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Tumor Inhibition by Metallocenes: Antitumor Activity of Titanocene Dihalides $(C_5H_5)_2TiX_2$ (X=F, Cl, Br, I, NCS) and Their Application in Buffered Solutions as a Method for Suppressing Drug-induced Side Effects*

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Tumorhemmung durch Metallocene: Antitumor-Wirksamkeit von Titanocen-dihalogeniden $(C_5H_5)_2TiX_2$ (X=F, Cl, Br, J, NCS) und deren Applikation in gepufferter Lösung als Methode zur Unterdrückung substanzbedingter Nebenwirkungen

Zusammenfassung. Die tumorhemmende Wirksamkeit von Titanocen-dihalogeniden $(C_5H_5)_2TiX_2$ mit X=F, Cl, Br, J, NCS wird an Ehrlich-Aszites-Tumor-tragenden CF 1-Mäusen untersucht. Verschiedene Dosen der Substanzen werden 24 h p.t.t. als einmalige i.p.-Injektionen sowohl in ungepufferten (pH 1.4–3.9) als auch in gepufferten (pH 4.2–5.9) Lösungen bzw. Suspensionen appliziert. Mit allen Verbindungen werden in optimaler Dosis Heilungsraten von 100% am 120. Tag p.t.t. erreicht. Dies entspricht Verlängerungen der mittleren Überlebensdauer (I.L.S.-Werte) um 600–750% gegenüber den unbehandelten Kontrolltieren. Einige substanzbedingte Nebenwirkungen, insbesondere das Auftreten postperitonitischer Symptome mehrere Wochen nach i.p.-Applikation höherer Dosen der Titanocen-dihalogenide, werden durch pH-Erhöhung in den Injektionslösungen deutlich zurückgedrängt.

Schlüsselwörter: Titanocen-dihalogenide – Tumorhemmende Aktivität – Ehrlich-Aszites-Tumor – Unterdrückung von Nebenwirkungen

Summary. The antitumor activity of titanocene dihalides $(C_5H_5)_2TiX_2$ with X=F, Cl, Br, I, NCS is investigated against Ehrlich ascites tumor in CF 1 mice. Varying doses of the compounds are applied as single i.p. injections 24 h p.t.t. both in non-buffered (pH 1.4–3.9) and buffered (pH 4.2–5.9) solutions or suspensions, all of the substances achieving in optimum doses cure rates of 100% on day 120 p.t.t. This corresponds to I.L.S. values of 600–750% referred to the untreated controls. Some drug-induced side effects, especially the appearance

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of postperitonitic symptoms several weeks after i.p. application of higher doses of titanocene dihalides, are strikingly reduced by pH elevation in the injected drug solutions.

Key words: Titanocene dihalides – Antitumor activity – Ehrlich ascites tumor – Suppression of side effects

Titanocene dichloride $(C_5H_5)_2TiCl_2$ represents the first metallocene for which cancerostatic activity has been established (Köpf and Köpf-Maier 1979). On varying the central atom M within the system $(C_5H_5)_2MCl_2$ we have demonstrated that several other metallocene dichlorides, especially those with M = V, Nb, and Mo, also reveal marked tumor-inhibiting properties, whereas others proved to be less active (M = Ta, W) or inactive (M = Zr, Hf) (Köpf-Maier and Köpf 1979; Köpf-Maier et al. 1979, 1980, in press).

An additional site to be modified in these molecules is occupied by the Cl ligands which can be replaced by other halide ligands X. This replacement was expected to possibly affect the cancerostatic activity as it is the case in a series of *cis*-diammineplatinum(II)dihalides $(NH_3)_2PtX_2$ (Cleare 1974). Therefore, in the present work the tumor-inhibiting potency of the titanocene derivatives $(C_5H_5)_2TiX_2$ with X = F, Cl, Br, I, and NCS was determined against Ehrlich ascites tumor in mice. In order to study the influence of reducing the acidity due to hydrolysis of the titanocene dihalides, an additional experimental series using buffered drug solutions was carried out.

Materials and Methods

a) Animals. Female CF 1 mice (Winkelmann, Paderborn) weighing 20–25 g served as test animals. Feeding (Altromin) and tap water were ad libitum.

Table 1. Mode of application of titanocene dihalides $(C_5H_5)_2TiX_2$ and pH ranges in the non-buffered and buffered solutions

X	Solution[a]	Total of animals[b]	Dose levels (mg/kg)	pH range[c]
F	n.	48	10, 20, 30, 40...120	3.9–3.3
	b.	48	10, 20, 30, 40...120	5.7–5.4
Cl	n.	72	10, 20, 30, 40...180	2.9–1.9
	b.	72	10, 20, 30, 40...180	5.5–4.7
Br	n.	76	10; 20, 40, 60...360	3.0–1.4
	b.	76	10; 20, 40, 60...360	5.5–4.4
I	n.	96	10, 20, 30, 40...240	3.1–1.9
	b.	96	10, 20, 30, 40...240	5.4–4.2
NCS	n.	52	10; 20, 40, 60...240	3.2–3.1
	b.	52	10; 20, 40, 60...240	5.9–5.5

[a] n. = Non-buffered; b. = buffered
[b] Four animals each per dose level
[c] The first value corresponding to the lowest, the second to the highest dose applied

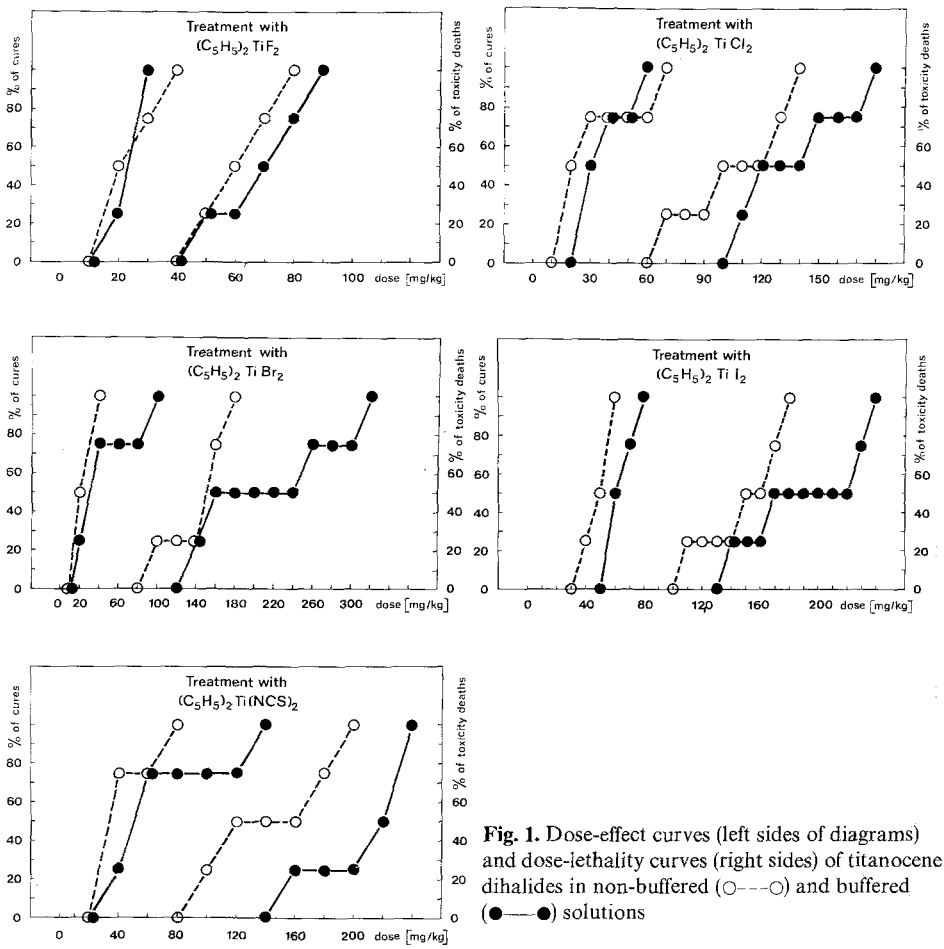


Fig. 1. Dose-effect curves (left sides of diagrams) and dose-lethality curves (right sides) of titanocene dihalides in non-buffered (○---○) and buffered (●——●) solutions

b) Substances. The titanocene dihalides $(C_5H_5)_2TiX_2$ with X = F, Cl, Br, I, NCS were prepared and purified according to literature methods (Wilkinson and Birmingham 1954; Samuel 1966). Elemental analyses (C, H, Ti) gave deviations $\leqq 0.5\%$ of the calculated values. The proton magnetic resonance and infrared spectra showed no evidence of impurities.

Samples corresponding to the single doses were dissolved or suspended in 0.05 ml DMSO by ultrasonic treatment for 5 min and filled up with saline to 0.5 ml. In the buffering experiments, aqueous 0.1 *M* $NaHCO_3$ was added to the similarly prepared DMSO-saline solutions in such amounts that the pH was elevated to the values given in Table 1 and a total volume of 0.5 ml was attained. Complete whitening of the mixtures, indicating extended hydrolysis by an excess of $NaHCO_3$, was avoided. The buffered solutions were injected *immediately* after $NaHCO_3$ addition.

c) Testing of Antitumor Activity. The ascites of donor mice bearing Ehrlich ascites tumor for 8–10 days was diluted with saline 1:7 (v/v). About $6 \cdot 10^6$ cells were transplanted i.p. into each animal on day 0 of the experiment. The i.p. application of the substances was performed 24 h p.t.t. Animal distribution, dose levels, and pH ranges are recorded in Table 1. Four additional animals served as untreated tumor-bearing controls in each of the 10 test groups. The controls obtained an injection of 0.5 ml of a DMSO-saline mixture 1:9 (v/v) without drug addition.

Table 2. Tumor-inhibiting and toxic properties of titanocene dihalides $(C_5H_5)_2TiX_2$

X	Solution[a]	Optimum dose[b] (mg/kg)	pH of drug solution at optimum dose	I.L.S. at optimum dose[d] (%)	LD_{50} (mg/kg)	LD_{100} (mg/kg)	T.I.[e]
F	n.	40 (1)	3.6	+659	60	80	2.0
	b.	30– 40 (2)	5.6	+684	65	90	2.6
Cl	n.	30– 60 (4)[c]	2.6–2.4	+620	100	140	3.3
	b.	60–100 (5)	5.3–5.1	+674	130	180	3.3
Br	n.	40– 80 (3)	2.4–2.0	+711	135	180	4.5
	b.	100–120 (2)	5.2	+567	200	320	4.9
I	n.	60–100 (5)	2.3–2.1	+614	145	180	2.6
	b.	80–130 (6)	5.4–5.3	+757	185	240	2.6
NCS	n.	80 (1)	3.1	+674	135	200	3.4
	b.	140 (1)	5.7	+614	200	220	3.4

[a] n. = Non-buffered; b. = buffered

[b] Cure rate 100% except X = Cl, n.

[c] Cure rate 75%

[d] All differences of mean survival times at optimum doses are significant compared to the control (2 $P < 0.05$, Wilcoxon-Mann-Whitney U-statistic)

[e] Defined as LD_{50}/ED_{75}

d) Evaluation of Experiments. Every day the animals were weighed and the deaths noted. Deaths within 8 days p.t.t. were defined as toxic, those occurring later as tumor deaths (all animals which had died later than 8 days p.t.t. showed macroscopical and histological evidence of tumor development). The key-date for determining the survival rate was day 120 p.t.t., the survival times of the control animals ranging from 11–20 (mean value 15.5 ± 2.0) days p.t.t. The I.L.S. was calculated by relating the mean survival time of the 4 animals of each dose level to that of the corresponding control group as percentage and by subtracting 100%. On day 120 p.t.t. all surviving animals were sacrificed. The peritoneal cavity was opened and macroscopically examined.

Results

The results of the antitumor-testing experiments using *non-buffered* solutions of the titanocene dihalides $(C_5H_5)_2TiX_2$ (X = F, Cl, Br, I, NCS) are summarized in Fig. 1 and Table 2. All dihalides are characterized by pronounced tumor-inhibiting properties causing cures of all animals treated with optimum doses, with exception of the dichloride accomplishing only an optimum cure rate of 75%[1]. The I.L.S. values until day 120 p.t.t. range at optimum doses between 614 and 711%.

On application in non-buffered solutions, all titanocene dihalides effect a similar dose-dependent pattern of reversible toxic symptoms, such as shagginess of the fur, abdominal retractions, hyposthenia, and neuromuscular disorder (Table 3). While the Cl and NCS derivatives evoke deteriorations of the general condition already in optimal doses, the F, Br, and I compounds cause the same phenomena only in dose ranges where toxic deaths appear.

A number of the surviving animals treated with high doses exhibited an irregularly contracted abdominal surface 6–8 weeks after therapy. Therefore, all animals

1 In an experiment using 10 animals per dose, non-buffered titanocene dichloride attained cure rates of 80–90% and I.L.S. values of 850–950% at day 180 p.t.t. (Köpf-Maier et al. 1980)

Table 3. General toxic symptoms after treatment with titanocene dihalides $(C_5H_5)_2TiX_2$

Dose level (mg/kg)	X and mode of application[a]									
	F		Cl		Br		I		NCS	
	n.	b.	n.	b.	n.	b.	n.	b.	n.	b.
20	−	−	−	−	−	−	−	−	−	−
30	−	−	−	−			−	−		
40	−	−	−	−	−	−	−	−	(+)	−
50	++	−	+	−			−	−		
60	++	(+)	+	−	−	−	−	−	+	−
70	+++	++	+	−			−	−		
80	+++	++	++	−	−	−	−	−	+	−
90	+++	++	++	−			−	−		
100	+++	++	++	−	−	−	−	−	+	−
110	+++	++	++	−			+	−		
120	+++	++	+++	−	++	−	+	−	+	−
130			+++	+			+	−		
140			+++	++	++	+	+	−	+	−
150			+++	++			+	−		
160			+++	++	+++	+	+	−	++	−
170			+++	++			+	−		
180			+++	++	+++	+	++	−	++	−
190							++	−		
200					+++	+	++	(+)	++	−
210							+++	(+)		
220					+++	+	+++	+	++	+
230							+++	+		
240					+++	+	+++	+	++	+

[a] n. = Non-buffered; b. = buffered

− No alteration of general condition

\+ Shagginess of the fur

++ Shagginess and abdominal retractions

+++ Shagginess, abdominal retractions, hyposthenia, diminished activity, and neuromuscular disorder

[ ] Optimum dose range

were laparotomized on day 120 p.t.t. and their peritoneal cavities were inspected. As indicated in Table 4, pathological findings only arise above the optimum dose range with all compounds excepted the di-iodide. The peritoneal symptoms aggravate with increasing doses and consist in fibrous thickenings of the parietal and visceral peritoneum and in circumscribed or extensive adhesions between the parietal peritoneum and the abdominal viscera. Remarkably, the peritoneal reactions are nearly absent with the NCS compound even in high doses.

In another experimental series we applied the titanocene dihalides in the same dose levels, but *buffered* with $NaHCO_3$ from the original pH values of 1.4–3.9 (dependent on the nature and concentration of the compound) to 4.2–5.9. The results

Table 4. Peritoneal symptoms after treatment with titanocene dihalides $(C_5H_5)_2TiX_2$

Dose level (mg/kg)	X and mode of application[a]									
	F		Cl		Br		I		NCS	
	n.	b.	n.	b.	n.	b.	n.	b.	n.	b.
20	–	–	–	–	–	–	–	–	–	–
30	–	–	–	–			–	–		
40	–	–	–	–	–	–	–	–	–	–
50	–	–	–	–			–	–		
60	+++	–	(+)	–	–	–	–	–	–	–
70	+++	(+)	+	–			–	–		
80	0	+	++	–	–	–	+	–	–	–
90	0	0	++	–			+	–		
100	0	0	+++	(+)	+	–	+	–	–	–
110	0	0	+++	(+)			++	–		
120	0	0	+++	+	++	–	+++	–	–	–
130			+++	+			+++	–		
140			0	+	++	–	+++	–	–	–
150			0	+			+++	–		
160			0	++	+++	–	+++	–	–	–
170			0	++			+++	(+)		
180			0	0	0	+	0	(+)	+	–
190							0	(+)		
200					0	+	0	+	0	–
210							0	++		
220					0	+	0	++	0	++
230							0	++		
240					0	+	0	0	0	0

[a] n. = Non-buffered; b. = buffered

– No peritoneal symptoms

\+ Peritoneal thickenings

++ Peritoneal thickenings and slight adhesions

+++ Considerable peritoneal thickenings and adhesions

0 No survivors

Optimum dose range

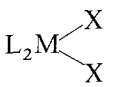
(Fig. 1, Tables 2–4) show that on pH elevation especially the toxic ranges are displaced to higher doses, that in most cases the optimum dose ranges are extended and that in the case of the titanocene dichloride the maximum cure rate is enlarged to 100% over a range of 5 dose levels. The T.I. values, however, increase after buffered treatment only with the difluoride and dibromide compounds and remain constant in all other cases.

Comparing the side effects under non-buffered and buffered conditions (Tables 3 and 4) it is obvious that the range of appearance of acute toxic symptoms like shagginess and abdominal retractions is shifted to higher dose levels by buffering and that the more serious symptoms like hyposthenia and neuromuscular dis-

order disappear. As an important result, the occurrence of postperitonitic findings is also considerably displaced to higher doses above the therapeutic ranges. Moreover, the degree of severity of the peritoneal symptoms is reduced so that in no dose level marked thickenings and adhesions are found.

Discussion

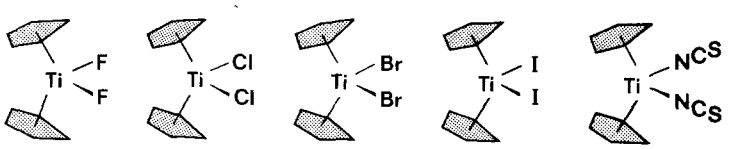
The structures of the metallocene dihalides and the well-known antitumor drug (Rosenberg 1973) *cis*-diamminedichloroplatinum(II) can both be simplified to the formula of a tetraco-ordinate neutral complex



where L represents the strongly bonded "carrier" ligands (C_5H_5 or NH_3, respectively) which facilitate the transfer of the metal moiety to the site of action in the organism, and X represents the dissociable adjacent *(cis)* ligands which by their replacement allow the active moiety to interact with the site of action, i.e., probably the nucleic acids (Roos 1977; Köpf-Maier et al. 1980; Köpf-Maier and Köpf submitted).

In the series of dihaloplatinum(II) complexes *cis*-$(NH_3)_2PtX_2$ (X = Cl, Br, I, SCN) only the Cl and – to a minor extent – the Br derivatives achieve antitumor activity, whereas the compounds with X = I and SCN effect no tumor inhibition (Cleare 1974). The author correlates these striking differences with the leaving abilities of X in this species and argues that only ligands with intermediate leaving ability like Cl and Br admit antitumor properties, whereas others like I and SCN which are bonded too strongly prevent any reaction within the organism.

The present work shows as a main result that in the titanocene dihalides $(C_5H_5)_2TiX_2$ there is an extensive possibility of varying X without loss of the cancerostatic potency. The investigated dihalides with X = F, Cl, Br, I including the bis(pseudohalide) with X = NCS[2]



are without exception strong tumor-inhibiting agents[3].

A possible explanation for this different behavior of the Pt and Ti compounds is given by the similarly intense ability of all titanocene dihalides including the I and NCS derivatives to cleave off their ligands X by hydrolysis forming an oxo-bridged Ti species (Samuel 1966; Thewalt and Schleußner 1978) and leading in

2 This ambident ligand is bonded via S in the thiocyanate *cis*-$(NH_3)_2Pt(SCN)_2$ (Cleare 1974) and via N in the isothiocyanate $(C_5H_5)_2Ti(NCS)_2$ (Villa et al. 1976)

3 Another bis(pseudohalide), the diazide $(C_5H_5)_2Ti(N_3)_2$, also exhibits antineoplastic properties (unpublished results)

aqueous solutions to pH values of 3.6 (F), 3.1 (NCS), and 2.7 (Cl, Br, I), respectively, in equimolar concentrations ($2 \cdot 10^{-3}$ *M*). This tendency to hydrolyze seems to be one of the suppositions for the tumor-inhibiting potency of the titanocene dihalides. Thus, the F and NCS compounds with the minor extent of hydrolysis show a limited optimal cancerostatic dose range, whereas with the more labile Cl, Br, and I ligands this dose range is more extended. Titanocenes containing very strongly bonded ligands X as in the case of the pentasulfide chelate $(C_5H_5)_2TiS_5$ (Köpf et al. 1968) effect no inhibition of Ehrlich ascites tumor.

It is, on the other hand, the acidity due to hydrolysis of the titanocene dihalide solutions which probably induces, after i.p. treatment with high doses, a chemical peritonitis giving raise to residual peritoneal thickening and adhesions. Other metallocene dichlorides with M = Nb, Mo and W, exhibiting in $2 \cdot 10^{-3}$ *M* aqueous solutions pH values of 2.6, 3.7 or 2.7, respectively, also provoke dose-dependent peritoneal alterations after i.p. application, whereas animals treated with $(C_5H_5)_2VCl_2$ (pH 4.0) show no peritoneal symptoms.

Another clue for the induction of these symptoms by acidity is the fact that pH elevation to 4–6 in the injected solutions of titanocene dihalides prevents to a high extent the occurrence of postperitonitic findings. The deteriorations of general condition following treatment are also reduced by buffering. Therefore, pH elevation appears to be a convenient method for suppressing drug-induced side effects of the metallocene dihalides. However, at $pH \geqq 7$ the cancerostatic activity of titanocene dihalides is diminished because of a proceeding hydrolytic cleavage of the C_5H_5 carrier ligands, the optimum pH range thus being limited to 4–6.

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Abbreviations

DMSO = dimethylsulfoxide; I.L.S. = increase in life span; i.p. = intraperitoneal(ly); p.t.t. = post transplantationem tumoris; T.I. = therapeutic index

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