl] titanium dichloride (Titanocene **X**), which has an IC_{50} value of 270 μmol/l when tested for cytotoxic effects on the LLC-PK cell line [17].

The cytotoxic effect was further increased by synthesizing the analogues achiral diarylmethyl substituted titanocenes, which can be obtained in a carbolithiation reaction of 6-arylfulvene with the corresponding aryl lithium species followed by a transmetallation with titanium tetrachloride [21,22]. With this new method, bis-[di-(*p*-*N*,*N*-dimethylaminophenyl)methylcyclopentadienyl] titanium (IV) (**2**) dichloride has been synthesized, which shows an IC_{50} value of 38 μmol/l when tested for cytotoxic effects on the LLC-PK cell line [21].

So far our most cytotoxic, Titanocene **Y**, was obtained through a third synthetic pathway, which has been recently published [23,24]: bis-[(*p*-methoxybenzyl)cyclopentadienyl] titanium(IV) dichloride (Titanocene **Y**), which has an IC_{50} value of 21 μmol/l when tested on the LLC-PK cell line, was synthesized from fulvene and super hydride ($LiBEt_3H$) followed by transmetallation with titanium tetrachloride. The structures of the three classes of titanocene mentioned titanocene are shown in Fig. 1.

The antiproliferative activity of Titanocene **X** and **Y** has been studied in 36 human tumor cell lines [25] and in four freshly explanted human tumors using Titanocene **X** [26]. These in-vitro and ex-vivo experiments showed that prostate, cervix and renal cell cancers are prime targets for these novel classes of titanocenes, whereas the IC_{50} values for the breast cancer cell lines were very promising

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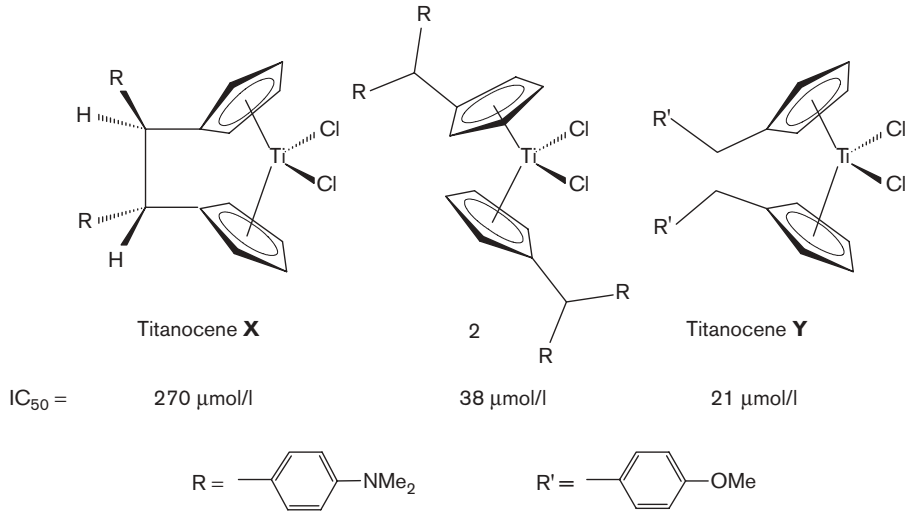
Fig. 1



Molecular structures of the titanocenes.

as well. These results were underlined by first mechanistic studies concerning the effect of these titanocenes on apoptosis and the apoptotic pathway in prostate cancer cells [27]. Furthermore, first animal studies have been recently published reporting the successful treatment of xenografted Caki-1 tumors and xenografted Ehrlich's ascites tumor in mice with Titanocene **X** and **Y** [28,29]. The effect of Titanocene **Y** against xenograft Caki-1 tumors in mice was shown to be superior to cisplatin.

In this paper, we present first ex-vivo and in-vivo results of our Titanocene **Y** tested on the hormone-dependent breast cancer cell line MCF-7. These experiments gain even more relevance under the surprising results of Jaouen *et al.* [30]. They observed an unexpected estrogenic effect when their titanocene derivative of tamoxifen was incubated in the presence of MCF-7. Even more surprisingly the same proliferative effect was shown for Cp_2TiCl_2 itself as well.

The experiments presented in this paper are based on a capillary soft agar cloning system, in which the antitumor activity of Titanocene **Y** on freshly explanted breast tumor cells was studied and compared with cisplatin. In addition, an MCF-7 xenograft model was used to allow a long-term treatment with 75% of the maximum tolerable dose of Titanocene **Y** to find a possible treatment regime.

Materials and methods

Antitumor agents

Titanocene **Y** was synthesized as described previously [23]. Solutions were prepared freshly in double-enriched CMRL medium including 9% dimethylsulfoxide. CMRL (2×) contains 500 ml CMRL 1066 medium (Gibco, Grand Island, New York, USA), 75 ml inactivated horse serum (Gibco), 10 ml fetal bovine serum (Gibco), 10 ml 2 mmol/l L-glutamine (Gibco), 6 ml 1 mmol/l nonessential amino acids (Gibco), 6 ml hydrocortisole (400 ng/ml) (Sigma, St Louis, Missouri, USA), 5 ml of 100 mmol/l sodium pyruvate (Gibco), 5 ml vitamin C (30 mmol/l) (Merck, Darmstadt, Germany), 5 ml penicillin/streptomycin solution (Gibco), 27 µl catalase solution (1 000 000 u/ml) (Serva, Heidelberg, Germany), 82 µl epidermal growth factor (EGF) solution (100 ng/ml) (Gibco), 5 ml 1 mol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid solution (Gibco), 5 ml 100 mmol/l sodium pyruvate (Gibco) and 7.5 ml asparagine solution (6.6 mg/ml) (Merck).

Titanocene **Y** was studied at final concentrations of 2.1, 21 and 210 µmol/l. Cisplatin was prepared as in clinical applications and was used at a final concentration corresponding to 0.1 of the clinically observed peak plasma concentration of 0.2 µg/ml, which corresponds to 1 µmol/l. A stock solution of cisplatin was stored at −80°C prior to use.

Capillary soft agar cloning system

For the human tumor cloning assay, breast cancer cells were obtained from a freshly biopsied breast tumor specimen that was achieved by sterile standard procedures as a part of routine clinical measures. The generation of single-cell suspensions followed instructions as described previously [31]. Human tumor cloning assay experiments were performed as described in [32,33]. In all investigations, tumor cells were exposed to Titanocene **Y** for 1 h. In negative controls, the

antitumoral compound was substituted by 0.9% sodium chloride. Positive controls contained 10^{-3} mol/l ammonium vanadate to inhibit cell growth. Colony formation was evaluated with an inverted microscope after an incubation period of 21–28 days at 37°C, 5% CO_2 and 100% humidity. Experiments were considered to be evaluable if positive controls demonstrated 30% or less colony formation compared to sodium chloride control.

Statistical analysis

Data were calculated as means and standard deviations of six replicates for each concentration and all controls. Percentage colony survival was calculated by determining the cells exposed to antitumor agent relative to calculated cells in untreated negative control. Inhibition was defined as significant if colony formation was 0.5 or less times the negative control.

MCF-7 xenografts

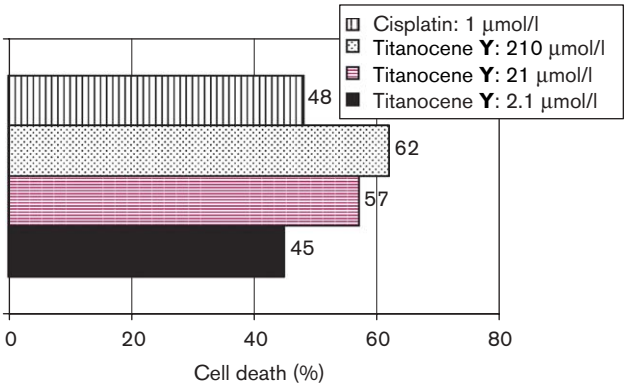
For in-vivo testing, 1×10^7 MCF-7 cells were injected subcutaneously to female nonobese diabetic (NOD)/severe combined immunodeficient mice (eight mice per group). When tumors were grown to a palpable size (5–6 mm diameter), treatment was initiated. Titanocene **Y** was dissolved in dimethylsulfoxide (final concentration 10%) and diluted with 0.5% Tween 80 in isotonic saline. It was injected intraperitoneally in doses of 30 mg/kg/day once, daily for 21 consecutive days. The control group of mice was treated with the solvent (negative control). Tumor size was measured with a caliper-like instrument. Tumor volumes were calculated as volume = length × width$^2 \times \pi/6$.

Results and discussion

The MCF-7 estrogen receptor-positive breast cancer cell line represents around 75% of the human breast cancer patients in the clinic with respect to the hormone response. Titanocene **X** and **Y** showed promising medium–high cytotoxicities *in vitro* with IC_{50} values of 116 and 76 µmol/l against MCF-7 [25]. These IC_{50} values were close enough to cisplatin (47 µmol/l) when tested on MCF-7 [25] and further investigations were especially encouraged by the fact that results by Top *et al.* [30] showed a proliferative effect of Cp_2TiCl_2 and their titanocene derivative of tamoxifen on the same cell line.

The *ansa*-Titanocene **X** was already investigated in a first ex-vivo study, where it showed a very reasonable cytotoxicity. As Titanocene **Y** showed in all cytotoxicity studies a higher antiproliferative potential, it was obvious to perform an ex-vivo experiment, in which a freshly explanted human tumor was tested against Titanocene **Y** in a capillary soft agar cloning system as well. As shown in Fig. 2, Titanocene **Y**, at a concentration of 210 µmol/l, induced a colony reduction of 62%, whereas a concentration of 21 µmol/l, which equals the IC_{50} value against LLC-PK [23], resulted in 57% of reduction. Even at the lowest concentration of only 2.1 µmol/l, Titanocene **Y** was able to reduce breast cancer cell colonies by 45%, which is nearly as effective as cisplatin given at 1.0 µmol/l. These results were even more promising than the corresponding in-vitro experiment, where the IC_{50} value for Titanocene **Y** was determined to be 76 µmol/l when tested on the MCF-7 breast cancer cell line.

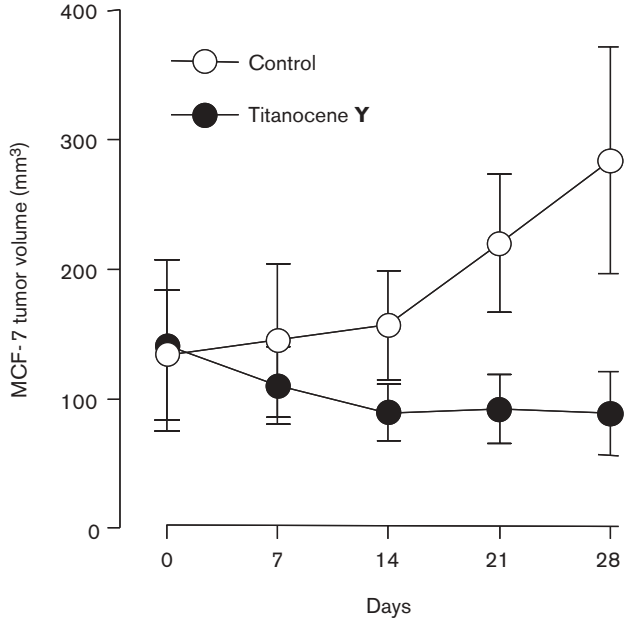
Fig. 2



Cytotoxicity data of Titanocene **Y** in comparison with cisplatin in human tumor cloning assay cell tests against breast cancer.

These very promising results in combination with the earlier xenograft results against Caki-1 [28,29] provided encouragement to do further xenograft mouse model experiments employing the well-established MCF-7 human breast cancer cell line. In this further experiment, two groups of eight mice each were treated intraperitoneally with solvent (control group) or with a dose of 30 mg/kg/day of Titanocene **Y** (treatment group, 75% of the maximum tolerable doses of Titanocene **Y**) for 21 consecutive days. Dose–response experiments were performed using the renal cell cancer cell line Caki-1 xenografted to female NMRI:*nu*/*nu* mice beforehand [28]. Therein the mice were treated with concentrations of 10, 20, 30, 40 and 50; and 40 mg/kg/day was evaluated to be the maximum tolerable dose. For this long-term experiment using MCF-7 xenografts, we decided to decrease the dose to 75% of the maximum tolerable dose per day, which corresponds to 30 mg/kg/day of Titanocene **Y**. Under these extremely long-term treatment conditions for 21 days, Titanocene **Y** induced a significant shrinkage in tumor size as shown in Fig. 3. In the control group, the tumors started at a volume of 134 mm^3 on day 0, reached 145 mm^3 on day 7 and 157 mm^3 on day 14. The tumors continued to grow as expected to 220 mm^3 and doubled their volume to 284 mm^3 on day 28 when compared to that on day 0. In the treatment group, the average tumor size reduced from 141 mm^3 on day 0 to 110 mm^3 on day 7. This trend continued on day 14 with a volume of 89 mm^3

Fig. 3



Tumor growth curves of MCF-7 xenografts in nude mice comparing a Titanocene **Y**-treated cohort against a control cohort.

and reached an almost stable value of 92 mm^3 on day 21, when the tumor shrinkage reached 35% compared to that on day 0. After the treatment was stopped on day 21, the tumor volume remained constant during the last week of the experiment leading to a stable disease on day 28 with a tumor volume of 89 mm^3. On day 28, a statistically highly significant difference was found between the control and the treatment group ($P < 0.01$), whereas the tumor reduction within the treatment was characterized by a strong trend ($P = 0.06$). In the earlier Caki-1 mouse model, Titanocene **Y** was given at concentrations up to 40 mg/kg/day for 5 consecutive days [28,29], which induced slower tumor growth (even compared to cisplatin), but no tumor shrinkage was observed. In addition, it is worthwhile to mention that none of the treated mice were lost during the application of Titanocene **Y**, which underlines the low general toxicity of Titanocene **Y** in comparison to many other chemotherapeutic drugs.

Conclusions and outlook

The ex-vivo experiment of Titanocene **Y** on the explanted human tumor shows that Titanocene **Y** has a cytotoxic effect comparable to cisplatin when tested on the explanted mammalian tumor. This result is especially promising with the background that Cp_2TiCl_2 shows a proliferative rather than an antiproliferative effect when tested *in vitro* on the MCF-7 cancer cell line [30]. This test was followed by the very successful in-vivo experiment, in which for the first time a shrinking of a tumor treated with a titanocene has been observed. To date, we saw a decrease in the tumor growth only when treated with Titanocene **Y**, but this time a decrease in the tumor volume itself was observed, and therefore these results are really novel and important.

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