BioMetals 1996, **9**, 267–271

Antitumor activity of titanocene amino acid complexes

Petra Köpf-Maier & I. C. Tornieporth-Oetting*

*Institut für Anatomie, Freie Universität Berlin, Berlin and *Institut für Anorganische und Analytische Chemie, Technische Universität Berlin, Berlin, Germany*

Received 27 November 1995; accepted for publication 6 February 1996

Seven ionic titanocene α-amino acid (aa) complexes $[(C_5H_5)_2Ti(aa)_2]^{2+}[X]_2^-$ with aa = glycine, L-alanine, 2-methylalanine, D-L-phenylalanine, D,L-4-fluorophenylalanine and X = Cl or AsF_6, were investigated for antitumor activity against fluid Ehrlich ascites tumor growing in CF1 mice. These complexes are the first stable model compounds of titanocene units with protein components, synthesized from a water-like, methanolic medium. All titanocene amino acid complexes induced antitumor activity which was manifested by maximum cure rates ranging from 30 to 70% and increases in life span from 78 to 276% in comparison with untreated control animals. The complexes containing chloride as anion X were more effective than the hexafluoroarsenate derivatives, which surprisingly showed a low substance toxicity. In all cases, the antitumor activity of the ionic titanocene amino acid complexes tested was less pronounced than that of the neutral parent compound $[(C_5H_5)_2TiCl_2]$.

Keywords: antitumor activity, Ehrlich ascites tumor, mechanism of action, titanocene amino acid complexes

Introduction

Titanocene dichloride $[(C_5H_5)_2TiCl_2]$ is a new organometallic cytostatic drug that has shown remarkable antitumor activity against numerous experimental tumors and heterotransplanted human carcinomas from the lung, the colon and the breast (Köpf-Maier & Köpf 1988, 1994, Köpf-Maier 1993). It is presently in the process of clinical development. A first clinical phase I trial was finished in December 1993 (Berdel *et al.* 1994, Korfel *et al.* 1995) and phase II studies were started some months ago. Titanocene dichloride is the first complex containing a transition metal other than platinum metals which has been introduced into clinical phase II trials (Köpf-Maier 1994).

Biological studies which were performed *in vivo* and *in vitro* pointed to nucleic acid metabolism as a probable site of molecular interaction of titanocene species or their consecutive metabolites as the basis for the antitumor activity of $[(C_5H_5)_2TiCl_2]$. This was suggested by a pronounced and irreversible depression of DNA synthesis after treatment with $[(C_5H_5)_2TiCl_2]$ (Köpf-Maier 1993), by clumping of the nuclear chromatin, the formation of atypical mitotic figures and giant cells (Köpf-Maier 1993), and the enrichment of titanium or titanium-containing species in the nuclear chromatin of treated carcinoma cells (Köpf-Maier 1990, Johnson *et al.* 1995). These results can be either explained by a primary attack of titanium-containing species at nucleic acid molecules or at proteins that are relevant for the structure, function and metabolism of nucleic acids such as histones, non-histone proteins or enzymes involved in the synthesis of nucleic acids.

Numerous groups have tried to synthesize model complexes of titanocene species with biologically relevant molecules, especially with nucleic acid components (Köpf-Maier & Köpf 1988, Köpf-Maier 1993 and references therein). Under physiological or similar conditions, this attempt was revealed to be much more difficult than was found previously with cisplatin. In aqueous or water-like solvents, there were certainly analytical and spectroscopic hints at an interaction between $(C_5H_5)_2Ti$ and C_5H_5Ti species with nucleic acid molecules (Pneumatikakis *et al.* 1988, McLaughlin *et al.* 1990, Murray and Harding 1994); stable model compounds, however, could not be isolated from aqueous media. On the other hand, the successful synthesis, isolation and characterization of stable titanocene α-amino acid complexes from the polar solvent methanol (Klapötke *et al.* 1994, Tornieporth-Oetting & White 1995) suggested that the complexation between titanocene species and proteins may be another possible mechanistic pathway leading to the antiproliferative activity of titanocene complexes.

In the present study we investigated the antitumor efficacy

Address for correspondence: P. Köpf-Maier, Institut für Anatomie, Freie Universität Berlin, Königin-Luise-Strasse 15, D-14195 Berlin, Germany. Fax: (+49) 30 838 3806.

of seven ionic titanocene α-amino acid (aa) complexes $[(C_5H_5)_2Ti(aa)_2]^{2+}[X]_2^-$ in order to determine the cytostatic potency of these molecules as compared with the parent compound $[(C_5H_5)_2TiCl_2]$, enfacing the possible role of these complexes as active species in the course of the antitumor action of titanocene dichloride.

Materials and methods

Substances

The ionic titanocene α-amino acid complexes $[(C_5H_5)_2Ti(aa)_2]^{2+}[Cl]_2^-$ with aa = glycine (**I**), L-alanine (**II**), 2-methylalanine (**III**), D,L-phenylalanine (**IV**), D,L-4-fluorophenylalanine (**V**) and the hexafluoroarsenate derivatives $[(C_5H_5)_2Ti(aa)_2]^{2+}[AsF_6]_2^-$ with aa = D,L-phenylalanine (**VI**) and D,L-4-fluorophenylalanine (**VII**) were synthesized according to the procedure described before (Klapötke *et al.* 1994, Tornieporth-Oetting & White 1995). In the meanwhile, the synthesis of complexes **I**–**V** could be improved in the following respects. The best results were obtained when the temperature during the synthesis was held at or below 20°C. At higher temperatures partial decomposition of the products formed took place causing lower yields of the desired titanocene amino acid complexes. On the other hand, the yield was increased by adding 10–15 ml $CFCl_3$ (R11) to the reaction mixture after the reaction was completed. By this method, the complexes **I**–**V** precipitated and could easily be isolated by filtration. Subsequent washing with R11 and drying *in vacuo* led to analytically pure products in high yields.

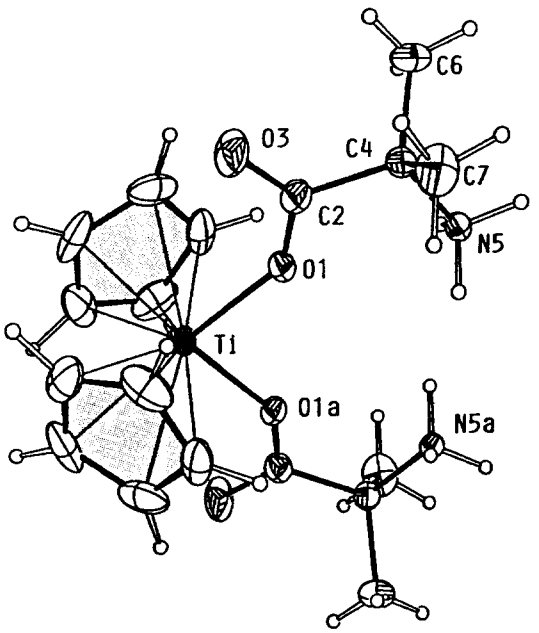

Figure 1. Molecular structure of the cation $[(C_5H_5)_2Ti(2\text{-methylalanine})_2]^{2+}$ in complex **III** determined by X-ray diffraction (modified according to Klapötke *et al.* 1994).

The complexes **I**–**VII** were characterized by elemental analysis (C, H, N), IR, NMR (^1H, ^{14}N, ^{19}F), mass and Raman spectra (Klapötke *et al.* 1994, Tornieporth-Oetting & White 1995). No impurities were detectable by these methods. The elemental analyses revealed deviations of 0.5% or less of the calculated values. In addition, complexes **III** and **VII** were confirmed by X-ray crystallography (Klapötke *et al.* 1994, Tornieporth-Oetting & White 1995). The crystal structure of **III** is illustrated in Figure 1.

For antitumor testing, the compounds were injected intraperitoneally (i.p.) at doses indicated in Table 1. The doses were administered in saline under addition of 10% dimethylsulfoxide (DMSO). The substance concentrations were so selected that each animal received a total volume of 0.4–0.5 ml (0.02 ml g^{-1} body weight). The preparations were injected i.p. within 30 min after dissolution. The untreated control mice only received the vehicle fluid, i.e. the mixture of DMSO and saline (1/9, v/v) without drug addition.

Animals

Female CF1 mice purchased from Harlan-Winkelmann (Paderborn, Germany) were kept under standard specific pathogen-free (SPF) conditions. They received food

Table 1. Pharmacological and toxicological data of titanocene amino acid complexes

Compounds	Applied doses (mg kg^{-1})	Optimum dose range[a] (mg kg^{-1})	Maximum cure rate (%)	Increase in life span at optimum dose[b]	LD_{20} (mg kg^{-1})	LD_{50} (mg kg^{-1})
I	10, 20, 40,... 160	100–120	50	185	140	>160
II	10, 20, 40,... 220	160	50	185	170	185
III	10, 20, 40,... 160	100–120	60	239	135	>160
IV	10, 20, 40,... 240	120–180	60	216	205	215
V	10, 20, 40,... 240	100–180	70	276	160	205
VI	10, 20, 40,... 380	—	30	78	375	>380
VII	10, 20, 40,... 380	340–360	50	196	380	>380
$[(C_5H_5)_2TiCl_2]$	10, 20, 30,... 180	30–70	100	380	75	100

[a] Defined as dose range with cure rates of 50% or above.
[b] ILS determined at day 90 after transplantation.

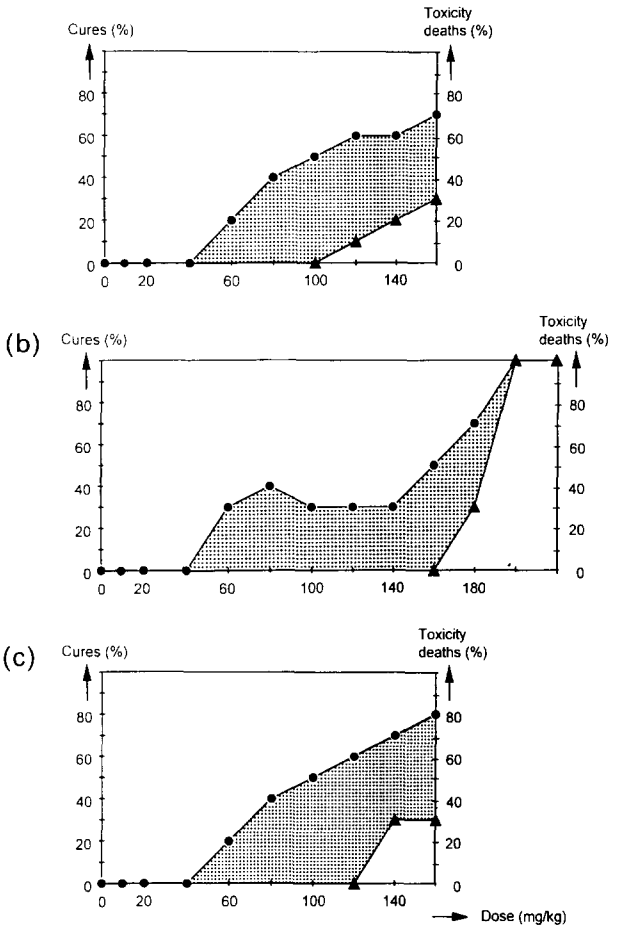

Figure 2. Dose–activity (left) and dose–lethality (right) relationships of **I** (a), **II** (b) and **III** (c) against fluid Ehrlich ascites tumor. The ordinate gives the number of animals that were either cured (left) or died as a result of substance toxicity (right). The shaded area indicates the range of surviving, cured animals.

(Altromin) and tap water *ad libitum*. At the beginning of the experiments, they were about 8–10 weeks of age and weighed 20–25 g.

Antitumor bioassay

The antitumor activity of the titanocene amino acid complexes **I–VII** was investigated against Ehrlich ascites tumor growing as a fluid tumor in the peritoneal cavity of mice. For tumor transplantation, the ascites of donor mice bearing the Ehrlich ascites tumor for 8 days were diluted with saline in a ratio of 1/8 (v/v). About 6×10^6 cells were then transplanted i.p. into each animal on day 0 of the experiment. The i.p. administration of the substances dissolved as described above and applied in single doses was performed 24 h later. Every dose group consisted of 10 animals. Another 70 mice (seven groups of 10 mice) served as untreated, tumor-bearing control animals. They were given 0.4–0.5 ml of the drug-free vehicle fluid on day 1.

The number of deaths was registered daily. Deaths occurring within 7 days of substance administration were related to drug toxicity and those noted later than day 8 after tumor transplantation were defined as tumor deaths. After day 8, all animals that died showed the macroscopic signs of massive tumor development within the peritoneal cavity. The key date for determining the number of surviving mice was day 90, at which time all surviving animals were free of any recognizable signs of tumor and were considered to be cured.

Results

All control animals which had been treated with the vehicle fluid without drug addition on day 1 after tumor transplantation developed tumor disease and died between days 14 and 24. In the seven control groups, the mean survival amounted to 18.5 ± 1.9, 19.7 ± 1.6, 20.1 ± 1.8, 20.3 ± 1.5, 19.0 ± 1.8, 18.7 ± 1.1 and 18.1 ± 1.9 days, respectively.

When tumor-bearing mice were treated on day 1 after transplantation with one of the complexes **I–VII** in a single dose ranging from 10 to 380 mg kg^{-1}, optimum cure rates of 30–70% were achieved. The main pharmacological and toxicological data characterizing the compounds are summarized in Table 1. The dose-dependent influence of treatment with complexes **I–VII** on the occurrence of tumor deaths, toxic deaths and cures is additionally illustrated in Figures 2–4.

None of the compounds tested achieved the antitumor potential of the parent compound titanocene dichloride in the fluid Ehrlich ascites tumor model (Table 1). The best activity was observed for the titanocene 4-fluorophenylalanine chloride complex **V** (Figure 3b) with a cure rate of 70% and an increase in life span (ILS) value of 277%,

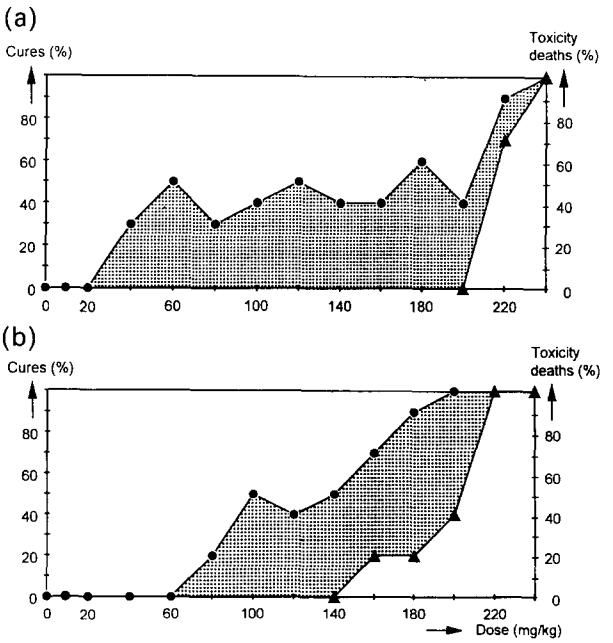

Figure 3. Dose–activity and dose–lethality relationships of **IV** (a) and **V** (b). For further explanations, see legend to Figure 2.

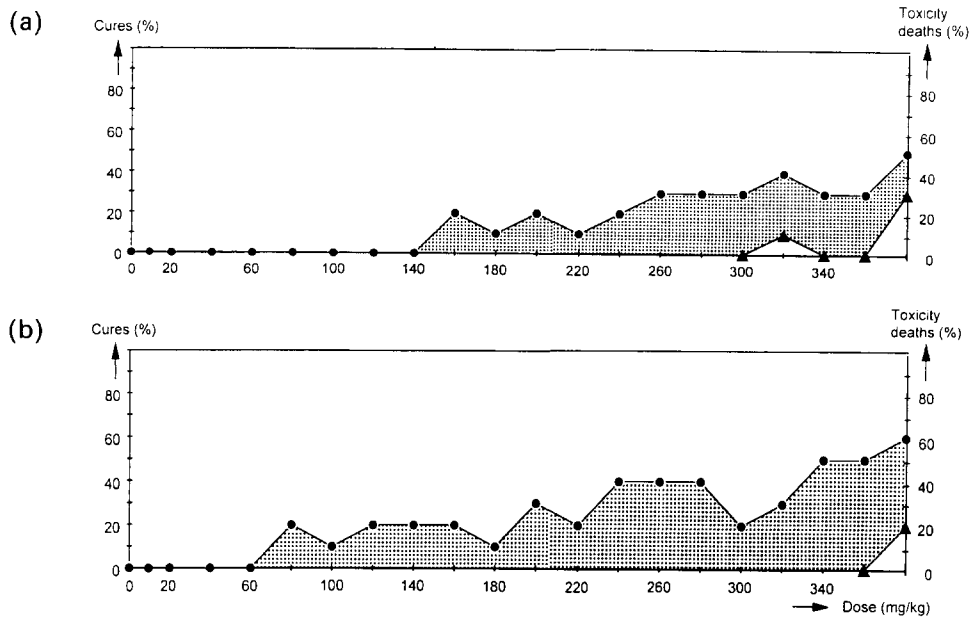

Figure 4. Dose–activity and dose–lethality relationships of **VI** (a) and **VII** (b). For further explanations, see legend to Figure 2.

followed by the methylalanine and phenylalanine analogs **III** (Figure 2c) and **IV** (Figure 3a), both effecting a maximum cure rate of 60% and (ILS) values of 239 and 216%, respectively. A cure rate of 50% was caused by the glycine and alanine complexes **I** (Figure 2a) and **II** (Figure 2b), and by the titanocene 4-fluorophenylalanine hexafluoroarsenate complex **VII** (Figure 4b). The lowest antitumor activity of all investigated titanocene amino acid complexes, finally, was with the phenylalanine hexafluoroarsenate derivative **VI** (Figure 4a), with a maximum cure rate of 30% and an ILS value of only 78%.

Comparing the results obtained with compounds **I–VII** among each other it becomes obvious that complexes **I–V**, which contain chloride as an anion, are characterized by a better antitumor efficacy at lower dose levels than the hexafluoroarsenate complexes **VI** and **VII**. On the other hand, the latter complexes showed a surprisingly low substance toxicity which manifested by LD_{20} values of 375 and 380 mg kg^{-1}, and LD_{50} values higher than 400 mg kg^{-1}.

Discussion

The results of the present study confirm good or moderate antitumor activity of the ionic titanocene α-amino acid complexes **I–VII** against fluid Ehrlich ascites tumor, which is a quite sensitive model for testing the antitumor potency of inorganic and organometallic compounds (Köpf-Maier & Klapötke 1992). Thus, titanocene species preserve growth-inhibiting efficacy after complexation with natural and artificial amino acids, albeit they are not as active as the parent compound titanocene dichloride. This result seems to contradict the hypothesis that titanocene amino acid complexes which are stable in water for at least several hours (Klapötke *et al.* 1994) are intrinsically active species of antitumor titanocene compounds. However, it should be considered that, in the titanocene amino acid complexes tested, the carboxyl group of the amino acids is occupied by the titanocene moiety (Köpf-Maier & Klapötke 1992, Klapötke *et al.* 1994, Tornieporth-Oetting & White 1995), so that the amino acids in the tested compounds can only bind monofunctionally to the N-terminal side of an amino acid or a peptide molecule, and cannot be integrated bifunctionally into a peptide or protein chain analogously to natural amino acids.

This may be one of the reasons for the reduced antitumor activity of titanocene α-amino acid complexes in comparison with titanocene dichloride. It cannot be excluded that, at least partially, the titanocene α-amino acid complexes tested in the present study unfold their antitumor activity by cleaving off the titanocene unit which then interacts directly or as mono(cyclopentadienyl)titanium species with biological macromolecules outside or inside of mammalian cells.

In another study performed previously, the antimicrobial behavior of complexes **IV–VII** was investigated in comparison with the free amino acids D,L-phenylalanine and D,L-4-fluorophenylalanine in cultures of *Escherichia coli* (Tornieporth-Oetting & White 1995). The results revealed only weak bactericidal activity of the two free amino acids, the parent compound $[(C_5H_5)_2TiCl_2]$, and the complexes **IV** and **V**. This activity was greatly enhanced after replacement of the chloride anions by the weakly basic $[AsF_6]^-$ anions in **VI** and **VII**. In the present study, a corresponding enhancement of antitumor efficacy could not be observed when complexes **IV** and **VII** were applied to tumor-bearing mice, suggesting that the mechanisms

resulting in antimicrobial and antitumor properties of the titanocene amino acid complexes are not fully identical.

Nevertheless, the successful synthesis, from a water-like, methanolic medium, of titanocene α-amino acid complexes containing Ti(IV) as a central metal atom is obviously an essential step towards the clarification of possible biochemical reactions of cyclopentadienyltitanium species and the mechanism of the antitumor activity of titanocene complexes since this kind of coordination of mono- or bis(cyclopentadienyl)titanium species could actually explain the antiproliferative and antitumor activity of titanocene complexes.

Acknowledgements

We gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft (KI 636/1-3).

References

Berdel WE, Schmoll HJ, Scheulen ME, *et al.* 1994 Phase I clinical trial of titanocene dichloride in adults with advanced solid tumors. *J Cancer Res Clin Oncol* **120** (Suppl), R-172.

Johnson AD, Mairs RJ, Gaze MN, Sass G, Huxham IM. 1995 Electron spectroscopic imaging of organic compounds using PC-based energy sequence imaging software. *Micros Microanal Microstruct* **6**, 65–77.

Klapötke TM, Köpf H, Tornieporth-Oetting IC, White PS. 1994 Synthesis, characterization, and structural investigation of the first bioinorganic titanocene(IV) α-amino acid complexes prepared from the antitumor agent titanocene dichloride. *Organometallics* **13**, 3628–3633.

Köpf-Maier P. 1990 Intracellular localization of titanium with xenografted sensitive human tumors after treatment with the antitumor agent titanocene dichloride. *J Struct Biol* **105**, 35–45.

Köpf-Maier P. 1993 Antitumor bis(cyclopentadienyl)metal complexes. In: Keppler BK, ed. *Metal Complexes in Cancer Chemotherapy*. Weinheim: VCH Verlagsgesellschaft; 259–296.

Köpf-Maier P. 1994 Complexes of metals other than platinum as antitumor agents. *Eur J Clin Pharmacol* **47**, 1–16.

Köpf-Maier P, Klapötke T. 1992 Ionic rhenocene derivatives with antitumor activity. *Cancer Chemother Pharmacol* **29**, 361–366.

Köpf-Maier P, Köpf H. 1988 Transition and main-group metal cyclopentadienyl complexes: preclinical studies on a series of antitumor agents of different structure type. *Struct Bond* **70**, 103–185.

Köpf-Maier P, Köpf H. 1994 Organometallic titanium, vanadium, niobium, molybdenum and rhenium complexes – early transition metal antitumor drugs. In: Fricker SP, ed. *Metal Compounds in Cancer Therapy*. London: Chapman & Hall; 109–146.

Korfel A, Schmoll HJ, Scheulen ME, *et al.* 1995 Klinische Phase-I-Studie mit Titanocen-dichlorid bei Patienten mit soliden Tumoren. *Akt Onkol* **84**, 51–59.

McLaughlin ML, Cronan JM, Schaller TR, Snelling RD. 1990 DNA–metal binding by antitumor-active metallocene dichlorides from inductively coupled plasma spectroscopy analysis: titanocene dichloride forms DNA–Cp_2Ti or DNA–CpTi adducts depending on pH. *J Am Chem Soc* **112**, 8949–8952.

Murray JH, Harding MM. 1994 Organometallic anticancer agents: the effect of the central metal and halide ligands on the interaction of metallocene dihalides Cp_2MX_2 with nucleic acid constituents. *J Med Chem* **37**, 1936–1941.

Pneumatikakis G, Yannopoulos A, Markopoulos J. 1988 Interactions of dichloro-bis(η^5-cyclopentadienyl)titanium(IV) with nucleosides. *Inorg Chim Acta* **151**, 125–128.

Tornieporth-Oetting IC, White PS. 1995 Ionic titanocene(IV) α-amino acid complexes of DL-phenylalanine and DL-4-fluorophenylalanine: synthesis, characterization, and investigation of the antimicrobial behavior toward *Escherichia coli*. *Organometallics* **14**, 1632–1636.