Two differently substituted fluorescent salen Ti(IV) complexes were developed. One was inactive on human cancer cells, whereas the other showed high cytotoxicity. Based on live cell imaging, both complexes penetrated the cell, but were not detected in the nuclei. Moreover, the inactive complex was trapped in endocytic vesicles, whereas the active complex accumulated in the perinuclear region and inflicted phototoxicity upon continuous irradiation.

Titanium(n) complexes are highly attractive candidates for anticancer therapy. After the platinum anticancer compounds, titanium(IV) complexes, in particular titanocene dichloride (Cp₂TiCl₂) and budotitane ([bzac]₂Ti(OEt)₂), were the first to enter clinical trials due to their high and wide activity in vitro and in vivo, accompanied by low toxicity, ultimately producing the non-toxic titanium dioxide in aqueous solutions. Nevertheless, these Ti(IV) compounds failed clinical trials due to their low hydrolytic stability in biological environments that led to the formation of undefined aggregates with low solubility, which also hampered mechanistic investigations.

Previously, we introduced anticancer Ti(IV) complexes based on phenolato ligands, which presented exceptional hydrolytic stability combined with high cytotoxicity and in vivo activity. In particular, Ti(IV) LTIX₂ type complexes based on diaminobis (phenolato) salan and salen ligands with two labile alkoxo groups were investigated, exhibiting activity often greater than that of cisplatin with slow hydrolysis to eventually yield highly stable and defined phenolato polynuclear clusters. Despite the different geometries of the salan and salen complexes, similar mechanisms were suggested based on their similar reactivity with a bidentate chelating ligand and similar hydrolytic behavior. Structure–activity studies indicated strong impact of the ligand substitution on complex performance: steric bulk decreased activity, and hydrophobicity decreased solubility and accessibility and by that, affected reactivity.

Very little is known about the mechanism of cytotoxicity of Ti(IV) complexes. Specifically, when analyzing potential cellular targets, various studies have been performed with older and newer compounds, the results of which are not all consistent. Although some studies pointed to possible interactions with DNA based on accumulation in nucleic acid-rich areas and various adducts identified, others suggested alternative mechanisms involving, for instance, enzyme inhibition. Herein, we utilize the photophysical features of the planar highly-conjugated salen system for the detection of Ti(IV) complexes in living cells using confocal fluorescence microscopy to shed light on the cellular biodistribution of the cytotoxic phenolato Ti(IV) complexes. The x-conjugated system of the bis-Schiff base salen ligands can be modulated by particular substitutions on the amine bridge and/or the phenolato rings to enhance fluorescence. Such salen chelating ligands were previously used as optical probes for cations such as Cu(n), Pt(II), Al(III), and Zn(n), and salen Zn(n) complexes were employed as fluorescent markers for cell imaging.

To enhance intramolecular charge transfer, diethylamino-substituted phenolato rings were employed, and two complexes were synthesized with cyano-substituted or phenylene-diamine bridges (Scheme 1). The ligands 1a and 2a were prepared according to a known procedure via a condensation reaction between substituted salicylaldehyde and the conjugated diamine. The Ti(n) complexes 1b and 2b were prepared as previously discussed for related compounds by reacting 1a and

Scheme 1  Fluorescent Ti(IV) salen complexes investigated.
2a, respectively, with one equiv. of titanium tetrakis(dimethylphenoxide) to obtain the Ti(IV) complexes in high yields. H and 13C NMR analyses of the products confirmed the formation of a single isomer with high symmetry due to the single set of signals for the phenolato moieties of the salen ligand and a single type of a labile ligand. Single crystals of 1b and 2b were obtained from diethyl ether and dichloromethane, respectively, at 35 °C. Although the structure of 1b was of low quality (Fig. S1†), Fig. 1 depicts the ORTEP drawing of 2b along with selected bond lengths and angles.† Both structures clearly feature mononuclear octahedral C2v-symmetrical complexes with trans binding of the two labile dimethylphenoxo ligands. Furthermore, the bond lengths and angles of 2b around the metal center are similar to those of related Ti(IV) salen complexes.4,5

The cytotoxicity of 1b and 2b was evaluated on human colon HT-29 and cervix HeLa cancer cell lines using the methylthiazolyldiphenyl-tetrazolium (MTT) assay to establish cell viability (Fig. 2).10 Complex 2b exhibited high antitumor activity with IC50 values ranging from 3.3 to 9.3 µM, which are among the lowest values obtained for this family.4,5 In contrast, 1b was inactive toward both cell lines tested. It was suspected that the electron withdrawing cyano groups in 1b reduce the stability or affect cellular penetration due to reduced lipophilicity. The free salen ligand 2a analyzed as the control showed significantly lower reactivity (Fig. S7†).

The comparative hydrolytic stability of 1b and 2b was evaluated upon the addition of 10% D2O (ca. 1000 equiv. relative to Ti) to d6-DMSO solutions of the complexes, as previously described for related Ti(IV) compounds (Fig. S2†).4,5,11 Spectra were collected every few minutes and the integration of selected signals of the labile 2,6-dimethylphenoxo ligands was measured relative to an internal standard. The hydrolytic stability of both complexes was similar with the t1/2 values for the labile ligand hydrolysis of 50 and 20 minutes for 1b and 2b respectively, ruling out reduced hydrolytic stability as the reason for the inactivity of 1b. Throughout the hydrolysis, single polynuclear species were separately obtained for each complex with no indication of salen ligand release even after 3 days. These products featured high symmetry, which indicated formation of either the di- or the tetra-nuclear product, as reported recently for related complexes,4,5,11 whereby MS analysis supported the specific formation of a dimeric structure as characteristic for the salen family of complexes (Fig. S3†).4,5 The spectra of the hydrolysis products did not change even upon the addition of 10 000 H2O equivalents to the parent complexes and stirring for 3 days, confirming the formation of a single defined cluster (Fig. S2c†).

The hydrolytic behaviour of the complexes implied that the active species derived from 2b is its defined hydrolysis product.4,5,12 Thus, to evaluate the possible reactivity of both hydrolysis products, complexes 1b and 2b were separately reacted with 10 000 equivalents of H2O and the hydrolysis products 1c and 2c (Scheme 1) were isolated (Fig. S4 and S2c†). Their cytotoxicity was measured on HT-29 and HeLa cells, as described above. Although the hydrolysis product 1c was inactive like its parent complex, 2c exhibited high cytotoxicity with IC50 values comparable to those of its monomeric precursor and even better for the HeLa cell line (Fig. 3). Previous reports presented the cytotoxic

![Fig. 1 ORTEP drawing of 2b† with 50% probability ellipsoids; H atoms and dichloromethane solvent molecule were omitted for clarity. Selected bond lengths [Å] and angles [°]: Ti(1)–N(2) 2.143(2), Ti(1)–O(1) 1.904(2), Ti(1)–O(2) 1.874(2), O(1)–Ti(1)–O(2) 89.17(7), O(1)–Ti(1)–O(1) 109.47(7), O(2)–Ti(1)–O(2) 177.7(1), N(2)–Ti(1)–N(2) 74.9(1), O(2)–Ti(1)–N(2) 90.25(7), and O(1)–Ti(1)–N(2) 87.88(7).](image)

| Cell line | IC50 [µM] | M[%] |
|-----------|----------|------|
| HT-29     | 3.3 ± 0.6 | 92%  |
| HeLa      | 9.3 ± 1.5 | 93%  |

![Fig. 2 Dependence of HT-29 and HeLa cell viability (based on MTT assay after 3 days incubation) on concentration of 1b and 2b, which is presented on a logarithmic scale.](image)

![Fig. 3 IC50 values (µM) of 2b and 2c on different cell types: HT-29 and HeLa cells.](image)
activities of such defined polynuclear hydrolysis products based on salan ligands but only in formulations to increase solubility and accessibility\(^{12a}\) or toward more sensitive murine cancer cell lines.\(^{12b}\) Thus, this first observation of high activity of a defined phenolato cluster toward a relatively resistant human cancer cell line indicates the enhanced solubility and accessibility of \(2c\), and supports the overall conclusion that the hydrolysis product is the active species in the cell.

Studies with fluorescent Ti(\(\text{IV}\)) complexes are scarce\(^{13}\) because fluorescence quenching often occurs due to the electron-poor d\(^0\) Ti(\(\text{IV}\)) metal, which involves strong ligand to metal charge transfer.\(^{13}\) Nevertheless, \(1b\) and \(2b\) are highly emissive in DMSO with red emission for \(1b\) and green-yellow emission for \(2b\), with similar features for their hydrolysis products \(1c\) and \(2c\), respectively (Fig. S5, S6 and Table S1†). Additionally, having two related complexes in hand, one cytotoxic and one inactive, provided an opportunity to compare the two with respect to cellular bio-distribution. Thus, viable cells treated with \(1b\) and \(2b\) were evaluated using confocal fluorescence microscopy. The treatment of human cervical HeLa cells separately with 5 \(\mu\)M of \(1b\) and \(2b\) for only a few minutes resulted in the development of a strong red emission under excitation at 561 nm for \(1b\) and green emission under excitation at 488 nm for \(2b\) in the cytoplasm (Fig. 4). Interestingly, despite being inactive, \(1b\) crossed the cell membrane relatively rapidly and also accumulated in the cytoplasm; therefore, its inactivity apparently does not result from its inability to enter the cell due to insufficient solubility/lipophilicity. Additionally, the defined hydrolysis products \(1c\) and \(2c\) were similarly analyzed at a concentration of 2 \(\mu\)M, and similar results and accumulation patterns were observed to those obtained for the parent complexes \(1b\) and \(2b\), respectively.

The subcellular accumulation of \(1b\) and \(2b\) and \(1c\) and \(2c\) was further investigated by staining the organelles with specific fluorescence probes: Hoechst\(^{\text{®}}\) for staining the nuclei blue and MitoTracker\(^{\text{®}}\) green or deep red for staining the mitochondria (applied for 1 or 2, respectively). Interestingly, no significant colocalization was detected for the Ti(\(\text{IV}\)) complexes, and in particular, no indication of entering the nuclei was observed.\(^{13}\) For the inactive \(1b\) and its hydrolysis product \(1c\) the signals appeared as spots and could be mapped as vesicles in the cytoplasm of the treated cells (Fig. 5, lines 1 and 2, respectively). These vesicles spread in the cell cytoplasm and their concentration increased with incubation time. This form of accumulation suggests that \(1b/1c\) enter the cells via endocytosis in the form of endocytic vesicles and are transported towards the cytoplasm, as observed previously for Ru(\(\text{II}\)) complexes.\(^{14}\) Interestingly, a different process was observed for the cytotoxic \(2b\) and its hydrolysis product \(2c\). These complexes crossed the membrane rapidly, possibly by diffusion, and accumulated in the perinuclear region, which could be associated with the endoplasmic reticulum (ER) domain or the Golgi apparatus, as presented in Fig. 5, lines 3 and 4, respectively. Some correlation was observed with MitoTracker\(^{\text{®}}\), which may result from mitochondria swelling. Thus, treatment with cytotoxic Ti(\(\text{IV}\)) compounds could induce mitochondrial dysfunction which eventually leads to apoptotic cell death. Interestingly, a previous study with Pt(\(\text{II}\)) complexes also showed accumulation in the ER region and induction of ER-stress and cell apoptosis,\(^{15}\) unlike the common observations of the nuclei as the target of Pt(\(\text{II}\)) complexes.\(^{16}\) Notably, the free ligand \(2a\), which was analyzed as the control, accumulated differently as defined spots near the nuclei (Fig. S8†).

Following the addition of 5 \(\mu\)M of \(2b\) to HeLa cells, as described above, and incubation under continuous irradiation (every 5–7 minutes), the viable cell population dramatically decreased, much more rapidly than expected (Fig. 6). After only 3 hours the cells underwent cell death and displayed cell shrinkage, loss of cell contact, and membrane blebbing (Fig. 2c, line 6), which are characteristic of early apoptosis.\(^{17}\) Despite the high cytotoxicity of \(2b\), such rapid cell death is not

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Fig. 4 Confocal images of the Ti(\(\text{IV}\)) complexes after incubation in HeLa cells for ca. 5 minutes: (a) \(1b\) excited at 561 nm; (b) merge image of the fluorescence with DIC image; (c) \(2b\) excited at 488 nm and (d) merge image of the fluorescence with DIC image.

Fig. 5 Fluorescence colocalization images merged with the DIC image of HeLa cells incubated for 1 hour with \(1b\) (5 \(\mu\)M with 0.2% DMSO in medium, line 1), \(1c\) (2 \(\mu\)M with 0.2% DMSO in medium, line 2), \(2b\) (5 \(\mu\)M with 0.2% DMSO in medium, line 3) and \(2c\) (2 \(\mu\)M with 0.5% DMSO in medium, line 4) and commercial dyes Hoechst\(^{\text{®}}\) (a) and MitoTracker\(^{\text{®}}\) (b), Ti(\(\text{IV}\)) compound (c) and merged images of (a)–(c) (d).
with additional detection techniques are required to unequivocally determine the biological target of these promising anticancer Ti(IV) complexes.

Conflicts of interest

There are no conflicts to declare.

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