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Carbonyl-Substituted Titanocenes: A Novel Class of Cytostatic Compounds with High Antitumor and Antileukemic Activity

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The outstanding success of cisplatin [*cis*-$Pt(NH_3)_2Cl_2$], one of the most broadly used chemotherapeutic agents,[1] has sparked tremendous interest in the development of related metal compounds with similar properties. Currently, complexes of platinum, iron,[2] ruthenium[3] and titanium[4] are in the center of attention. Of the titanium compounds derivatives of titanocene dichloride [Cp_2TiCl_2] have emerged as the most promising candidates for further investigations.[5] However, the progress of this fascinating field of research has been severely hampered by the lack of a general approach to structurally and functionally diverse complexes. Thus, the highly desirable screening of wide regions of chemical space[6] could not be realized.

Recently, we have described the first modular approach to exactly these compounds, that relies on the use of titanocenes with pending carboxylic acid chlorides.[7] In this manner oxygen and nitrogen nucleophiles could be acylated to yield a large number of structurally and functionally diverse titanocene complexes possessing ester and amide functionality. Besides the possibility to vary the nucleophile another crucial issue for the biological activity of the titanocenes can be addressed. Due to coordination of the carbonyl group, the amide complexes are soluble and stable in water or DMSO. Here, we report on the first synthesis of ketone-substituted titanocenes and the activity of our complexes against a variety of malignant cells.

As in the case of the amides our synthetic targets were cationic and water soluble complexes. However, ketones are noticeably weaker ligands than amides. Thus, we looked for a synthetic sequence that allows the conversion of the carboxylic acid chloride to electron-rich ketones as well as the abstraction of one chloride ligand of titanium. Thus, a general access to a large number of cationic complexes can be envisioned. All complexes discussed here were obtained as racemates, only one enantiomer is shown.

Friedel–Crafts acylations[8] of donor-substituted arenes in the presence of $ZnCl_2$ emerged as especially well-suited for our purposes. The desired complexes could be isolated in satisfactory to high yields as the tetrachloro zincates (Table 1).

An example of the coordination of the ketone at the cationic titanium center is depicted in Figure 1.

In addition to the variation of the aryl substituents and the straightforward modification of the *gem*-dialkyl group attached to the upper cyclopentadienyl ligand our synthesis of the carboxylates allows the alteration of the distance of the keto group from this ligand and the introduction of the substituents at the lower cyclopentadienyl ligand. A single additional methylene group prevents coordination of the ketone in **2f** as the result of the highly unfavorable formation of a strained ring. The structural modifications (Table 1, Scheme 1) should be essential for the biological activity of the complexes for three reasons. First, the polarity of the compounds can be tailored. Second, the electronic and steric properties of all substituents will have an influence on the exact coordination geometry of titanium. Third, the overall three-dimensional shape of the titanocenes can be varied in a straightforward manner. This can turn out to be crucial regarding the binding ability to enzymes and receptors.

To understand the biological activity of our titanocenes, we investigated a broad spectrum of both solid tumors and leukemia cell lines. These include BJAB cells (lymphoma),

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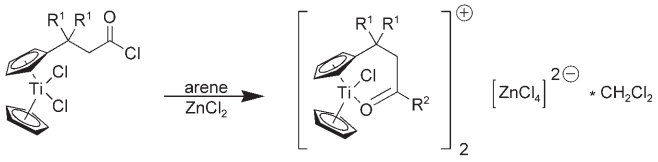
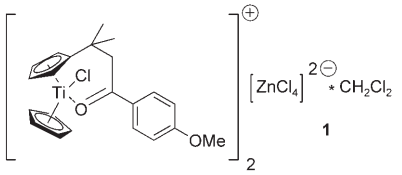
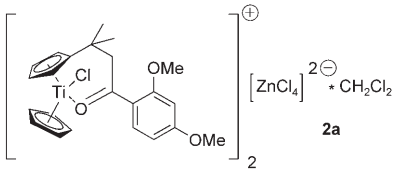
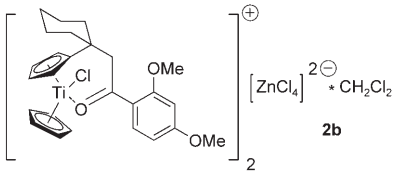
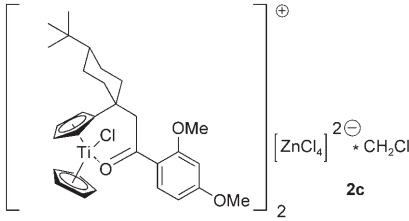
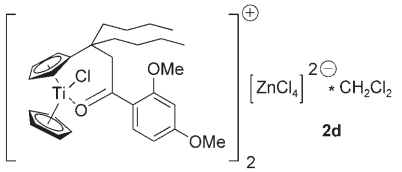
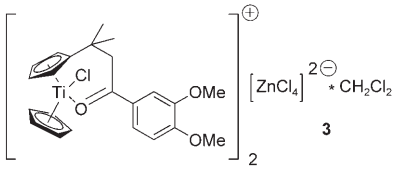
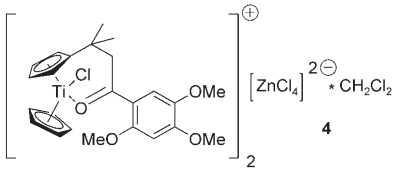
Table 1. Synthesis of ketone-substituted titanocenes via the Friedel–Crafts acylation.



Product	Yield [%]
 **1**	97
 **2a**	90
 **2b**	60
 **2c**	49
 **2d**	78
 **3**	86
 **4**	26

MelHO and A375 cells (melanoma), MCF-7 cells (mammacarcinoma), Nalm-6 and Jurkat cells (leukemia) as well as primary human tumor cells of relapsed rhabdomyosarcoma and nephroblastoma. We also investigated primary human ALL (acute lymphoblastic leukemia) und AML (acute myeloblastic leukemia) cells. In all malign cells an unusually high concentration dependant induction of apoptosis was observed. Such a high specific induction of apoptosis is of tremendous relevance for therapeutic applications,[9] since a damage of healthy human cells that are not in proliferation by necrosis can in principle be minimized.[10]

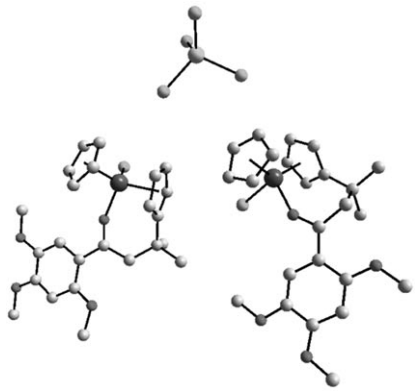
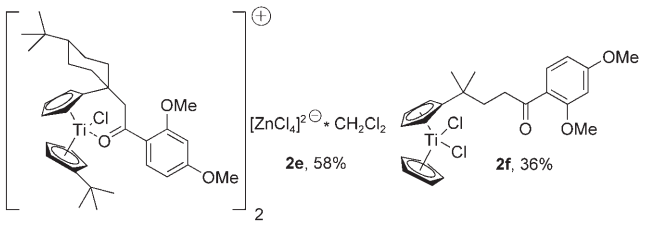


Figure 1. Cationic coordination of titanium in the crystal structure of **4** (CH_2Cl_2 omitted for clarity).



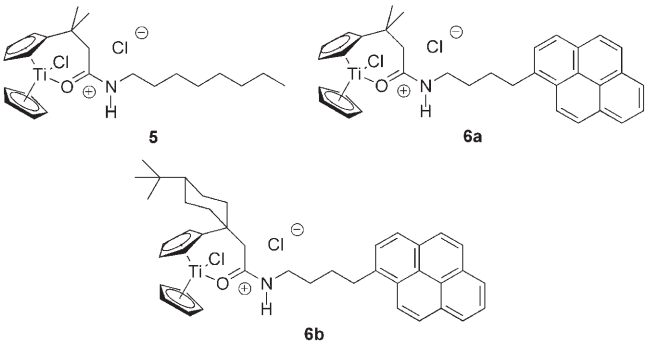
Scheme 1. Further synthetic modifications of the titanocenes' substitution pattern.

Here, we discuss our results of the lymphoma cell line BJAB as a typical example. BJAB cells express the cell surface transmembrane receptor CD95 through which receptor-mediated apoptosis can be induced. Hence, they are able to die via the intrinsic as well as the extrinsic apoptosis-signalling pathway.[11] For this reason, the BJAB cells are exceptionally well-suited for investigating the induction of apoptosis by titanocenes.[12]

Since cytotoxic drugs develop their effect by specific induction of apoptosis, it is reasonable to measure precisely this induction and not the unspecific cytotoxicity that is usually expressed by the LC50 values. Therefore, for our investigation of the structure–activity relationship of our titanocenes we determined the AC50 value, which is the concentration where in 50% of lymphoma cells specific apoptosis is induced. The AC50 value, which is measured by detecting all cells with damaged membrane, will always be lower than the corresponding LC50. This is because in the DNA fragmentation assay only the apoptotic cells at a defined point of time in the cell death cascade are measured.

Of the amide-substituted complexes the ones shown in Scheme 2 turned out to be most active. Because they are not cationic, all ester-substituted titanocenes were ineffec-

tive. The activities of complexes **1**–**4**, **5**, **6a** and **6b** against the BJAB cell line are summarized in Table 2. The ketone complexes **1**–**4** were soluble in DMSO and DMSO/water mixtures.



Scheme 2. Highly active cationic amide complexes.

Table 2. Activity oft the carbonyl-substituted titanocenes against the BJAB cell line.

Complex	2a	2b	2c	2d	2e	2f	5	6a	6b
AC50 [μM]	>100	>100	12.5	35	80	>100	55	50	30

The AC50 of the most active compound **2c** in BJAB cells is 12.5 μM (Figure 2). For measuring this value the cells (10^5 BJAB cells per mL) were incubated with various concentrations of **2c** for 72 h at 5% CO_2 and 37 °C. After dying with propidium iodide the DNA fragmentation was then detected by flow-cytometric determination via FACS analysis. A concentration dependent induction of apoptosis of more than 71% of the cell population was measured. Only 3% of the cells were necrotic.

An unspecific cytoxic effect of the titanocenes via necrosis[13] could be excluded by determination of extra cellular lactate dehydrogenase (LDH) disposal via ELISA-reader detection after incubation for 3 h (Figure 3).

The unspecific necrosis that is mediated through membrane toxicity is observed within short periods of time (1 h). Therefore it can be detected by measuring the concentration

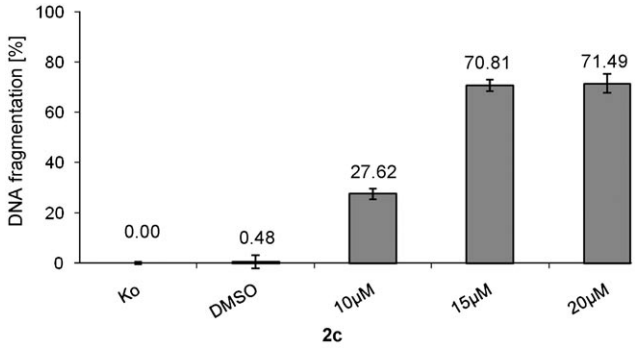


Figure 2. Induction of apoptosis by **2c**.

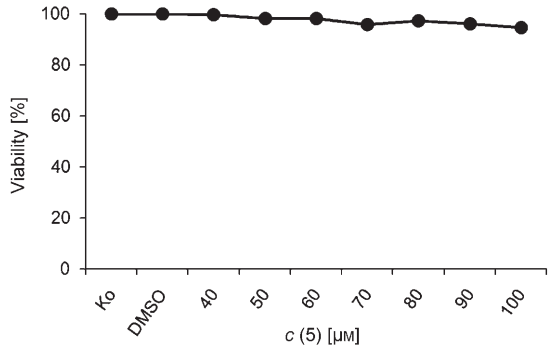


Figure 3. Exclusion of unspecific cytotoxic effects (such as necrosis) of **5** via detection of LDH release through photometrical measurement via ELISA-reader. The average of two measurements is shown. The experiment was repeated twice, the deviation of the values of the three independent measurements was below 3%.

of large otherwise intracellular proteins such as LDH.[14] Our results therefore clearly indicate that the cyctotoxic effect of the carbonyl-substituted titanocenes is selectively mediated through the induction of apoptosis. The unspecific damage by **5** is so low that even at the highest concentration the viability of the cell population is more than 94%.

After treatment with the titanocenes for 72 h a microscopic investigation shows the cellular changes typical for apoptosis. In Figure 4A) represents the control sample after 72 h (incubation: intact lymphoma cells in dense colonies) and B) the induction of apoptosis after incubation with **5** (75 μM). Besides the fragments of some apoptotic cells, a small number of lymphoma cells with the typical morphological changes and the characteristic apoptosis induced "blebbing" are observed.[15]

These results have been supported by first in vivo experiments. The titanocenes are well tolerated in concentrations of up to 75 mg kg^{-1} weight.

The data presented in Table 2 show a clear dependency of the biological activity of our titanocenes on their structure. As the ester-substituted complexes as well as **2f** are not effective in inducing apoptosis, cationic coordination at the titanium center is essential. Introduction of substituents at the lower cyclopentadienyl ligand results in a noticeable decrease of activity. For further screening of the complexes it is important to note that the most potent amide- and ketone-substituted complexes contain distinctly different

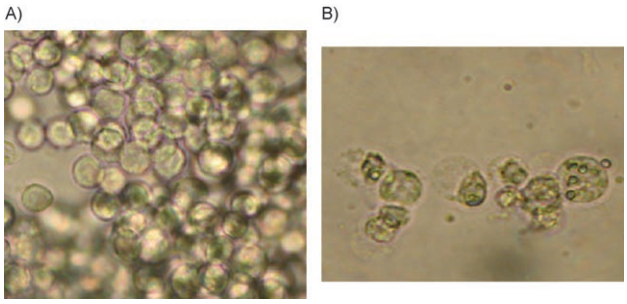


Figure 4. Microscopic view of apoptosis induction by **5** in BJAB cells.

substituents at the carbonyl group. Whereas 2,4-dimethoxyphenyl substituted ketones give the best biological results, all aniline and benzylamine scrutinized, which contain amides, are inactive. Only introduction of long alkyl chains, preferentially with terminal polycondensed aryl groups leads to complexes with high induction of apoptosis. The *gem*-dimethyl group adjacent to the upper cyclopentadienyl moiety in **2a**, **5** and **6a** proved to be less than ideal. The activities observed against BJAB cells were not sufficient. Also, the introduction of the cyclohexyl entity in **2b** did not increase the activity. Only the even more sterically demanding substituents, such as 4-*tert*-butyl cyclohexyl (**2c** and **6b**) or di-*n*-butyl (**2d**) lead to satisfactory results. Complexes **2c** and **6b** are the most potent titanocenes in the induction of apoptosis described in the literature to date.[4,5,16] In the last study cited it was also demonstrated that titanocenes can induce more apoptosis than cisplatin in a number of cancer cells.

In order to achieve a high biological activity a very bulky substituent on the upper cyclopentadienyl ligand is highly desirable. With these results a general design principle for biologically active titanocenes seems at hands for the first time.

In conclusion, our carbonyl-substituted titanocenes constitute a novel class of compounds resulting in exceptionally high levels of induction of apoptosis in various tumor and leukemia cells. Therefore, they are attractive candidates for the treatment of a number of malignant diseases. First in vivo experiments demonstrate a significant inhibition in tumor growth in SCID mice with human lymphomas. Currently, we are working on establishing the mechanism of apoptosis induction and on identifying the target of our novel compounds.

Experimental Section

Synthesis of 2c: A solution of the carboxylate (1.24 g, 2.5 mmol) in $SOCl_2$ (4.5 mL, 61.5 mmol) was stirred for 2 h. Then, $SOCl_2$ was removed in vacuo (1 mbar) for 3 h at 80 °C. The resulting solid was dissolved in CH_2Cl_2 (10 mL), and the red solution was added to $ZnCl_2$ (1.70 g, 12.5 mmol) and 1,3-dimethoxybenzene (5.15 g, 37.5 mmol). After stirring over night, the solvent was evaporated and **2c** was isolated by recrystallization from CH_2Cl_2/cC_6H_{12} as a red solid (1.44 g, 86 %).

Acknowledgements

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Keywords: antitumor agents · apoptosis · metallocenes · titanium

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