Antiproliferative activity of Titanocene Y against tumor colony-forming units

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Bis-[(*p*-methoxybenzyl)cyclopentadienyl] titanium dichloride, better known as Titanocene Y, is a newly synthesized titanium-based anticancer drug. We studied the antitumor activity of Titanocene Y with concentrations of 2.1, 21 and 210 μmol/l against a range of freshly explanted human tumors, using an in-vitro soft agar cloning system. The sensitivity against Titanocene Y was highly remarkable in the case of renal cell, ovarian, nonsmall cell lung and colon cancer. In particular the surprisingly good response of nonsmall cell lung cancer and colon cancer against Titanocene Y at its lowest concentration of 2.1 μmol/l was well comparable or better with respect to cisplatin, given at a concentration of 1.0 μmol/l. Further clinical development of Titanocene Y appears to be warranted because of the broad cytotoxic activity shown and the specific activity of Titanocene Y against renal cell cancer. *Anti-Cancer Drugs* 18:317–321

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Introduction

Titanium-based reagents have significant potential against solid tumors. Budotitane [(*cis*-diethoxybis (1-phenylbutane-1,3-dionato)titanium (IV))] looked very promising during its preclinical evaluation, but did not go beyond phase I clinical trials, although a Cremophor EL-based formulation was found for this rapidly hydrolyzing molecule [1]. Much more robust in this aspect of hydrolysis is the titanocene dichloride (Cp_2TiCl_2), which shows medium antiproliferative activity *in vitro*, but promising results *in vivo* [2,3]. Titanocene dichloride reached clinical trials, but the efficacy of Cp_2TiCl_2 in phase II clinical trials in patients with metastatic renal cell carcinoma [4] or metastatic breast cancer [5] was too low to be pursued.

Recently, novel methods starting from fulvenes [6–15] and other precursors [16–18] allow direct access to the highly substituted titanocenes via reductive dimerization, carbolithiation or hydridolithiation of the fulvene followed by transmetallation in the last two cases. By using the reductive dimerization of fulvenes with titanium dichloride [1,2-di(cyclopentadienyl)-1,2-di-(4-*N*,*N*-dimethylaminophenyl)-ethanediyl], titanium dichloride (Titanocene **X**) was synthesized, which has an IC_{50} value of 270 μmol/l when tested for cytotoxic effects on the LLC-PK cell line [8]. This was a significant progress, because Cp_2TiCl_2 exhibits an IC_{50} value of only 2000 μmol/l against LLC-PK, which explains partly the failed phase II clinical trials against the renal cell carcinoma.

So far, our most cytotoxic titanocene, Titanocene **Y**, was obtained through a further synthetic pathway, which has been recently published [12]: 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 budotitane and the three mentioned titanocenes are shown in Fig. 1.

The antiproliferative activity of Titanocene **X** and **Y** has been studied in 36 human tumor cell lines [19], and in four freshly explanted human tumors using Titanocene **X** [20]. These in-vitro and ex-vivo experiments showed that prostate, cervix and renal cell cancer are prime targets for these novel classes of titanocenes, whereas the IC_{50} values for the breast cancer cell lines were very promising 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 [21]. Furthermore, first animal studies have been published recently reporting the successful treatment of xenografted

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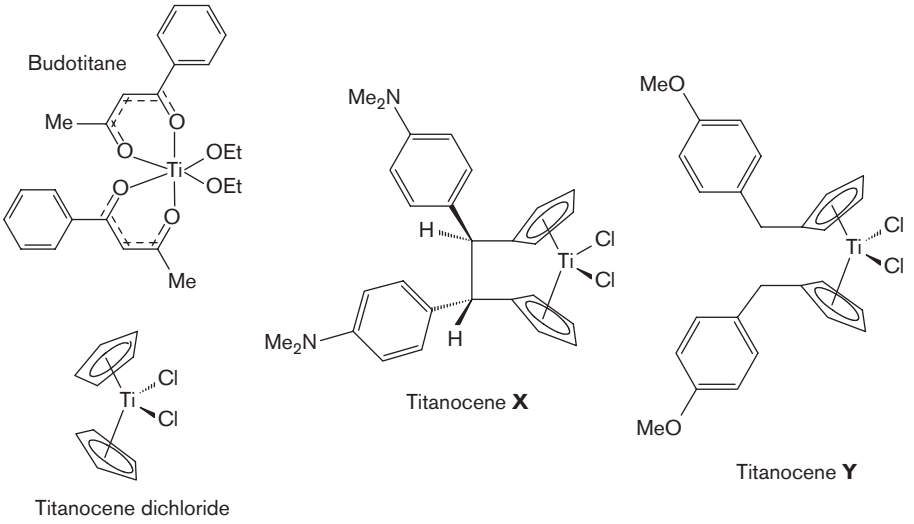
Fig. 1



Molecular structures of budotitane and three titanocenes.

Ehrlich's ascites tumor in mice with Titanocene **X** [22] and xenografted Caki-1 tumors with Titanocene **Y** [23]. The effect of Titanocene **Y** against xenograft Caki-1 tumors in mice was shown to be superior to cisplatin.

The purpose of our present study was to evaluate the extent of cytotoxicity and antitumor activity of Titanocene **Y**. Using a capillary soft agar cloning system, we studied the antitumor activity of Titanocene **Y** on a variety of freshly explanted tumor cells and compared the activity of Titanocene **Y** with regard to cisplatin.

Materials and methods

Antitumor agents

Titanocene **Y** was synthesized as described previously [12]. 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 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 the 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.

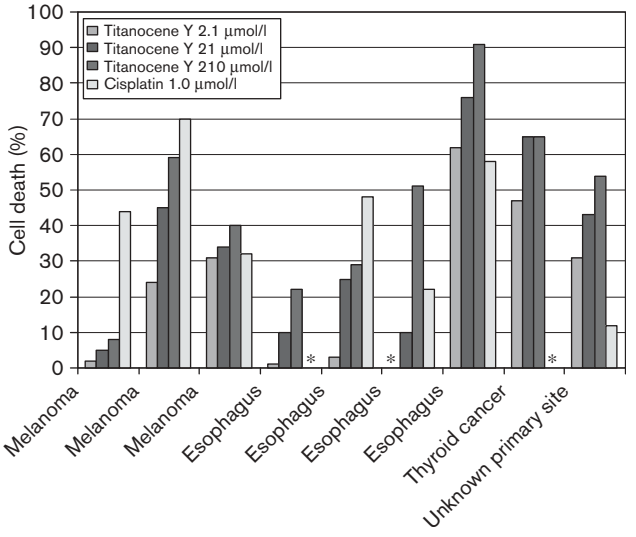
Capillary soft agar cloning system

For the human tumor cloning assay, cells were obtained from freshly biopsied tumor specimens that were achieved by sterile standard procedures as part of the routine clinical measures. The generation of single-cell suspensions followed the instructions as described previously [24]. Human tumor cloning assay experiments were performed as described in [25,26]. In all the 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 the 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 the positive controls demonstrated 30% or less colony formation compared with NaCl control.

Statistical analysis

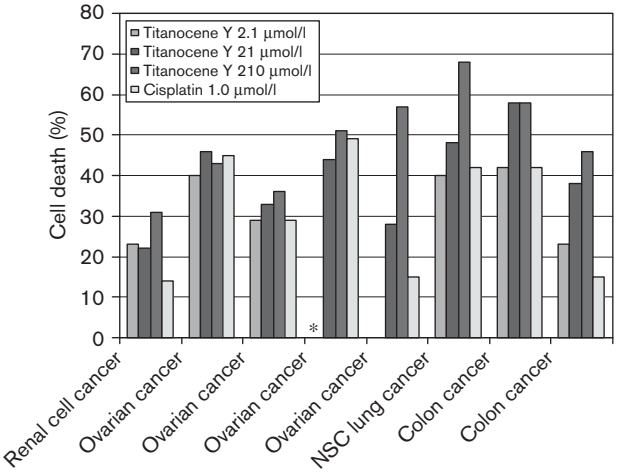
Data were calculated as the means and standard deviations of six replicates for each concentration and all controls. Percentage colony survival was calculated by determining the cells exposed to antitumor agent relative to the calculated cells in untreated negative control.

Fig. 2



Cytotoxicity data of Titanocene **Y** in comparison to cisplatin in HTCA cell tests against melanoma, oesophagus, thyroid cancer and an unknown primary site. HTCA, human tumor cloning assay; *value missing.

Fig. 3



Cytotoxicity data of Titanocene **Y** in comparison to cisplatin in HTCA cell tests against renal cell, ovarian, nonsmall cell (NSC) lung and colon cancer. HTCA, human tumor cloning assay; *value missing.

Inhibition was defined as significant, if the colony formation was 0.5 times or less than the negative control.

Results and discussion

The antitumor effect of Titanocene **Y** was studied in a total of 17 tumors after 1-h short-term exposure. The lesser-affected tumors are presented in Fig. 2, whereas the most promising results are summarized in Fig. 3. The first three results shown in Fig. 2, starting from the left, are melanoma tumors: at 2.1 µmol/l of Titanocene **Y**, 2% of the melanoma cells died, whereas the medium concentration of 21 µmol/l induced a 5% cell death. Following the highest concentration of 210 µmol/l, 8% of the melanoma cells were killed, in comparison with 44% cell death which was induced in the control experiment using 1 µmol/l cisplatin solution. In the second melanoma sample, colony survival decreased in a concentration-dependent manner (24% cell death at 2.1 µmol/l, 45% at 21 µmol/l, 59% at 210 µmol/l), which was still significantly less effective than cisplatin control at 1 µmol/l (70% cell death). The last melanoma sample demonstrated a higher sensitivity to Titanocene **Y**, with 31% cell death at 2.1 µmol/l, 34% at 21 µmol/l and 40% at 210 µmol/l compared to that of control of 1 µmol/l cisplatin solution, which induced 32% cell death. A similar mixed picture was observed for the three oesophagus samples: the first one showed little induction of cell death (1% at 2.1 µmol/l, 10% at 21 µmol/l, 22% at 210 µmol/l), whereas the cisplatin value was not available owing to the shortage of cell material. The second oesophagus experiment gave a similar trend of 3% cell death at 2.1 µmol/l, 25% at 21 µmol/l and 29% at 210 µmol/l compared with the 1 µmol/l cisplatin solution, which was already able to kill 48% of the cells. The third oesophagus tumor showed again little response against Titanocene **Y**: 10% cell death at 21 µmol/l and 51% at 210 µmol/l were induced, while cisplatin would kill 22% of the colonies (the value for 2.1 µmol/l Titanocene **Y** is missing). In the fourth oesophagus case, at a low concentration, 62% of tumor colony-forming units were killed already, and at a medium and high concentration a decrease of 76 and 91% in colony formation was noticed, which is superior to the cisplatin (1 µmol/l) with 58% cell death. In addition, one sample of thyroid cancer was available, which responded with 47, 65 and 65% to the low, medium and high concentrations of Titanocene **Y**. Unfortunately, the cisplatin value is missing and does not allow comparison. The last tumor in Fig. 2 originated from an unknown primary site, which responded superior to Titanocene **Y** when compared with cisplatin. Here the cell death ranges from 31% at 2.1 µmol/l to 43% at 21 µmol/l and 54% at 210 µmol/l of Titanocene **Y** solution, whereas cisplatin killed only 12% of the cells at 1 µmol/l.

The results presented in Fig. 3 show generally good response against Titanocene **Y**: for renal cell cancer, 23% of the colony-forming units died at a low concentration, and 22 and 31% of the cells were killed at the medium and high concentrations, whereas cisplatin killed only 14% of the cells [27]. The first ovarian cancer sample showed almost comparable results to cisplatin (40% cell death at 2.1 µmol/l, 46% at 21 µmol/l, 43% at 210 µmol/l of Titanocene **Y** and 45% at 1 µmol/l of cisplatin), whereas the second sample responded equally well to the lowest

concentration of Titanocene **Y** as to the cisplatin solution (29% cell death at 2.1 µmol/l, 33% at 21 µmol/l, 36% at 210 µmol/l of Titanocene **Y** and 29% at 1 µmol/l of cisplatin). The third ovarian cancer sample cannot be evaluated fully, as the cell death value for the lowest Titanocene **Y** concentration is missing. At the medium and high concentration, 44 and 51% cell death were induced, which compares to 49% when treated with cisplatin. The last ovarian cancer is special: the response against cisplatin at 1 µmol/l with 15% cell death is very low; here also Titanocene **Y** is not impressive with 0% cell death at 2.1 µmol/l, 28% at 21 µmol/l and 57% at the highest concentration of 210 µmol/l. It is encouraging to report that the only nonsmall cell lung cancer sample showed an impressive result of 40% cell death at 2.1 µmol/l, 48% at 21 µmol/l and 68% at 210 µmol/l of Titanocene **Y** compared to that of 42% cell death when treated with the 1 µmol/l cisplatin solution. Another unexpected new target for titanocenes seems to be colon cancer: the first sample responded equally well to the lowest concentration of Titanocene **Y** as to the cisplatin solution (42% cell death at 2.1 µmol/l, 58% at 21 µmol/l, 58% at 210 µmol/l of Titanocene **Y** and 42% at 1 µmol/l of cisplatin), while the second colon cancer sample showed superior results to cisplatin (23% cell death at 2.1 µmol/l, 38% at 21 µmol/l, 46% at 210 µmol/l of Titanocene **Y** and 15% at 1 µmol/l of cisplatin).

The inhibitory effect of Titanocene **Y** was directly compared with cisplatin at 10% of the clinically achievable peak concentration of 0.2 µg/ml, which equals to 1 µmol/l. By doing so, Titanocene **Y**, at 2.1 µmol/l was compared against cisplatin at 1 µmol/l, but this factor of around 2 should not influence the comparability too much. In most samples of renal cell, ovarian, nonsmall cell lung and colon cancer, Titanocene **Y** used at the lowest concentration of 2.1 µmol/l showed superior or similar inhibition when compared to that of cisplatin. In selected cases of melanoma and oesophagus cancer, this comparable behavior was seen as well, while thyroid cancer needs to be reinvestigated. The differences in cell death induced by Titanocene **Y** did not change dramatically when the concentrations reached 21 or 210 µmol/l, which are probably unachievable concentrations with respect to clinical experiments.

Conclusions and outlook

The ex-vivo experiments of Titanocene **Y** on the explanted human tumors demonstrate that the compound has a significant cytotoxic effect comparable to cisplatin when tested on the explanted renal cell, ovarian, nonsmall cell lung and colon tumors. The primary application of Titanocene **Y** against the renal cell cancer owing to a lack of chemotherapy against this cancer is underlined by the above-mentioned findings. Our results encourage further preclinical xenograft investigations to determine the antitumor spectrum of Titanocene **Y** in order to prepare for a clinical phase I study.

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References

1 Schilling T, Keppler BK, Heim ME, Niebch G, Dietzfelbinger H, Rastetter J, *et al.* Clinical phase I and pharmacokinetic trial of the new titanium complex budotitane. *Investig New Drugs* 1995; **13**:327–332.
2 Melendez E. Titanium complexes in cancer treatment. *Crit Rev in Oncol/Hematol* 2002; **42**:309–315.
3 Caruso F, Rossi M. Antitumor titanium compounds and related metallocenes. *Metal Ions Biol Syst* 2004; **42**:353–384.
4 Lummen G, Sperling H, Luboldt H, Otto T, Rubben H. Phase II trial of titanocene dichloride in advanced renal-cell carcinoma. *Cancer Chemother Pharmacol* 1998; **42**:415–417.
5 Kröger N, Kleeberg UR, Mross KB, Edler L, Saß G, Hossfeld DK. Phase II clinical trial of titanocene dichloride in patients with metastatic breast cancer. *Onkologie* 2000; **23**:60–62.
6 Eisch JJ, Owuor FA, Xian S. New syntheses of *ansa*-metallocenes or unbridged substituted metallocenes by the respective reductive dimerisation of fulvenes with group 4 metal divalent halides or with group 4 metal dichloride dihydrides. *Polyhedron* 2005; **24**:1325–1339.
7 Fox S, Dunne JP, Tacke M, Gallagher JF. Novel derivatives of *ansa*-titanocenes procured from 6-phenylfulvene: a combined experimental and theoretical study. *Inorg Chem Acta* 2004; **357**:225–234.
8 Tacke M, Allen LT, Cuffe LP, Gallagher WM, Lou Y, Mendoza O, *et al.* Novel titanocene anti-cancer drugs derived from fulvenes and titanium dichloride. *J Organomet Chem* 2004; **689**:2242–2249.
9 Rehmann FJK, Cuffe LP, Mendoza O, Rai DK, Sweeney N, Strohfeldt K, *et al.* Heteroaryl substituted *ansa*-titanocene anti-cancer drugs derived from fulvenes and titanium dichloride. *Appl Organomet Chem* 2005; **19**:293–300.
10 Tacke M, Cuffe LP, Gallagher WM, Lou Y, Mendoza O, Müller-Bunz H, *et al.* Methoxy-phenyl substituted *ansa*-titanocenes as potential anti-cancer drugs derived from fulvenes and titanium dichloride. *J Inorg Biochem* 2004; **98**:1987–1994.
11 Rehmann FJK, Rous AJ, Mendoza O, Pampillon C, Strohfeldt K, Sweeney N, *et al.* Novel substituted *ansa*-titanocene anti-cancer drugs. *Polyhedron* 2005; **24**:1250–1255.
12 Sweeney N, Mendoza O, Müller-Bunz H, Pampillón C, Rehmann FJK, Strohfeldt K, *et al.* Novel benzyl substituted titanocene anti-cancer drugs. *J Organomet Chem* 2005; **690**:4537–4544.
13 Pampillón C, Mendoza O, Sweeney N, Strohfeldt K, Tacke M. Diarylmethyl substituted titanocenes: promising anti-cancer drugs. *Polyhedron* 2006; **25**:2101–2108.
14 Pampillón C, Sweeney N, Strohfeldt K, Tacke K. Diheteroarylmethyl substituted titanocenes: a novel class of potential anti-cancer drugs. *Inorg Chem Acta* 2006; **359**:3969–3975.
15 Sweeney NJ, Müller-Bunz H, Pampillón C, Strohfeldt K, Tacke M. Novel heteroaryl substituted titanocene anti-cancer drugs. *J Inorg Biochem* 2006; **100**:1479–1486.
16 Allen OR, Croll L, Gott AL, Knox RJ, McGowan PC. Functionalized cyclopentadienyl titanium organometallic compounds as new antitumor drugs. *Organometallics* 2004; **23**:288–292.
17 Causey PW, Baird MC. Synthesis, characterization, and assessment of cytotoxic properties of a series of titanocene dichloride derivatives. *Organometallics* 2004; **23**:4486–4494.
18 Meyer R, Brink S, van Rensburg CEJ, Jooné GK, Görls H, Lotz S. Synthesis, characterization and antitumor properties of titanocene derivatives with thiophene containing ligands. *J Organomet Chem* 2005; **690**:117–125.
19 Kelter G, Sweeney N, Strohfeldt K, Fiebig HH, Tacke M. *In vitro* anti-tumor activity of bridged and unbridged benzyl-substituted titanocenes. *Anticancer Drugs* 2005; **16**:1091–1098.
20 Oberschmidt O, Hanauske AR, Rehmann FJK, Strohfeldt K, Sweeney N, Tacke M. Preclinical activity of [1,2–di(cyclopentadienyl)-1,2-di(*p-N,N*-dimethylaminophenyl)-ethanediyl] titanium dichloride against tumor colony forming units. *Anticancer Drugs* 2005; **16**:1071–1073.
21 O'Connor K, Gill C, Tacke M, Rehmann FJK, Strohfeldt K, Sweeney N, *et al.* Novel titanocene anti-cancer drugs and their effect on apoptosis and the

apoptotic pathway in prostate cancer cells. *Apoptosis* 2006; **11**: 1205–1214.
22 Valadares MC, Ramos AL, Rehmann FJK, Sweeney NJ, Strohfeldt K, Tacke M, *et al.* Antitumor activity of [1,2–di(cyclopentadienyl)-1, 2-di(*p-N*,*N*-dimethylaminophenyl)-ethanediyl] titanium dichloride in xenografted Ehrlich's ascites tumor. *Eur J Pharmacol* 2006; **534**:264–270.
23 Fichtner I, Pampillón C, Sweeney NJ, Strohfeldt K, Tacke M. Antitumor activity of titanocene Y in xenografted Caki-1 tumors in mice. *Anticancer Drugs* 2006; **17**:333–336.
24 Vogel M, Hilsenbeck SG, Depenbrock H, Danhauser-Riedl S, Block T, Nekarda H, et al. Preclinical activity of taxotere (RP56976, NSC628503) against freshly explanted clonogenic human tumor cells: comparison with taxol and conventional antineoplastic agents. *Eur J Cancer* 1993; **29A**:2009–2014.
25 Hanauske A-R, Hanauske U, Von Hoff DD. The human tumor cloning assay in cancer research and therapy. *Curr Probl Cancer* 1985; **9**:1–50.
26 Hanauske A-R, Hilsenbeck SG, von Hoff DD. Human tumor screening. In: Teicher BA, Andrews PA, editors. *Cancer drug discovery and development: anticancer drug development guide: preclinical screenings, clinical trials, and approval*. 2nd ed. Totowa: Humana Press; 2004. 63–76.
27 Kurbacher CM, Nagel W, Mallmann P, Kurbacher JA, Saß G, Hübner H, *et al. In vitro* activity of titanocenedichloride in human renal cell carcinoma compared to conventional antineoplastic agents. *Anticancer Res* 1994; **14**:1529–1533.