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Highly Selective Apoptotic Cell Death Induced by Halo-Salane Titanium Complexes

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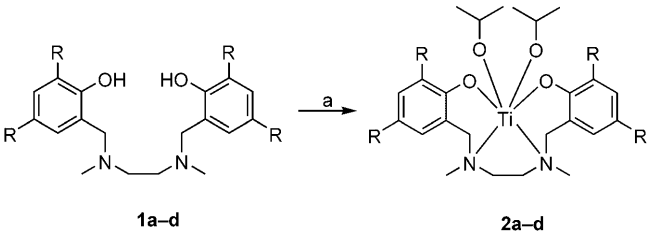
Following the accidental discovery of cisplatin by Rosenberg and co-workers[1,2] and its enormous success as chemotherapeutic agent, there has been a growing interest in the investigation of other platinum-based compounds as well as non-platinum metal-based systems. Among these, titanium(IV) complexes showed encouraging antitumor activity in various cell lines.[3–7] Nearly all complexes investigated were derivatives of either titanocene dichloride[8] or budotitane,[9,10] the only titanium complexes reaching clinical trials so far. Titanocene dichloride showed promising results in phase I trials and was further investigated in phase II studies. Unfortunately, no objective clinical responses were observed.[11,12] The main disadvantages of titanocene complexes are their fast hydrolysis under physiological conditions and the formation of unidentified metabolites. The hydrolysis of the first chloride of titanocene dichloride occurs within seconds and the cyclopentadienyl ligands are hydrolyzed after 2–3 days. This hampers identification of the active species and the investigation of mechanistic detail.[13] Furthermore, little is known about the cellular uptake of titanium complexes or their exact mechanism of action. It has been shown that titanocene dichloride is enriched in areas near the nuclear chromatin, covalently binds to DNA and inhibits DNA synthesis.[14,15] Interestingly, the binding to DNA occurs via the phosphate backbone rather than the nucleobases.[16,17] Titanocene dichloride was also reported to inhibit human topoisomerase II.[18]

Concerning the cellular uptake, it seems that transferrin, as well as albumin, plays a role in transferring Ti^{IV} into the cell. While stripping of the ligands is required for trafficking of Ti^{IV} via transferrin,[19] a route via albumin could leave the complexes intact. An adduct of the complex stabilized by albumin might then enter the cell.[20] This would promote a more active role for these drugs in contrast to the prodrug role proposed for the transferrin delivery mechanism. Consistent with these findings, two methyl substituted titanium salane complexes were recently reported to be cytotoxic independent of transferrin.[21]

We herein report the synthesis of halogen-substituted titanium salane complexes **2a–d** and their detailed biochemical evaluation in two different tumor cell lines. The salane ligands **1a–d** were accessible by simple refluxing the appropriate phenol, *N*,*N*′-dimethylethylenediamine and formaldehyde in methanol.[22] Metalation with titanium tetraisopropoxide (Ti(O*i*Pr)$_4$) finally gave the racemic C_2 symmetrical complexes[23,24] (Scheme 1), which could be recrystallized from *n*-hexane or toluene.



Scheme 1. Preparation of complexes **2a–d** (**a**: R = F; **b**: R = Cl; **c**: R = Br; **d**: R = Me). *Reagents and conditions*: a) Ti(O*i*Pr)$_4$, toluene, RT.

Single crystals of **2a** (Figure 1) were obtained from toluene at room temperature.

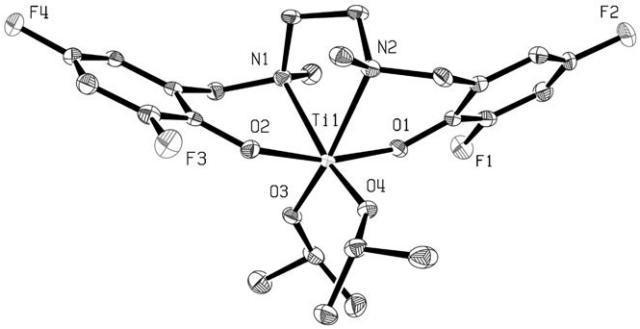


Figure 1. ORTEP diagram of **2a** at 50% probability ellipsoids.

The cytotoxicity of complexes **2a–d** was studied using the AlamarBlue assay in the human cervix carcinoma cell line HeLa S3 and in Hep G2 cells, an established human hepatocarcinoma cell line with epithelial morphology. This assay was reported to be highly reproducible and more sensitive than the MTT assay.[25] Cells were incubated for 48 h with different concentrations of the complexes. AlamarBlue was added and converted by living cells to the red fluorescent dye resorufin.

Measuring the fluorescence and comparing it to a negative control gave the relative number of cells that survived the treatment.[26] The resulting dose-response curves for the HeLa S3 cell line are shown in Figure 2. The IC_{50} values—the concentration at which 50% of cells remained viable with respect to

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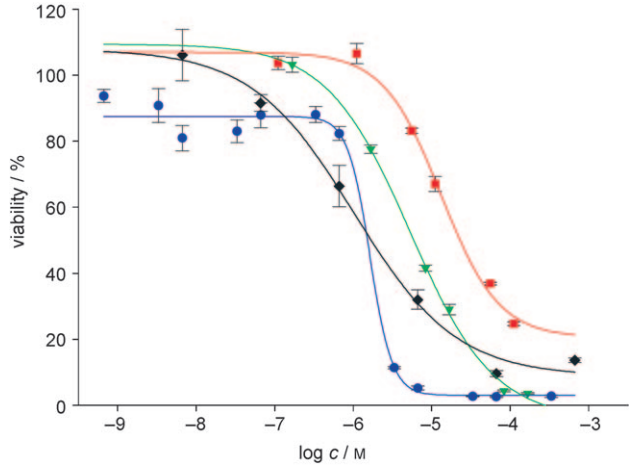

Figure 2. Loss of cell viability (HeLa S3) as a function of treatment with varying concentrations of cisplatin or complexes **2a–c** after 48 h of incubation. Complexes: **2a**, ●; **2b**, ▼; **2c**, ■; cisplatin, ◆.

Table 1. IC_{50} values of complexes **2a–d** and cisplatin in HeLa S3 and Hep G2 cells.[a]

Complex	IC_{50} [μM]	
	HeLa S3	Hep G2
2a	1.6 ± 0.1	2.2 ± 0.2
2b	5.3 ± 0.2	4.0 ± 0.2
2c	13 ± 1	40 ± 6
2d	2.2 ± 0.1	2.1 ± 0.2
cisplatin	1.2 ± 0.4	3.0 ± 1.3

[a] values measured after 48 h of incubation.

controls—of the titanium complexes in both cell lines are summarized in Table 1. Cisplatin was also tested as a reference compound.

The IC_{50} values were clearly dependent on the steric demand of the halogen. The cytotoxicity of halogen-substituted complexes **2a** (R = F) and **2b** (R = Cl) is similar to that observed for the methyl-substituted complex **2d**. This observation indicates that electronic effects play only a minor role in the overall cytotoxicity. Complexes **2a** and **b**, with IC_{50} values comparable to cisplatin, are among the most active titanium complexes found to date.[27] NMR studies recently conducted by our group employing a [D8]THF/D_2O mixture revealed remarkable robustness towards hydrolytic cleavage. With a typical half-life for the loss of the isopropoxy groups of ~7 h, salane complexes **2a–d** are much more stable compared with titanocenes complexes.

Anticancer drugs are effective due to their specific induction of apoptosis,[28] therefore, we also studied the overall cytotoxicity measured by the AlamarBlue assay; the distribution of cell death based on either apoptosis or necrosis. In contrast to necrosis, which is a form of traumatic cell death resulting from acute cellular injury, apoptosis is programmed cell death. In principle, antitumor agents should predominantly induce apoptosis as, in contrast to necrosis, apoptosis does not cause inflammatory responses, which could cause severe side effects.

We followed the fate of HeLa S3 cells upon incubation with complexes **2a**, **2b** and **2d** at 8 μM, 10 μM and 10 μM, respectively—that is, concentrations two- to fivefold greater than the IC_{50} values for loss of cell viability (Table 1)—via double staining with propidium iodide and fluorescein isothiocyanate-labeled annexin V (Figure 3). Because of its significantly lower cytotoxicity bromo-substituted complex **2c** was omitted from these measurements.

A differentiation between apoptotic and necrotic cell death is possible as propidium iodide only stains necrotic and late-apoptotic cells with ruptured cell membranes, whereas annexin V binds to both apoptotic and necrotic cells. The proportions of apoptotic and necrotic cells were determined using flow cytometry.

We found that the halogen-substituted complexes **2a** and **2b** nearly exclusively killed the cells through apoptosis induction (Figure 3). Metal-free ligands **1a** and **1b** were also tested in a control experiment; no significant toxicity was observed even with four times higher concentrations. Notably, after incubation with complex **2b** (R = Cl), cell death was caused almost exclusively by apoptosis (> 96%, Table 2). Few other titanium complexes are known to be as highly selective for the induction of apoptosis[29,30] and the IC_{50} values of the complexes reported herein are at least one order of magnitude higher than those of comparable antitumor agents such as cisplatin.

Table 2. Percentage of dead Hela S3 cells killed via apoptosis upon incubation with **2a**, **b** and **d**.

Complex	Concentration [μM]	Apoptotic/dead cells [%]
2a	8.0	90.1 ± 4.4
2b	10.0	95.5 ± 2.6
2d	10.0	48.8 ± 9.0

To the best of our knowledge, complex **2b** is the first titanium complex that combines high cytotoxicity with a selective induction of apoptosis. Selective induction of apoptosis is of enormous therapeutic relevance as nonspecific damage to healthy cells by necrosis can be minimized. Interestingly, the ratio of apoptosis to necrosis was, in contrast to the overall cytotoxicity, not as significantly affected by the steric demand of the ligands. A noticeable difference was found between the halogen-substituted complexes **2a** and **2b** (> 90% apoptotic cell death) and the methyl-substituted complex **2d** (< 50% apoptotic cell death). Here, electronic effects might play a role.

Currently, we are studying further aspects of the mechanism of action of those salane complexes to better understand their striking selectivity.

Experimental Section

The Supporting Information contains full experimental details for the synthesis of the salane complexes **2a–d** outlined in Scheme 1. Details of the cytotoxicity studies conducted can also be found in the Supporting Information. Crystallographic data can be obtained

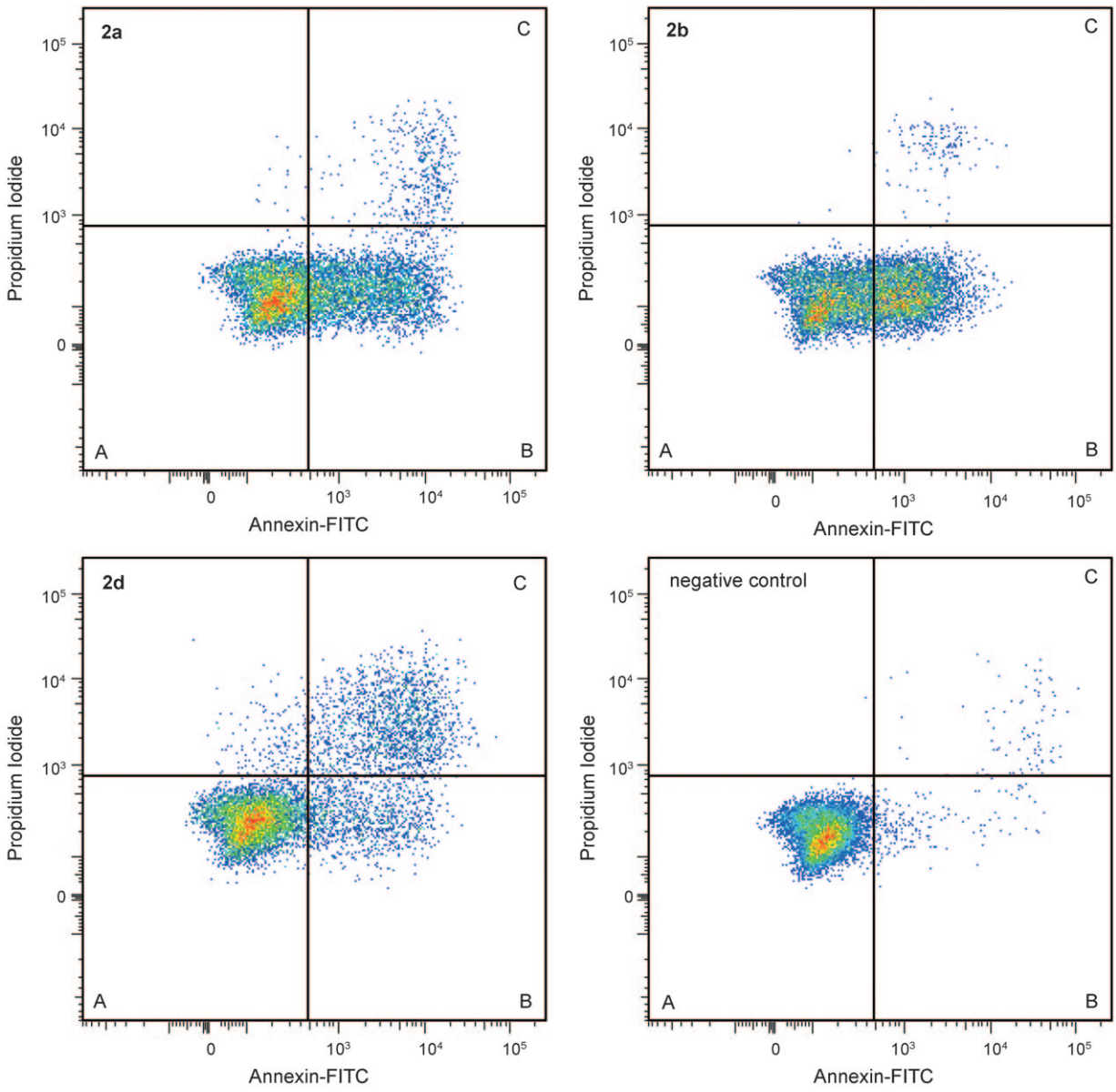

Figure 3. Quantification of viable (quadrant A), apoptotic (quadrant B) and necrotic (quadrant C) HeLa S3 cells after 24 h of incubation with complexes **2a** (top left), **2b** (top right), **2d** (bottom left), or control medium (bottom right).

free of charge from the Cambridge Crystallographic Data Centre (CCDC 719706, www.ccdc.cam.ac.uk/data_request/cif).

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Keywords: antitumor agents · apoptosis · cytotoxicity · structure–activity relationships · titanium complexes

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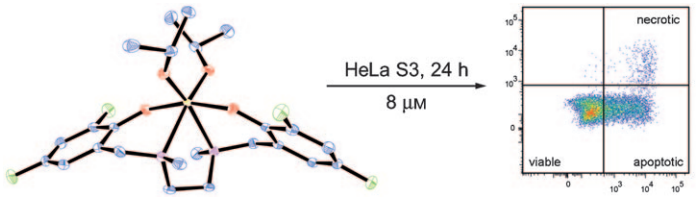

Metal-based antitumor agents: Halogen-substituted titanium salane complexes showed IC_{50} values comparable to cisplatin. In contrast to their alkyl-substituted congeners, they almost exclusively induced apoptotic cell death. This unique combination of very low IC_{50} values and pronounced preference for apoptosis makes them promising therapeutic agents.

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