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Review

Modern cytotoxic titanium(IV) complexes; Insights on the enigmatic involvement of hydrolysis

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Contents

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ABSTRACT

In this paper, we describe our recent results with three families of Ti(IV) complexes, elaborating on their cytotoxic activity, hydrolytic behavior, and the potential connection between the two. Complexes of diamine bis(phenolato) ligands, particularly those of ligands of sequential arrangement of donor atoms, demonstrate appreciable activity towards colon HT-29 and ovarian OVCAR-1 cell, which is higher than that of titanocene dichloride, budotitane and cisplatin. Strong dependence of activity on the particular ligand is observed, where small modifications relating to steric bulk at various locations, electronic effects, ligand lability, general symmetry of the complex and more, have a major effect on the cytotoxic activity. An interesting correlation between cytotoxic activity and hydrolytic behavior is observed. Complexes of cytotoxic activity lead upon addition of water to defined O-bridged clusters within several hours,

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which is a particularly slow period for such processes, where the bulk of the substituents located near the metal site affects the rate of hydrolysis. In contrast, complexes that are inactive biologically either decompose rapidly, or possess steric bulk too large to form the O-bridged cluster. Additional mechanistic investigations revealed that the activity is independent of transferrin, and that the cell penetration of the active species by an alternative mode occurs relatively rapidly prior to its decomposition to give inactive complexes, among which, the O-bridged cluster. Thus, a relatively inert ligand is required to allow this process and stabilize the active species, while hydrolysis of the more labile groups seems to be essential for its formation. These conclusions are also supported by additional studies conducted with hydroxylamino-triazine compounds, which proved them to be excellent ligands for Ti(IV) forming especially short Ti–N coordinative bonds, thus leading to exceptionally high hydrolytic stability. Some cytotoxic activity was observed for highly inert homoleptic Ti[ONO]$_2$ complexes of this family, while lack of activity was obtained with analogous substantially more rigid [ONNO]Ti(OiPr)$_2$ complexes featuring weaker ligand binding and thus hydrolytic instability. Well-defined Ti(IV) complexes of bis(carboxylato) ligands are more difficult to obtain due to the ability of the carboxylato groups to bind in a bidentate mode and bridge two metal centers, nevertheless, such complexes are in general more hydrolytically instable and lack cytotoxic activity.

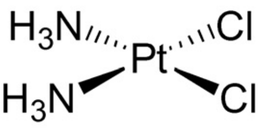
1. Introduction

Bioinorganic chemistry is a rapidly evolving area of research [1–5]. Transition metal atoms take part in a variety of biological processes due to their characteristic electronic features, which generally involves their binding to electron-rich biological components, such as proteins and DNA. It is thus reasonable to propose that metal ions may be incorporated into drugs, with the main goal being interacting in a controlled manner with biological systems.

One of the first inorganic complexes discovered to exhibit biological activity is cisplatin (Scheme 1) [6–9]. Cisplatin operates by interacting with DNA, mainly via cross-linking to two adjacent guanine residues, following the loss of the two labile chloride ligands, which hampers cell growth. Cisplatin is considered one of the most efficient drugs for the treatment of certain types of cancer; however, drug toxicity and resistance limit its utilization for a broader range of diseases. Therefore, various analogues of cisplatin are constantly studied.

In recent years, there has been a growing interest in the development of non-platinum-based anti-cancer therapeutics [10–22]. The main goal is to increase the variety of potential drugs, which may perhaps achieve higher activity enabling the administration of lower dose, attack different types of tumor cells, overcome drug resistance problems, and exhibit better selectivity and lower toxicity. Other metal complexes may introduce numerous options for coordination numbers, oxidation states, electron affinity, etc., and thus may operate by different mechanisms.

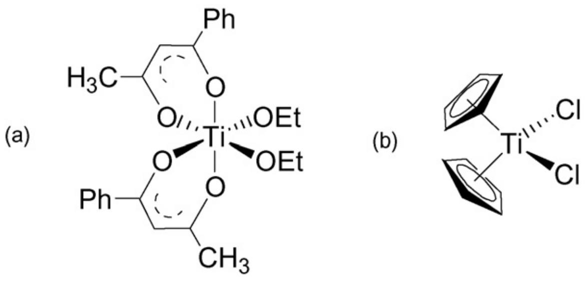
Several transition metals have led to biologically active compounds, such as ruthenium, rhodium, gallium, vanadium, tin, etc. [10–22]. Our work focuses on complexes of titanium(IV) [23,24]. As titanium is present in many biomaterials, such as in food in the form of whitening pigment, it is not unreasonable to conceive that it may be incorporated into drugs and into living systems, with particularly low toxicity. In fact, as an oxophilic metal, it is logical to envision that Ti(IV) species would form strong bonds with the negatively charged and acidic DNA component, as well as with other biological molecules. However, this oxophilicity also makes complexes of Ti(IV) of organic and inorganic ligands highly susceptible to hydrolysis, a characteristic that should be taken into account when biological applications are considered. Throughout the hydrolysis process, the ligands bound to the Ti(IV) center are replaced with water molecules according to their particular lability, giving various O-bridged aggregates of different possible sizes and nuclearities [25–27], with the final thermodynamic product of hydrolysis being titanium dioxide.



Scheme 1. Cisplatin.

Research of Ti(IV) complexes for biological applications thus far has been restricted to two families of compounds: the budotitane (bzac)$_2$Ti(OEt)$_2$ (Scheme 2, a) [27–30], the titanocene dichloride Cp$_2$TiCl$_2$ (Scheme 2, b) [31–35] and their various close derivatives; namely, complexes of Cp or diketonato ligands. Budotitane was the first non-platinum complex to enter clinical trials for its activity towards colon tumor cells. This compound showed the greatest promise out of several analogues tested, and the observed dependence of activity on the planarity of the substituents suggested that the mechanism of its activity involves DNA intercalation. Titanocene dichloride demonstrated activity towards cisplatin-resistant cells with different and improved toxicity pattern, with higher in vivo than in vitro activity [36], and thus entered clinical trials as well. DNA was also proposed to be the biological target of titanocene dichloride [37], although no unequivocal evidence was obtained.

The main disadvantage of budotitane and titanocene dichloride, which ultimately lead to their failure in clinical trials, is their rapid hydrolysis to give unidentified aggregates [24,29,38]. As the various hydrolysis products may include both active and inactive species, low efficacy was observed in terms of activity vs. toxicity ratio [39]. It was established that for both compounds, the labile ligands (OEt, Cl) hydrolyze first within seconds, followed by the hydrolysis of the inert groups (diketonato, Cp) within hours. Ti–O clusters are consequently obtained, with the final thermodynamic product being titanium dioxide. As some polynuclear O-bridged Ti(IV) complexes of diketonato ligands that are close derivatives of budotitane showed cytotoxic activity, it was proposed that such clusters, particularly of short Ti–O bonds and wide Ti–O–Ti angles, might be the active species in the biological environment [29,40]. This



Scheme 2. Budotitane (a) and titanocene dichloride (b).

observation supports the notion that some labile ligand hydrolysis is essential for activity, especially when considering the mechanism of action of cisplatin (Scheme 1), which involves hydrolysis of the chloro ligands in the cell and binding to DNA through their coordination sites. Nevertheless, substantial mechanistic details of activity for both compounds, including the identity of the biological target, the exact nature of the active species, and the role and participation of the ligands and the particular lability required [30,41], are still lacking.

Additional recent studies pointed to the interaction of titanocene dichloride with the serum protein transferrin [42–47]. Transferrin [48] is a blood serum carrier protein that regularly binds Fe(III) ions in the serum and releases them in the cell. Several metallo-drugs were proposed to be delivered into cells via binding to transferrin, such as the anti-tumor agents based on ruthenium and bismuth [49,50]. Thus, transferrin leads to complete ligand stripping from the Ti(IV) center and binds the ion in the same active site that it normally utilizes to bind Fe(III). In this manner, the instability problem of the complexes at physiological solutions is bypassed. The advantage of this mode of drug delivery is that transferrin receptors, located on the surface of cells, are expressed in a substantially elevated level in malignant cells due to their excessive necessity for iron for metabolism and growth. This affords some selectivity and may decrease toxicity concerns. Nevertheless, early loss of the most inert Cp ligands abolishes their potential influence on the interaction with the biological target and thus questions the contribution and influence of the particular ligand to the cytotoxic activity. Recent studies, however, suggested that potential binding of Ti(IV) to the serum protein albumin may also be achieved in the form of ligand-bound complex [51], which restores the interest in particular ligand design and in structure–activity relationship studies by ligand modification.

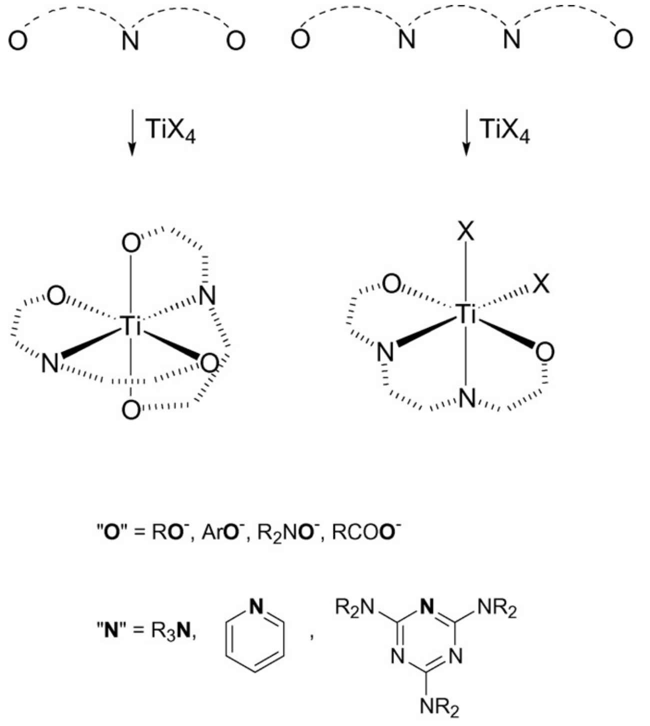
The great promise of Ti(IV) complexes manifested by their cytotoxic activity against both cisplatin-sensitive and cisplatin-resistant cells, as well as the significantly lower toxicity and less acute side effects of Ti(IV) relative to other transition metals, make complexes of Ti(IV) highly attractive to further explore for therapeutic application, with one main objective being elucidating their hydrolytic behavior and develop complexes of well defined hydrolysis processes. In this review, we describe our recent study with several new families of Ti(IV) complexes designed particularly to follow several requirements that are of interest, the focus being on decreasing the susceptibility to hydrolysis. We observed several interesting structural effects on both hydrolytic behavior and cytotoxic activity, which are not unrelated despite the different patterns of structure dependence observed.

2. Reference measurements: is every labile source of Ti(IV) active?

The hydrolytic instability of the cytotoxic complexes budotitane (Scheme 2, a) and titanocene dichloride (Scheme 2, b) and the evidence of transferrin binding to the Ti(IV) ion following complete ligand hydrolysis from titanocene dichloride and its possible role in delivering the stripped ion to the biological target, put in question the actual need for a carefully designed inert ligand for the formation of cytotoxic Ti(IV) complexes. In order to examine whether every labile source of Ti(IV) ion can lead to activity, we measured the cytotoxicity of $Ti(OiPr)_4$ and $TiCl_4(THF)_2$, two labile complexes that we normally apply as starting materials to synthesize Ti(IV) complexes. We measure cytotoxicity on two types of tumor cells: HT-29 colon cells and OVCAR-1 ovarian cells, which are considered relatively resistant cells. The cell viability is measured based on the MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay, according to the procedure previously discussed in detail [52]. This is a commonly used assay to assess cell viability, which is indicative of cellular metabolic activity. This assay involves a chemical transformation of the MTT to a compound of a purple color upon interaction with enzymes that are present in viable cells, and thus an evaluation of the color intensity by a spectrophotometer enables estimation of the percentage of viable cells in solution. Both $Ti(OiPr)_4$ and $TiCl_4(THF)_2$ did not show any cytotoxic activity towards the colon and ovarian cells analyzed, even when apotransferrin was added to the biological medium. We also applied titanium dioxide, the final thermodynamic product of hydrolysis of the Ti(IV) complexes, and no activity was observed, which is not surprising considering its poor water solubility and high inertness. We thus conclude that the labile Ti(IV) complexes form unreactive aggregates or even hydrolyze all the way to titanium dioxide too rapidly to demonstrate any activity or to allow binding of the Ti(IV) ion to transferrin. It is thus obvious that a carefully designed ligand of some inertness is essential for stabilizing some sort of an active species and for obtaining cytotoxic compounds based on a Ti(IV) center.

3. Ligand and complex design

When designing proper ligands for Ti(IV) that may lead to interesting insights regarding the cytotoxic activity of their complexes, we generally sought for ones that should lead to a relatively high hydrolytic stability. Thus, we aimed at formation of octahedral metal centers, with anionic O-donors suitable for the oxophilic Ti(IV) metal, and basic N-donors for coordinative bonds to complete the coordination sphere, with a preference for chelate ligand binding. One parameter we were interested to explore is whether monodentate labile ligands (Cl, OR) are required to obtain cytotoxic activity. Therefore, we studied both tridentate dianionic $[ONO]^{2-}$ ligands that lead to homoleptic $Ti[ONO]_2$ complexes with no particularly labile monodentate groups, as well as tetradentate dianionic $[ONNO]^{2-}$ ligands leading to $[ONNO]TiX_2$ type complexes featuring two monodentate (X) ligands, preferably in a *cis* configuration to allow chelate binding to an appropriate target (Scheme 3).



Scheme 3. Schematic presentation of ligand and complex design; one potential isomer of each complex type is depicted.

The ligands we employed thus far include aliphatic and aromatic O-donors, as well as aminohydroxo and carboxylato covalent ligands. The N-coordinative donor can also be either aliphatic or aromatic. The different families of compounds will be discussed, focusing on their cytotoxicity, their hydrolytic behavior, and the possible relation between the two.

4. Ti(IV) complexes of amine bis(phenolato) ligands

4.1. Synthesis and structure

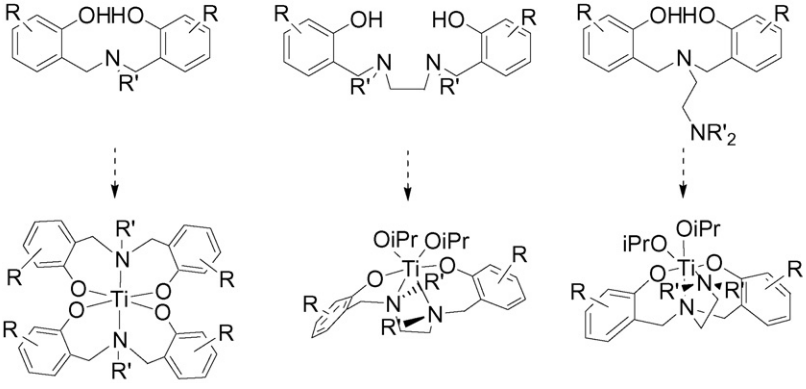
Amine bis(phenolato) ligands and their Ti(IV) complexes have been known for several years, studied mostly for catalysis purposes [53,54]. For instance, high activity and stereo-selectivity is observed for complexes of this family in α-olefin polymerization [55–59]. Tridentate ligands of this family give homoleptic complexes where steric crowding allows (Scheme 4), which exhibit *trans*-phenolato binding for both ligand units in a *mer-mer* geometry [60]. Two general types of tetradentate diamine bis(phenolato) ligands are known, which vary in the arrangement of donor atoms (Scheme 4), and thus Ti(IV) complexes of different symmetries are obtained upon reaction with a Ti(IV) precursor; hereby we employ $Ti(OiPr)_4$. Symmetrical ligands of sequential donor connectivity mainly wrap around the metal to produce C_2-symmetrical complexes [61–63], while branched ligands that include one coordinative donor on a side arm rather than in the central part of the ligand lead to formation of C_s-symmetrical complexes when binding of the side donor to the metal is achieved [60,62]. Complexes of both types are of high structural similarity; they both feature octahedral geometry; they both include similar donor types, namely, two phenolato O-donors and two N-coordinative bonds; they both feature a *trans* configuration of the phenolato ligands and a *cis* configuration of the isopropoxo groups. The C_2-symmetrical complexes are the closer analogues to budotitane (Scheme 2, a), as not only do they include similar covalent O-donors and exhibit similar coordination number, but they also feature similar general wrapping of the ligands around the metal, since the symmetrically substituted analogue of budotitane also exhibits a C_2-symmetry. Nevertheless, the advantage of the phenolato complexes is in the increased stability expected of a complex that includes binding of a single dianionic tetradentate ligand relative to a complex featuring two bidentate monoanionic ligands, and in the clean synthesis where the phenolato complexes are obtained as a single (racemic) isomer, unlike budotitane that needs to be separated from different inactive isomers obtained throughout the synthetic procedure [28,64].

The high availability of the bis(phenolato) complexes makes them highly convenient to study for the evaluation of the effect of various parameters on reactivity. Ligands of this family are synthesized very conveniently by a single step Mannich condensation from an amine or diamine, formaldehyde, and an appropriately substituted phenol [60,65], all are starting materials that are commercially available with numerous different substituents that may impose both steric and electronic effects. The complexes are obtained mostly in quantitative yields as single isomers at RT from the reaction of $Ti(OiPr)_4$ with the ligand precursor under an inert atmosphere due to the instability of the titanium precursor [61–63]. We have thus far investigated several complexes of this family, while attempting to elucidate the effect of steric bulk, electronic features, general geometry, and ligand nature on their hydrolytic behavior and cytotoxic activity. Some of the complexes studied and the parameters evaluated are depicted in Schemes 5 and 6. We examined steric effects by incorporating substituents of various bulk at different positions both on the aromatic rings and on the N-donor. We evaluated electronic effects by incorporating electron donating or withdrawing groups on the aromatic rings. We evaluated the effect of the lability of the isopropoxo groups by replacing the two *cis*-ligands with a single bidentate catecholato ligand. We compared the C_2- and C_s-symmetrical complexes and thus evaluated the effect of the general geometry. We also compared bis(isopropoxo) complexes of tetradentate ligands to homoleptic ones of tridentate ligands, and assessed the effect of the aromatic rings by studying an aliphatic analogous complex (Schemes 5 and 6).

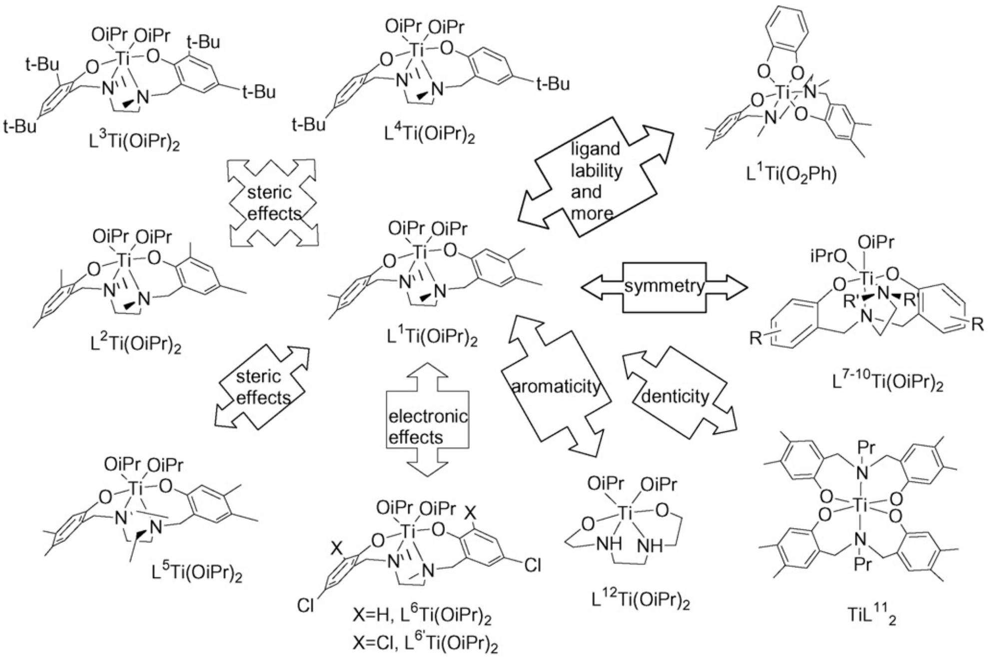
ORTEP drawings of the crystal structures of some of the complexes investigated are given in Fig. 1. In general, all of the Ti(IV) complexes obtained directly upon the reaction of $Ti(OiPr)_4$ with tetradentate ligands of sequential arrangement of donor atoms feature a highly similar structure, with *trans*-phenolato ligands, *cis*-isopropoxo groups, and a general symmetry of C_2, as known to occur for this family of compounds [62,63,66,67].

Of particular interest is the rather similar Ti–N bond in $L^1Ti(OiPr)_2$ (2.34 Å) and in $L^5Ti(OiPr)_2$ (2.37 Å) (Fig. 1), featuring dimethyl and diethyl N-substituents, respectively. This feature indicates that the slight increase of N-donor steric bulk does not result in significant change in the solid-state structure. In addition, the aliphatic analogue $L^{12}Ti(OiPr)_2$ possesses highly similar coordination sphere to those of the phenolato complexes, with two *cis*-isopropoxo groups and *trans*-alkoxo ligands, giving overall another C_2-symmetrical complex.

A distinctive feature is observed for the catecholato complex. Replacing the two *cis*-isopropoxo ligands with a single bidentate catecholato ligand gave a C_1-symmetrical complex with a *cis* configuration of the phenolato groups [67], which is suggested to result from an associative mechanism of ligand replacement. Therefore, following binding of the first phenolato group of the catecholato unit, ligand rearrangement occurs. Thus, $L^1Ti(O_2Ph)$ differs from $L^1Ti(OiPr)_2$ by two parameters, the ligand lability and the general geometry.



Scheme 4. Amine- and diamine bis(phenolato) ligands and their Ti(IV) complexes.



Scheme 5. Complexes investigated.

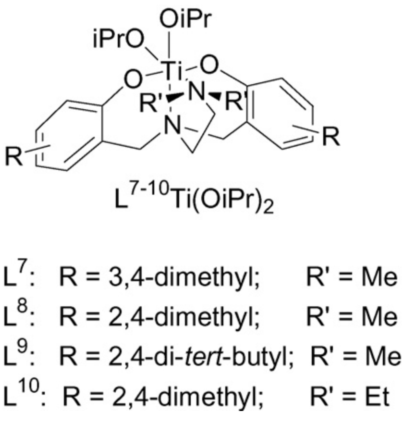
The X-ray structure of $L^{10}Ti(OiPr)_2$, a representative of a Ti(IV) complex of a tetradentate ligand of branched donor connectivity, features a C_s-symmetrical complex as normally obtained from ligands of this class [60]. Thus, the two isopropoxo groups are in a *cis* configuration and a longer Ti–N distance to the side arm donor of 2.46 Å relative to the 2.33 Å value for the central N-donor is obtained. This complex is otherwise of high similarly to its C_2-symmetrical analogues, as it also exhibits octahedral geometry, two O-covalent donors, two N-coordinative donors, *trans*-phenolato ligands, and *cis*-isopropoxo groups.

4.2. Cytotoxicity

Cytotoxicity was measured on colon HT-29 and ovarian OVCAR-1 cells based on the MTT assay according to the procedure previously discussed in detail [52]. This is a commonly used assay to assess cell viability, which is indicative of cellular metabolic activity. In general, similar patterns of activity were observed for both cell types.

4.2.1. C_2-Symmetrical complexes: steric effects

We evaluated the effect of steric bulk on the cytotoxic activity of the complexes by incorporating substituents of varying sizes at different positions of the aromatic rings. We evaluated the effect of large groups both when located near the metal site, as well as when located on a peripheral part of the complex. Steric bulk was found to inflect a major influence on cytotoxicity, both when located proximal to the metal site and when located on the complex periphery. $L^1Ti(OiPr)_2$ and $L^2Ti(OiPr)_2$, both feature two methyl substituents on the aromatic rings (Scheme 5) possess appreciable cytotoxic activity towards the cells analyzed, which is higher than that of the known Ti(IV) complexes, as well as higher than that of cisplatin (Fig. 2; Table 1), with their free ligands studied as a reference being inactive. Despite their different *ortho* groups, namely H for $L^1Ti(OiPr)_2$ and Me for $L^2Ti(OiPr)_2$, their activities are rather similar. However, when incorporating two larger *t*-Bu substituents, one being *ortho* to the binding oxygen atoms, the activity is completely lost (Fig. 2). Placing only a single *t*-Bu substituent on each aromatic ring in a position that is distant from the metal binding site is sufficient to diminish significantly the cytotoxic activity. Thus, $L^4Ti(OiPr)_2$ exhibits cytotoxic activity that is in between that of $L^{1,2}Ti(OiPr)_2$ and that of $L^3Ti(OiPr)_2$ (Fig. 3). Thus, not only steric interference to the metal interactions near its binding site are problematic for cytotoxic activity, but also the total bulk of the complex is



Scheme 6. C_s-symmetrical complexes.

Table 1
IC_{50} (μM) values for diamine bis(phenolato) bis(isopropoxo) complexes on HT-29 and OVCAR-1 cells and comparison to known compounds with or without a supplement of apo-transferrin (Tr).

	Reagent	Tr	HT-29	OVCAR-1
1	Cp_2TiCl_2	–	710 ± 120	780 ± 90
2	Cp_2TiCl_2	+	460 ± 40	520 ± 30
3	$(bzac)_2Ti(OiPr)_2$	–	53 ± 1	53 ± 1
4	$(bzac)_2Ti(OiPr)_2$	+	56.9 ± 0.6	65.0 ± 0.6
5	$L^1Ti(OiPr)_2$	–	12 ± 1	14 ± 1
6	$L^1Ti(OiPr)_2$	+	16 ± 3	15 ± 3
7	$L^2Ti(OiPr)_2$	–	12 ± 1	12 ± 1
8	$L^2Ti(OiPr)_2$	+	20 ± 3	39 ± 4
9	$L^3Ti(OiPr)_2$	–	Inactive	Inactive
10	$L^3Ti(OiPr)_2$	+	Inactive	Inactive
11	$L^4Ti(OiPr)_2$		–	–
12	$L^5Ti(OiPr)_2$		Inactive	Inactive
13	$L^6Ti(OiPr)_2$		–	24 ± 6
14	$L^1Ti(O_2Ph)$		20 ± 2	40 ± 4
15	Cisplatin	–	33 ± 3	17 ± 4

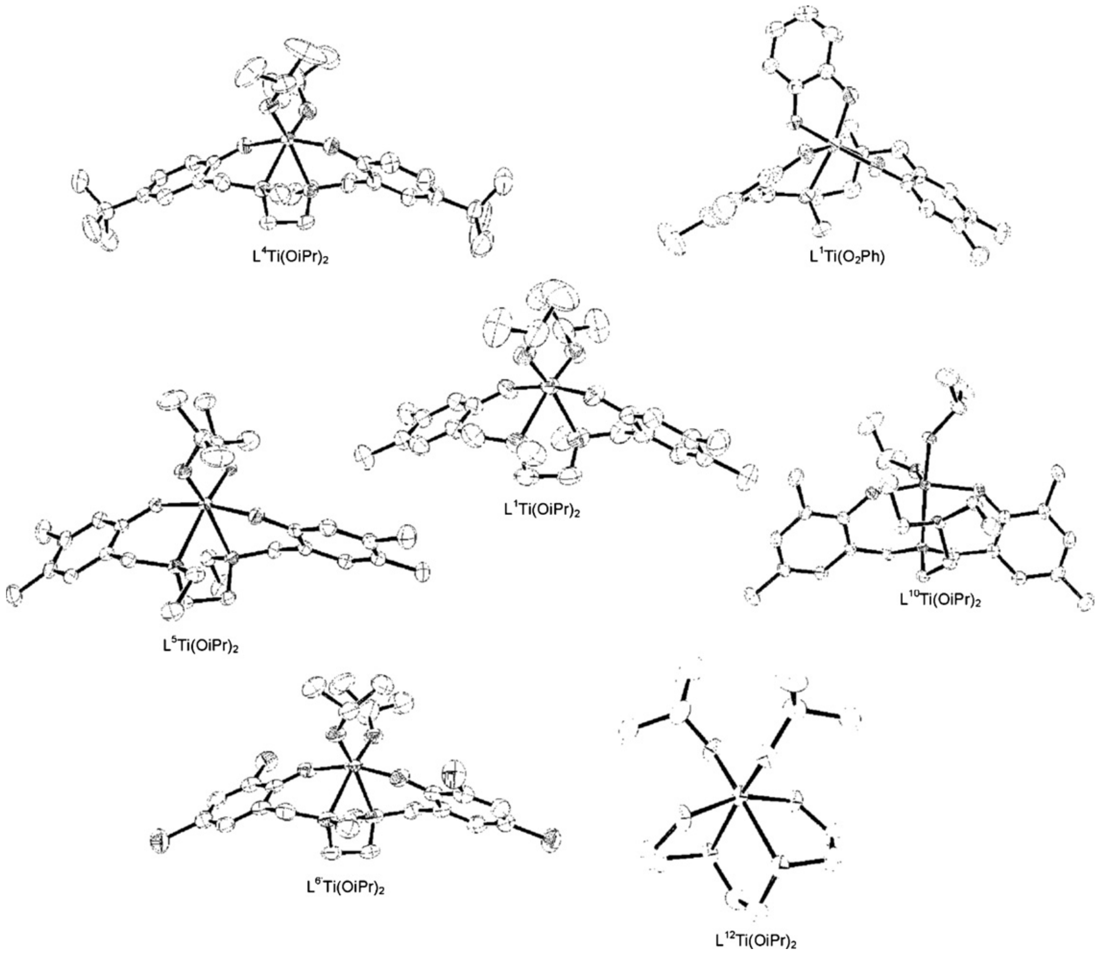


Fig. 1. ORTEP drawings of complexes investigated [53,54].

of significance. The latter may influence DNA intercalation if indeed DNA is the biological target as suggested for budotitane and its derivatives [28], or alternatively, may point to the involvement of indirect effects concerning solubility and cell penetration rate, as the total hydrophobicity of the complex is altered. A more drastic negative effect on cytotoxicity is observed when larger substituents are incorporated directly to the N-donor. Despite the high similarity in the X-ray structures of $L^1Ti(OiPr)_2$ and $L^5Ti(OiPr)_2$ (Fig. 1), $L^5Ti(OiPr)_2$ featuring only slightly larger Et substituents on the N-donor demonstrates complete lack of cytotoxic activity towards both cell types studied (Fig. 3). This result points mainly to the requirement of a strongly bound chelating ligand (*vide infra*), as it is reasonable that despite the similar solid-state structures, weaker Ti–N binding occurs in solution.

4.2.2. C_2-Symmetrical complexes: electronic effects

We recently began to evaluate electronic effects on the cytotoxicity of the C_2-symmetrical complexes by incorporating electron withdrawing chloro substituents in the *para* positions to the binding phenolato oxygen donors. $L^6Ti(OiPr)_2$ possess somewhat higher activity than does $L^4Ti(OiPr)_2$ featuring bulkier *t*-Bu substituents in replacement of the chloro atoms; however, lower cytotoxic activity is observed for the chlorinated compound relative to the dimethyl analogue $L^1Ti(OiPr)_2$ (Fig. 4). Thus, having established the negative

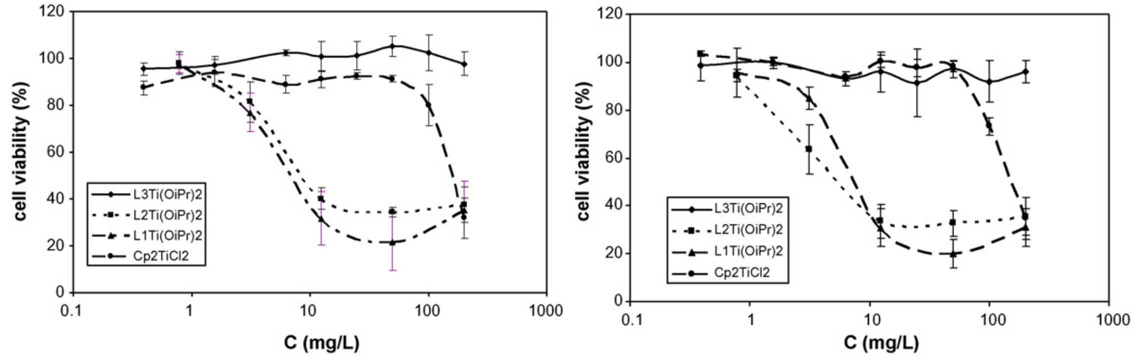


Fig. 2. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^{1-3}Ti(OiPr)_2$ and Cp_2TiCl_2 presented in a logarithmic scale [53,54a].

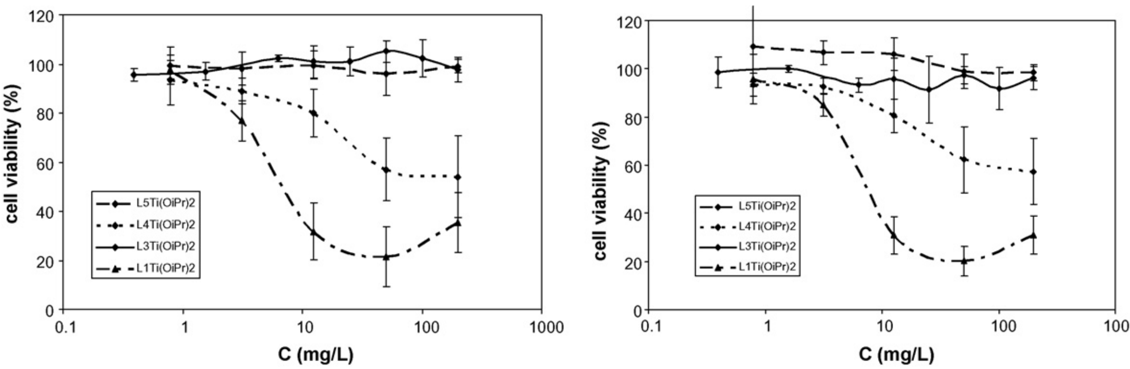


Fig. 3. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^{1,3-5}Ti(OiPr)_2$ presented in a logarithmic scale [54a].

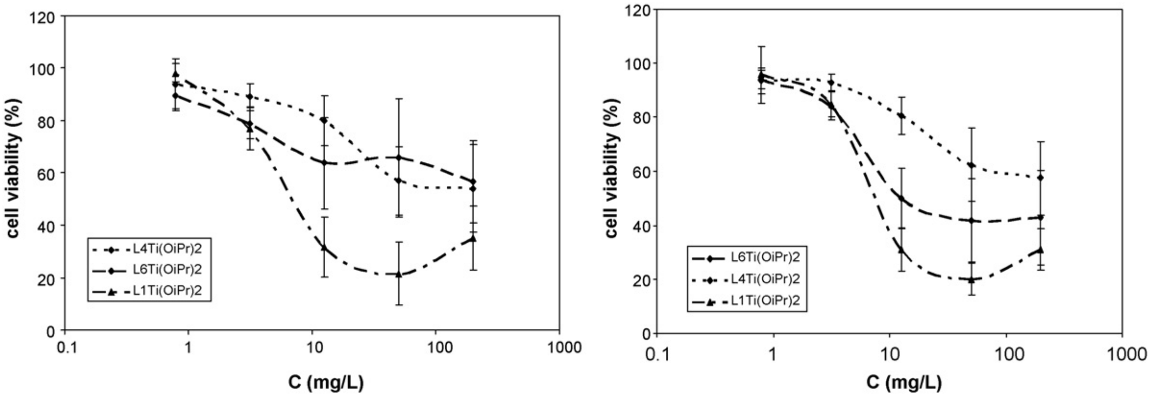


Fig. 4. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^{1,4,6}Ti(OiPr)_2$ presented in a logarithmic scale [54].

effect of large steric bulk all around the complex, it appears that electron withdrawing groups have a negative effect on cytotoxicity which further emphasizes the need for an electron-rich ligand that may bind strongly to the Ti(IV) center in a rather inert fashion.

4.2.3. *Effect of labile ligand type: C_1-symmetrical complexes*

The single catecholato ligand in replacement of two isopropoxo groups should reduce the lability of the complex; however, it also produces a complex of a different symmetry (Fig. 1). The cytotoxicity of $L^1Ti(O_2Ph)$ is somewhat lower than that of the bis(isopropoxo) analogue $L^1Ti(OiPr)_2$ (Fig. 5; Table 1). This may point to the involvement of the hydrolysis of the most labile groups in the cytotoxicity mechanism, a process that is quite likely to occur considering our knowledge of the mechanism of other metal-based drugs, such as that of cisplatin. It is also possible that the different arrangement of the bis(phenolato) ligand around the metal and the total different symmetry play a role in the different activity. Considering possible initial hydrolysis of the catecholato ligand in the biological environment, we believe that the geometry of the ligand is of lesser importance as rearrangement can occur, as did upon the original formation of the catecholato substituted complex from the original $L^1Ti(OiPr)_2$ complex of C_2-symmetry.

4.2.4. *Effect of symmetry and general geometry: C_s-symmetrical complexes*

Despite the very high similarity in the coordination sphere of the C_s-symmetrical complexes of the ligands of branched donor connectivity in comparison to the C_2-symmetrical complexes, significantly lower activity is observed for the formers towards both cell types analyzed (Fig. 6). Nevertheless, for this family of compounds,

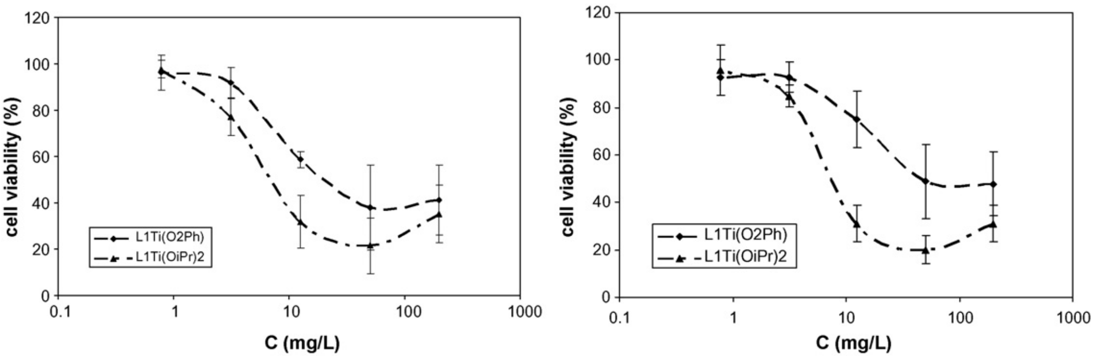


Fig. 5. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^1Ti(OiPr)_2$ and $L^1Ti(O_2Ph)$ presented in a logarithmic scale [54a].

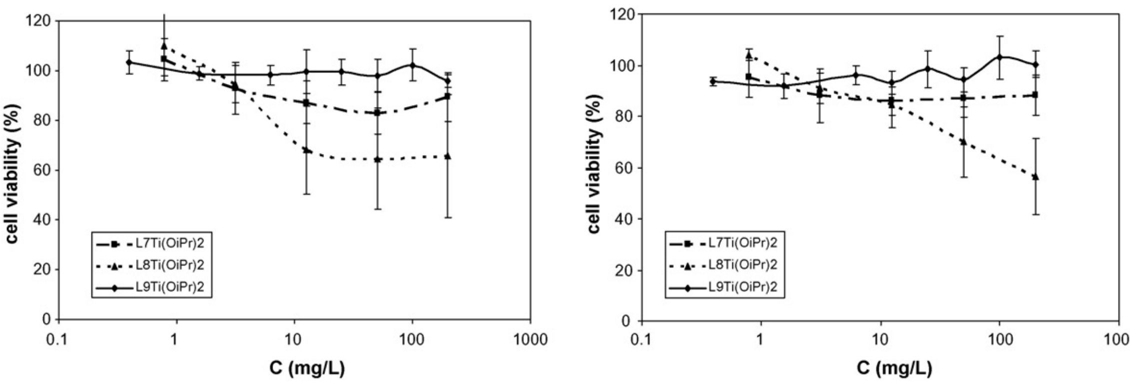


Fig. 6. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^{7-9}Ti(OiPr)_2$ presented in a logarithmic scale [54b].

rather similar patterns of steric bulk dependence are observed. Very mild activity is demonstrated for the complexes $L^{7,8}Ti(OiPr)_2$ (Scheme 6) featuring two methyl substituents at positions similar to those of $L^{1,2}Ti(OiPr)_2$, with perhaps slightly higher activity for $L^8Ti(OiPr)_2$ featuring Me substituents at positions *ortho* to the binding phenolato groups. In addition, the bulky complex $L^9Ti(OiPr)_2$, an analogous complex to $L^3Ti(OiPr)_2$ featuring two *t*-Bu substituents, is completely inactive (Fig. 6). Additionally, comparing the activity of $L^{10}Ti(OiPr)_2$ (Scheme 6; Fig. 1) to that of its *N*,*N*-dimethyl analogue reveals similar negative effect of the larger donor substituents (Fig. 7). Altogether, we can say that the negative effect of steric bulk is well established, and in addition, the particular ligand type and the exact complex geometry and symmetry play an immense role in the cytotoxic activity. Two possible reasons for the reduced activity of the C_s-symmetrical complexes are the following: (a) the different geometry affects the three-dimensional interaction with the biological target; for instance if DNA intercalation is required [28], the arrays of the aromatic rings in $L^{7-10}Ti(OiPr)_2$ are less approachable; (b) as the side arm donor generally binds to the Ti(IV) center in a weaker fashion, it is plausible that weaker ligand binding brings about reduced activity, as observed with $L^5Ti(OiPr)_2$ featuring larger N-substituents that may also weaken ligand binding (*vide supra*).

4.2.5. Homoleptic complexes

The homoleptic complex $TiL^{11}{}_2$ (Scheme 5) is completely inactive against both cell types analyzed. This result is not surprising since regardless of the absence of monodentate ligands, very rapid hydrolysis in air is observed within seconds, and thus similarly to the results obtained for the labile $Ti(OiPr)_4$ and $TiCl_4(THF)_2$, inactive aggregates are obtained too rapidly to demonstrate activity (*vide infra*). Thus, the extra stability granted by the tetradentate ligands vs. the tridentate ones is essential for survival of an active species for an appropriate time to demonstrate activity.

4.2.6. The role of the aromatic rings

Having established that the C_2-symmetry of the complexes comprised of ligands with sequential donor connectivity is an important factor based on the substantially lower activity observed for the analogous C_s-symmetrical complexes, an analogous C_2-symmetrical aliphatic complex with similar coordination sphere only lacking the planarity of the aromatic rings was investigated. $L^{12}Ti(OiPr)_2$ (Fig. 1) is also completely inactive towards the cells employed. This observation may support the involvement of DNA intercalation in the cytotoxicity mechanism requiring planar moieties; however, may also relate to the reduced hydrolytic stability of this compound, a factor that seems to be important in determining activity (*vide infra*).

4.3. Hydrolysis

4.3.1. C_2-Symmetrical complexes

Due to the hydrolytic instability generally observed for Ti(IV) complexes, including for the biologically active complexes budotitane and titanocene dichloride (Scheme 2), special attempts were made to gain some insights on the hydrolytic processes of the family of highly activity phenolato complexes presented herein, and to evaluate whether their hydrolytic behavior is at all related to in their cytotoxic activity. Hydrolysis studies were thus conducted by several techniques, including UV–vis, NMR, and X-ray crystallography.

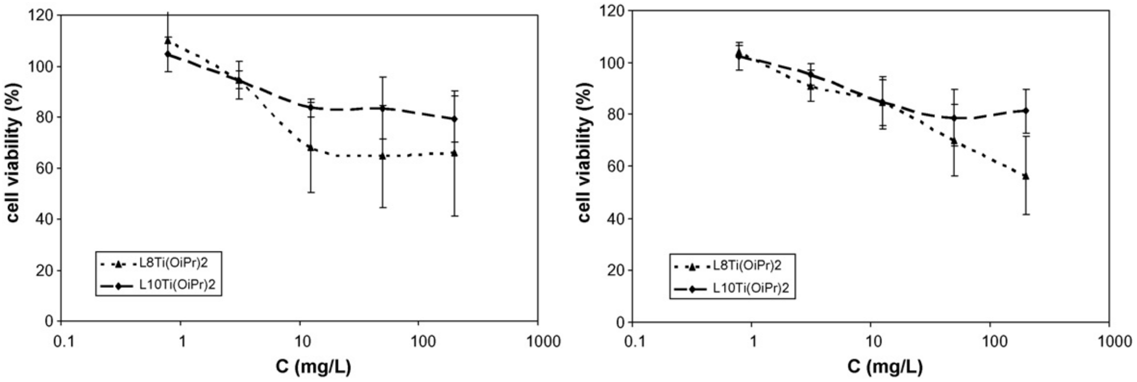


Fig. 7. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^{8,10}Ti(OiPr)_2$ presented in a logarithmic scale [54b].

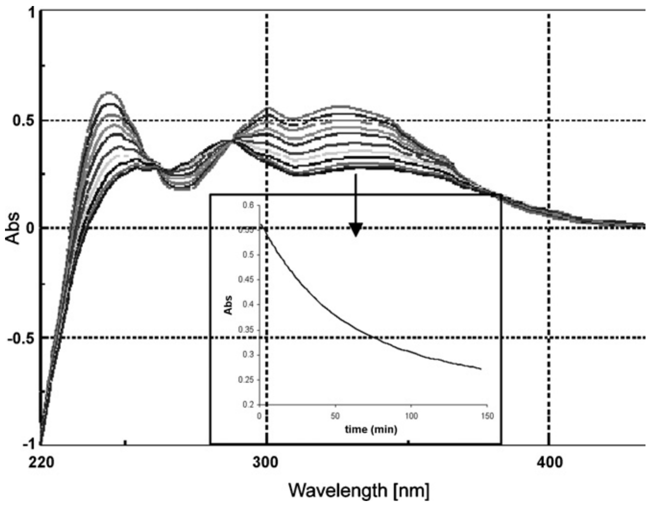


Fig. 8. UV–vis absorption overtime for $L^1Ti(OiPr)_2$ upon addition of water [54a].

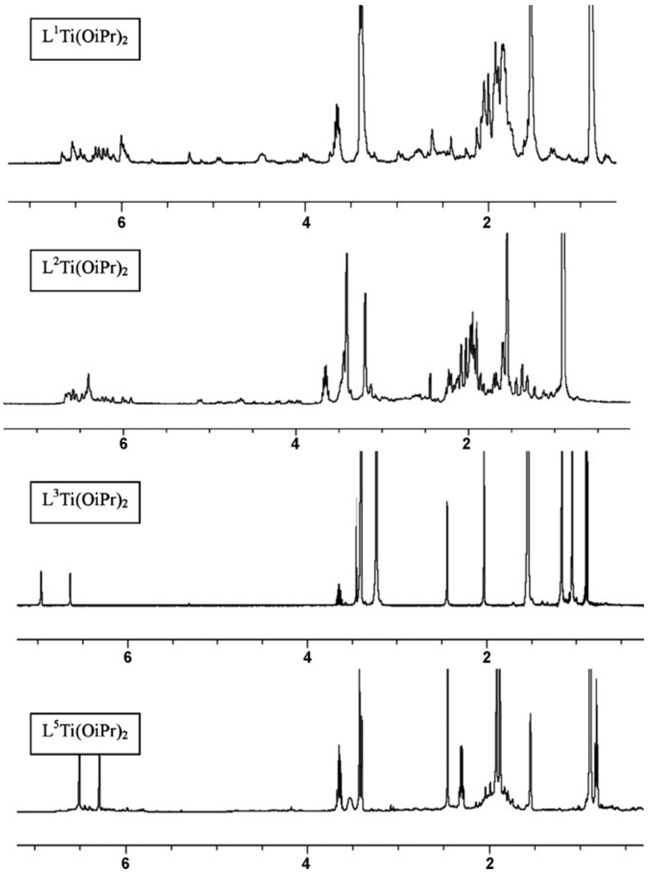


Fig. 9. ^1H NMR of $L^{1-3,5}Ti(OiPr)_2$ in d_8-THF/D_2O after completion of hydrolysis reaction [54a].

Measurements of the UV–vis absorbance change particularly at λ = 320–350 nm characteristics of the LMCT band of the chelating ligand with time following water addition to a THF solutions of the complexes suggested that a new species with bound chelating ligand forms with a time scale of several hours for biologically active complexes. Thus, the LMCT absorbance does not decay to zero and a shift in the band is detected for $L^{1,2,4}Ti(OiPr)_2$ (Fig. 8). In contrast, inactive complexes in general do not follow this behavior; $L^3Ti(OiPr)_2$ reveals simple decay of the LMCT band within hours while $L^5Ti(OiPr)_2$ demonstrates a significantly more rapid hydrolysis, within minutes, where also no shift in the LMCT band was detected. Stopped flow measurements verified that no rapid changes occur in the first seconds of water addition for $L^{1-3}Ti(OiPr)_2$.

^1H NMR measurements overtime upon addition of D_2O to d_8-THF solutions of the complexes shed light on the nature of the species formed upon hydrolysis. Indeed, a new species of low symmetry characterized by multiple signals was obtained within several hours for bis(isopropoxo) complexes of at least some cytotoxic activity ($L^{1,2,4}Ti(OiPr)_2$) (Fig. 9). Plotting the integration of various signals overtime (Figs. 10 and 11) revealed the following: (a) the biologically active complexes indeed lead to the formation of the new compound of low symmetry in significant amounts, ca. 80%, while ca. 20% of the tetradentate ligand is released as free bis(phenol) compound; (b) all of the bound isopropoxo groups are accounted for in the isopropanol formed; (c) release of free bis(phenol), that of isopropanol, and formation of the new species all occur with a similar time scale, of several hours. This process is surprisingly slow for these complexes, as normally traces amounts of water are enough to form O-bridged Ti(IV) clusters from isopropoxo complexes or equivalent (*vide infra*) [25,68–71]. Different behavior is observed for the biologically inactive complexes. The bulky complex $L^3Ti(OiPr)_2$ apparently cannot form the new species due to steric inhibition [72], and thus only release of free bis(phenol) ligand and isopropanol are observed within several hours (Fig. 9), where all signals representing bound phenolato ligand in the original complex are accounted for in the formation of the free bis(phenol) compound (Figs. 10 and 11). Interestingly, similar timescale for hydrolysis is observed for both the isopropoxo groups and for the tetradentate ligand, despite the expected difference in their lability. Consistently with the results obtained from the UV–vis measurements, the ^1H NMR studies also revealed that the *N*,*N*-diethyl complex $L^5Ti(OiPr)_2$ hydrolyzes substantially more rapidly than do $L^{1-4}Ti(OiPr)_2$ (Fig. 10). Thus, free bis(phenol) ligand is obtained within minutes, with a similar timescale of hydrolysis to those of the isopropoxo groups, all accounted for in the isopropanol formed. The more rapid hydrolysis is presumably resulting from a more electron-poor Ti(IV) center due to weaker binding of the tetradentate ligand in solution despite the similar solid state structure to that of the *N*,*N*-dimethyl analogue, a parameter which appears to also contribute to its lack of cytotoxic activity (*vide supra*). Nevertheless, rapid formation of smaller amounts of a new species is also observed upon exposure of $L^5Ti(OiPr)_2$ to water (Fig. 9). Comparing not just the general hydrolytic behavior but also the actual hydrolysis rates of the different complexes reveals strong dependence of the hydrolysis rates on the steric bulk particularly at positions proximal to the metal binding site, a structural dependence which is different than that of the cytotoxic activity (Table 2). Thus, $L^1Ti(OiPr)_2$ and $L^2Ti(OiPr)_2$ exhibiting similar cytotoxicity, both featuring two methyl substituents on each aromatic ring but at different positions, demonstrate substantially different $t_{1/2}$ values for hydrolysis: 5 h for $L^1Ti(OiPr)_2$ featuring *ortho* H atoms and 31 h for $L^2Ti(OiPr)_2$ featuring *ortho* Me groups. In addition, $L^1Ti(OiPr)_2$ and $L^4Ti(OiPr)_2$ both exhibiting H atoms at positions *ortho* to the binding phenolato donors exhibit hydrolysis rates with a similar order of magnitude despite their different cytotoxicity. Different

Table 2
$t_{1/2}$ values for hydrolysis of $L^{1-5}Ti(OiPr)_2$.

Complex	$t_{1/2}$
$L^1Ti(OiPr)_2$	5 h
$L^2Ti(OiPr)_2$	31 h
$L^3Ti(OiPr)_2$	10 h
$L^4Ti(OiPr)_2$	3 h
$L^5Ti(OiPr)_2$	5 min

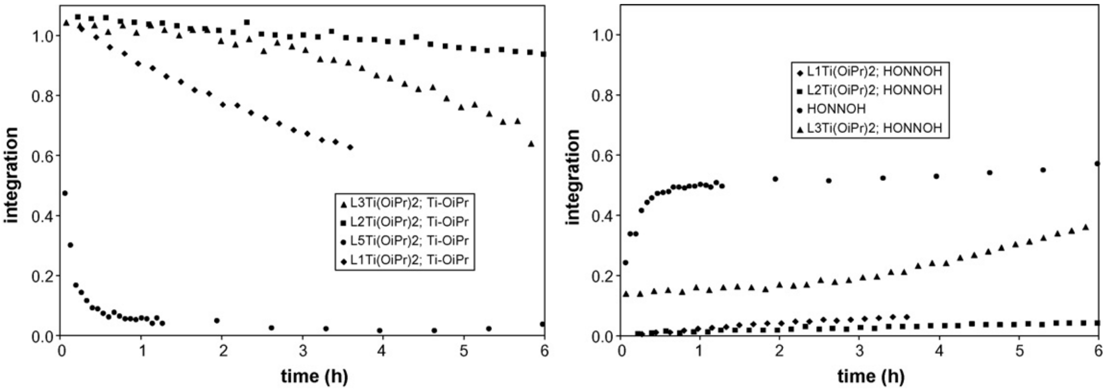


Fig. 10. Integration of the bound isopropoxo signal (left) and free bis(phenol) ligand signal (right) in the ^{1}H NMR of $L^{1-3,5}Ti(OiPr)_2$ vs. time following addition of D_2O to the d_8-THF solution at RT [54a].

pattern is observed for $L^2Ti(OiPr)_2$ and $L^3Ti(OiPr)_2$ featuring Me and *t*-Bu *ortho* groups, respectively; however, these two complexes lead to different hydrolysis processes: the former leads to formation of a new species while the latter results in simple bis(phenol) ligand release. Altogether, these observations also support the conclusion regarding faster hydrolysis resulting from weaker ligand binding where larger N-donor substituents are employed, as their proximity to the metal binding site should have had an opposite effect if indeed strong binding had been achieved.

Structural characterization of the new species formed upon hydrolysis of $L^1Ti(OiPr)_2$ was conducted by X-ray crystallography. Reacting the bis(isopropoxo) complex with 50–100,000 water equivalents and allowing the reaction to stir for 3 days gave the hydrolysis product which crystallized from diethylether. Smaller number of water equivalents and/or shorter reaction times did not afford the product and only crystals of the starting bis(isopropoxo) complex were obtained, which is consistent with this reaction occurring rather slowly. As evident by its crystal structure, the new species indeed includes bound bis(phenolato) ligands as suggested by the LMCT band shift in the UV–vis, and is formed upon release of isopropanol by water molecules giving an O-bridged compound, as also observed by the ^{1}H NMR measurements. Altogether the structure features a trinuclear Ti(IV) core with three bridging oxo groups, where each Ti(IV) center is bound to a single bis(phenolato) ligand (Fig. 12). Only one of the three Ti(IV) centers includes *trans*-phenolato ligands as in the starting complex, while the other two possess *cis*-phenolato ligands [67], similarly to the geometry of $L^1Ti(O_2Ph)$ (Fig. 12). This observation suggests similar ligand replacement mechanisms for the two complexes, presumably of an associative nature, upon which, ligand rearrangement occurs following the first new ligand binding. Altogether, the complex exhibits a low C_1-symmetry, which explains the multiple signals observed in the ^{1}H NMR. It is also notable that the shortest C_{Ar}–C_{Ar} distance between two carbon atoms *ortho* to the binding phenolato O-donors of two different chelating ligands in the trinuclear cluster is 3.7 Å, with the distance between their corresponding H atoms being 3.4 Å. This explains the substantially longer reaction times required for formation of the analogous cluster from $L^2Ti(OiPr)_2$ featuring two Me *ortho* groups, as well as the inability to form this particular structure with the *ortho* *t*-Bu substituted analogue $L^3Ti(OiPr)_2$.

4.3.2. *Other complexes*

The catecholato complex $L^1Ti(O_2Ph)$ demonstrates substantially higher hydrolytic stability and releases only approximately 10% of the catecholato ligand as free catechol and similar amounts of the bis(phenolato) ligand as free bis(phenol) compound, with no further increase in integration of the free ligands signals following that point. As this complex demonstrates reduced cytotoxicity relative to $L^1Ti(OiPr)_2$ despite the additional planar moiety, it is not unreasonable that hydrolysis of the isopropoxo groups is an essential stage in the cytotoxic activity, especially considering formation of O-bridged clusters particularly for the active C_2-symmetrical complexes which involves isopropoxo hydrolysis to give isopropanol. Following the catecholato hydrolysis, similar L^1 ligand as in $L^1Ti(OiPr)_2$ and its steric and electronic requirements should afford decent binding and enable formation of the O-bridged cluster, and thus should also allow for the cytotoxic activity to be demonstrated. It should also be taken into account that other factors may also influence the cytotoxic activity of $L^1Ti(O_2Ph)$, such as the general different chelating ligand arrangement in the catecholato complex relative to the bis(isopropoxo) analogue, although

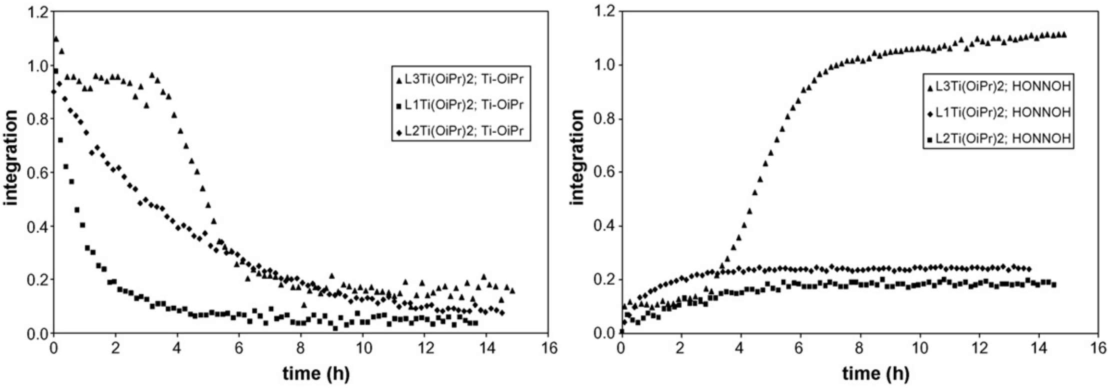


Fig. 11. Integration of the bound isopropoxo signal (left) and free bis(phenol) ligand signal (right) in the ^{1}H NMR of $L^{1-3}Ti(OiPr)_2$ vs. time following addition of D_2O to the d_8-THF solution at 37 °C [54a].

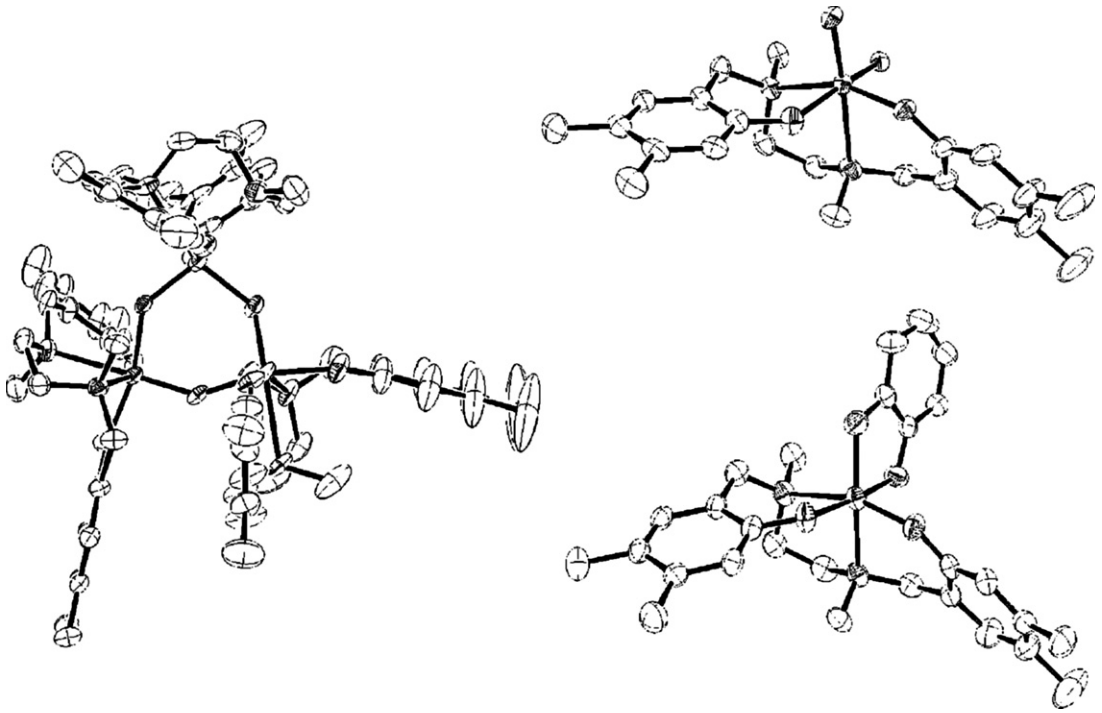


Fig. 12. ORTEP drawing of $L^1{}_3Ti_3O_3$ (left), that of one of its Ti(IV) centers of *cis*-phenolato ligands (right; top), and that $L^1Ti(O_2Ph)$ (right; bottom) [54a].

we suspect that this parameter does not have a major influence since throughout a tentative catecholato hydrolysis, ligand rearrangement can occur (*vide supra*).

$TiL^{11}{}_2$ demonstrates significantly more rapid hydrolysis than do the bis(isopropoxo) complexes of tetradentate ligands with small N-donor substituents, which occurs within seconds. Therefore, the thermodynamic parameter relating to the additional strong coordinative bond afforded by a tetradentate ligand plays a significant role in substantially enhancing the electron density of the metal and thus the hydrolytic stability of the complex, while the more inhibited approach to the metal center when bound to two tridentate ligands is of lesser significance. This observation supports the conclusions made for $L^5Ti(OiPr)_2$ featuring larger N-donor substituents, where rapid hydrolysis is attributed to weaker binding of the coordinative N-donors. $L^{12}Ti(OiPr)_2$ also demonstrates significantly more rapid hydrolysis than that of the bis(phenolato) complexes which also occurs within seconds pointing to stronger binding of O-donors of an aromatic nature as expected. Inspecting the hydrolytic behavior of the C_s-symmetrical complexes of branched tetradentate ligands, we also observed more rapid hydrolysis relative to that of the C_2-symmetrical complexes, which we attribute to the weaker binding of the donor located on a side arm relative to those on the ligand backbone, consistently with the observations with $TiL^{11}{}_2$ and $L^5Ti(OiPr)_2$.

Overall, the hydrolytic instability of both $TiL^{11}{}_2$ and $L^{12}Ti(OiPr)_2$ is the most likely explanation to their lack of cytotoxic activity, as labile complexes such as $Ti(OiPr)_4$ and $TiCl_4(THF)_2$ are also inactive. The particular enhanced stability afforded by the phenolato vs. alkoxo ligand is therefore of high significance for the cytotoxic activity, as is the stability gained by the additional donor of the tetradentate ligand. The faster hydrolysis of the C_s-symmetrical complexes is also a possible reason for their reduced cytotoxicity. It is also plausible that due to their generally reduced hydrolytic stability, the bigger the steric bulk at positions *ortho* to the binding phenolato groups the better it is for enhancing hydrolytic resistance, as these groups have the biggest effect on the hydrolytic stability, as long as a cluster can still form. Perhaps this notion may serve as an explanation to the higher activity observed for this family of compounds for $L^8Ti(OiPr)_2$ relative to that of $L^7Ti(OiPr)_2$ (Scheme 6).

4.4. *Additional mechanistic studies*

4.4.1. *Transferrin effect*

In order to evaluate the potential role of the protein transferrin in the delivery of the Ti(IV) to the cells, the cytotoxic activity was measured upon addition of apo-transferrin to the biological medium. Whereas clear positive effect on activity was observed for titanocene dichloride in agreement with previous reports [42–47], no positive effect was observed for $L^{1,2}Ti(OiPr)_2$ as well as for $(bzac)_2Ti(OiPr)_2$ (Fig. 13; Table 1) [47]. Thus, an alternative penetration mode exists for these complexes which is not less effective, and which allows for the rather inert bis(phenolato) ligand to remain bound to the metal and to strongly affect the cytotoxic activity, as observed throughout the structure–activity relationship studied described above. This is in agreement with studies on the cytotoxic activity of titanocene and budotitane derivatives towards HT-29 cells, which also indicated that transferrin is not required for protection of the Ti(IV) center from hydrolysis where a strongly bound ligand exists instead [47]. No activity was observed for $L^3Ti(OiPr)_2$ in the presence of the protein as well, supporting strong binding of the chelating ligand.

4.4.2. *Cytotoxicity dependence on incubation times*

Time dependence measurements were conducted for the two most active compounds $L^{1,2}Ti(OiPr)_2$ to shed some light on the nature of the active species and its potential formation or decomposition with time in the biological environment. The activity was found to increase with time within a frame of 3 days incubation in the presence of cells. However, when the Ti(IV) complexes were exposed to the biological medium for 2 days prior to cell administration, and consequently were incubated with the cells for an additional day, the activity was completely lost (Fig. 14). This observation suggests that either the original complex or some other active species that is formed in the biological medium undergoes cell penetration relatively rapidly, and once in the cells,

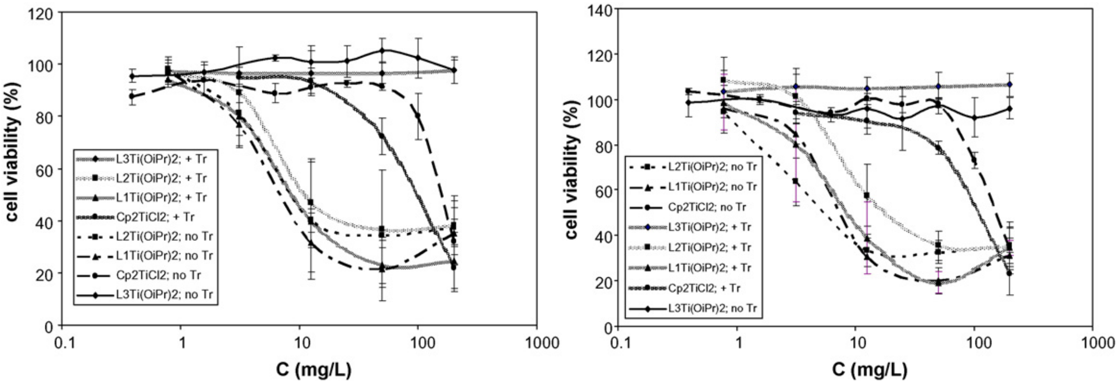


Fig. 13. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^{1-3}Ti(OiPr)_2$ and Cp_2TiCl_2 presented in a logarithmic scale with or without added apo-transferrin (10 μg/mL) [53].

the reactivity is retained, while in the absence of cells, decomposition occurs. The cell penetration rate is thus a crucial stage, and therefore, hydrolysis and/or decomposition should occur relatively slowly to allow for the cytotoxic activity to be demonstrated.

4.4.3. What is the active species?

As all cytotoxic complexes of this family lead to formation of O-bridged clusters upon water addition, and as studies conducted with budotitane and derivatives suggested analogous clusters to be the active species [29,40], the trinuclear complex obtained from $L^1Ti(OiPr)_2$ (Fig. 12) was suspected as a potential active species in the present system as well. Cytotoxicity measurements towards the colon and ovarian cells employed revealed no activity for this cluster whatsoever (Fig. 15) unlike observations with budotitane derivatives. As this cluster is itself stable for days in the presence of water, and as we observed loss of activity after 2 days in the biological medium in the absence of cells suggesting overall low stability of the active species, this observation is not surprising. It is thus plausible that the biologically active species is either the original complex, some intermediate in the cluster formation, or an alternative complex that is formed throughout the initial period of water exposure, and is able to penetrate the cells quite rapidly and demonstrate activity prior to cluster formation. Another interesting insight was obtained from studies involving addition of the biological medium (without phenol red) to the bis(isopropoxo) complexes as well as to the trinuclear cluster and monitoring the LMCT band in the UV–vis spectrophotometer with time. For all complexes, including the cluster that is itself stable for days in pure water, simple decay of absorbance was observed to release free ligands under these biologically more relevant conditions in the absence of cells. This result is in agreement with formation of the active species prior to that of the cluster, which once is formed, activity is lost. Nevertheless, the correlation observed between the ability to form the cluster and the cytotoxic activity of the complexes to the very least defines the steric requirements for fruitful biological interactions. As lower cytotoxic activity is also observed for the catecholato complex $L^1Ti(O_2Ph)$ (Fig. 15), it is certainly reasonable to suspect that hydrolysis of the isopropoxo groups needs to occur for formation of the active species, as also occurs for the cluster formation. Further studies are required and are currently underway to gain more information on the nature of the active species, its exact cellular target, and the mechanism of action of these biologically active complexes.

5. Hydroxylamino-triazine compounds and their Ti(IV) complexes

5.1. Tridentate ligands and their homoleptic complexes

Throughout our quest towards ligands that should bind strongly to the Ti(IV) center and afford hydrolytically stable complexes [73–75], we stumbled across the melamine derivatives. The distinctive electronic features of these compounds as presented by their resonative structures make the triazine nitrogen atoms especially good ligands due to their high electron density (Scheme 7) [76–82]. Two additional O^- groups make these compounds tridentate dianionic $[ONO]^{2-}$ type ligands. Thus, upon reaction with $Ti(OiPr)_4$, homoleptic TiL_2 complexes are formed. The X-ray structures of two such complexes (Fig. 16) support large contribution of the resonative structure of negatively charged N-donors. This is pronounced first by high planarity of the melamine-like moieties, which indicates sp^2 character of the melamine nitrogen atoms, and in particular, by the 2.0 Å distance of the Ti–N bonds, which is substantially shorter than average coordinative bonds and similar to Ti–N covalent bond distances. Thus, strong ligand binding

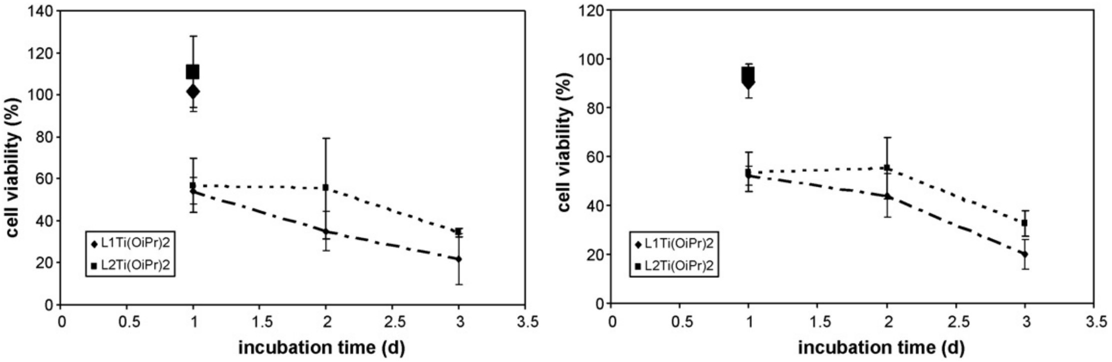


Fig. 14. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] on incubation time with (top) or without (bottom) 2 days exposure to the biological medium prior to cell administration of $L^{1,2}Ti(OiPr)_2$ at 50 mg/L [54a].

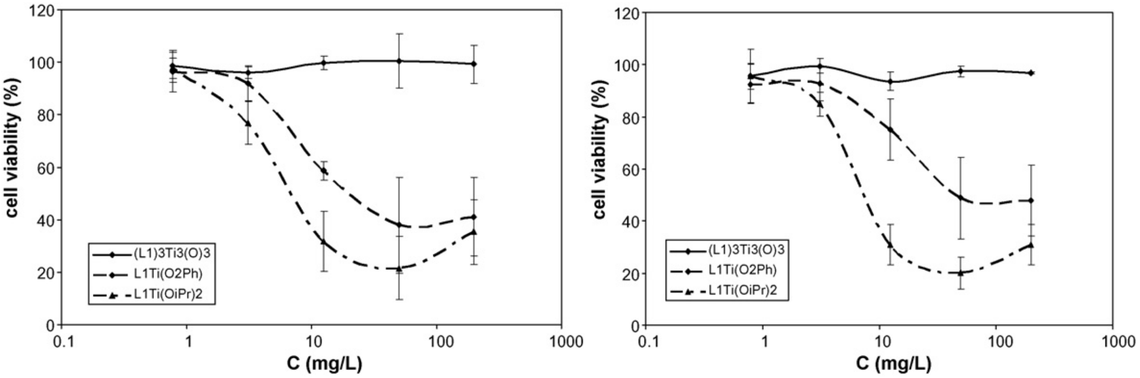
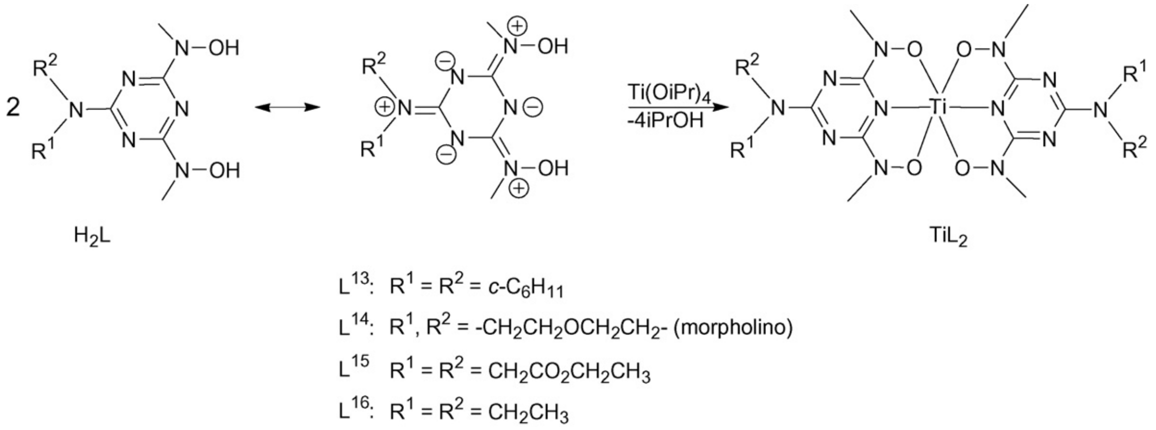


Fig. 15. Dependence of HT-29 (left) and OVCAR-1 (right) cell viability based on the MTT assay [52] after 3 days incubation period on administered concentration of $L^1Ti(OiPr)_2$, $L^1Ti(O_2Ph)$, and $L^1{}_3Ti_3(\mu_2\text{-}O)_3$ presented in a logarithmic scale [54a].



Scheme 7. Hydroxylamino-triazine compounds and their homoleptic complexes.

is obtained due to its high electron density. These complexes feature an octahedral geometry with *mer-mer* binding of the tridentate ligands, similar to the geometry of $TiL^{11}{}_2$ (*vide supra*) [60].

5.1.1. Hydrolysis

Hydrolysis of $TiL^{13-16}{}_2$ was measured based on UV–vis, monitoring the LMCT absorbance vs. time upon addition of water with or without a buffer at various pH and temperature conditions. Exceptional hydrolytic stability was observed, especially under conditions relevant to the biological environment, namely, at 37 °C and pH of 5.0–7.4 (Table 3); no decomposition is observed for days, which is particularly remarkable considering the rapid hydrolysis observed for $TiL^{11}{}_2$, the homoleptic complex of tridentate bis(phenolato) ligands, featuring similar geometry, symmetry and donor atoms. It is thus obvious that the electron-rich triazine nitrogen atoms play a significant role in the substantial hydrolytic stability of these complexes. Faster hydrolysis is observed under particularly acidic conditions (pH 1.9) or basic ones (pH 11.5). Overall, the pH dependence of hydrolysis suggests that around neutral pH, the basic mechanism of hydrolysis is dominant, which is similar to the observations with other Ti(IV) complexes previously reported [83,84].

Table 3
$t_{1/2}$ values for hydrolysis of $TiL^{15}{}_2$.

pH	*T*	$t_{1/2}$ (h)
1.9	RT	24
5.0	RT	>400
7.1	RT	>250
11.5	RT	14
5.0	37 °C	>300
7.1	37 °C	>150

5.1.2. Cytotoxicity

$TiL^{15}{}_2$ is of the highest water solubility and was thus employed for cytotoxicity measurements. Despite the high inertness of this complex, some mild activity was measured towards colon HT-29 (cell growth inhibition of 30%) and ovarian OVCAR-1 cells (cell growth inhibition of 40%) (Fig. 17; left). Since the free

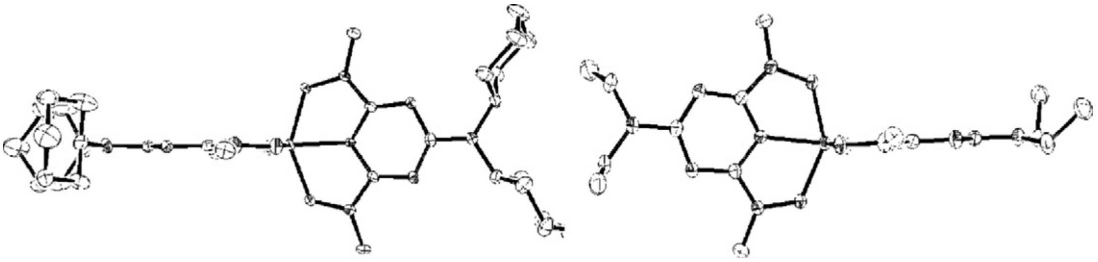


Fig. 16. ORTEP drawing of $TiL^{13}{}_2$ (left) and $TiL^{16}{}_2$ (right) [73,75].

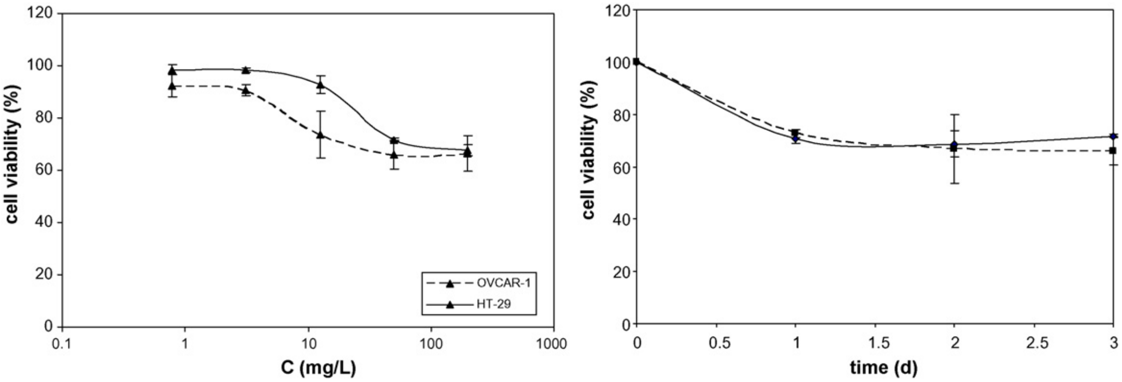


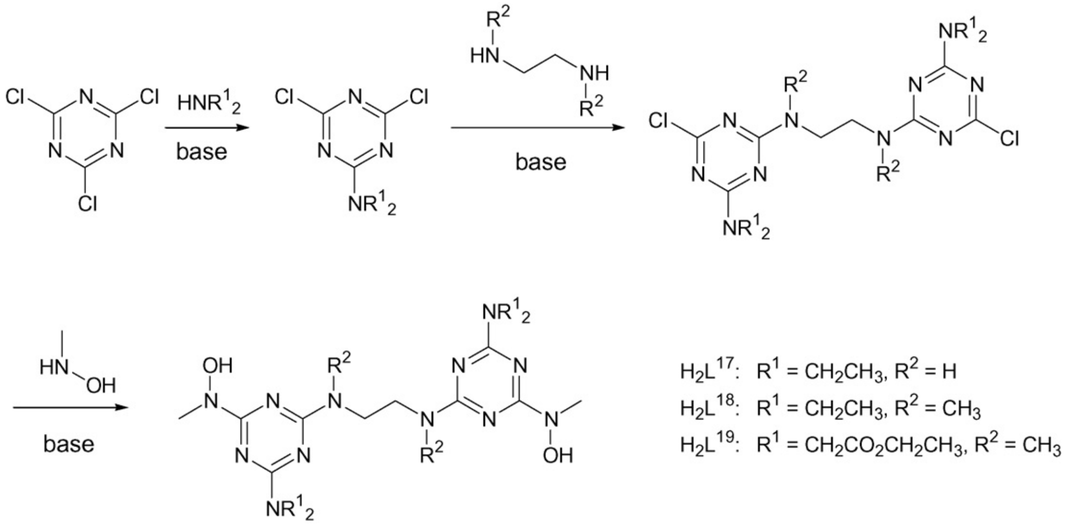
Fig. 17. Dependence of cell viability based on the MTT assay [52] on administered concentration of TiL^{15}_2 presented in a logarithmic scale after 3 days incubation period (left) and on incubation time at 50 mg/L (right) [74].

ligand H_2L^{15} demonstrates high cytotoxic activity towards both cell types analyzed, time-dependence measurements were conducted to establish whether the activity observed for the complex results from traces of hydrolyzed ligand in biological environment despite the complex inertness in pure water. Weaker dependence of activity on incubation time was observed for the complex relative to that of the free ligand, and in addition, exposure of the complex to the biological medium for 2 days prior to insertion of cells completely abolished the complex activity (Fig. 17; right) similarly to the results obtained with bis(phenolato) complexes (Fig. 14). It was thus concluded that the activity of the complex results from the metal center and not from dissociated free ligand, as the concentration of the ligand in solution should increase with long incubation times and long exposure to the biological medium. It is thus logical that under biological conditions in the cellular environment, partial ligand release is obtained upon interaction with the biological target despite the complex inertness in pure water, a conclusion which is also supported by the various mechanistic measurements and structure activity relationship studies conducted with the Ti(IV) complexes of bis(phenolato) ligands. Nevertheless, formation of higher coordination numbers upon biological interaction while binding of the triazine ligands remaining intact cannot be completely ruled out at this point.

It is thus obvious that some inertness of the ligands is required for activity, as weakly binding ligands lead to inactive complexes, while complexes that we consider inert in the presence of pure water are able to demonstrate at least some activity under biological conditions. This conclusion is based on the results obtained with TiL^{11}_2 vs. TiL^{15}_2, and $L^1Ti(OiPr)_2$ vs. $L^1Ti(O_2Ph)$ and $L^5Ti(OiPr)_2$. Two factors may be associated with the rather mild activity observed for the triazine complexes are the following: (a) too high inertness and/or coordinative saturation inhibits biological interaction, especially considering the reduced activity of $L^1Ti(O_2Ph)$ relative to that of $L^1Ti(OiPr)_2$; the biological interaction is either achieved through additional coordination numbers or, more likely, through partial ligand hydrolysis to open a coordination site for the interaction with the proper biological target, as discussed; (b) insufficient solubility in water does not allow for the entire dose to reach the cellular target and thus the actual activity may be higher than that measured. We are currently working on the development of triazine derivatives of enhanced water solubility.

5.2. Tetradentate ligands and their bis(isopropoxo) complexes

Bis(triazine)-based ligands were synthesized in order to incorporate an additional coordinative N-donor with the unique characteristics of high cytotoxic density, with the attempt to produce $LTiX_2$ type complexes of strongly bound tetradentate L ligands on one hand, and two additional monodentate “labile” X groups on the other. Several compounds that include two triazine rings linked together by a diaminoalkyl bridge with an O^- donor on each ring that can function as a covalent ligand, were synthesized in three steps. A schematic representation of the synthesis is given in Scheme 8.



H_2L^{17}: $R^1 = CH_2CH_3$, $R^2 = H$
H_2L^{18}: $R^1 = CH_2CH_3$, $R^2 = CH_3$
H_2L^{19}: $R^1 = CH_2CO_2CH_2CH_3$, $R^2 = CH_3$

Scheme 8. Synthesis of bis(triazine) compounds to be used as tetradentate ligands.

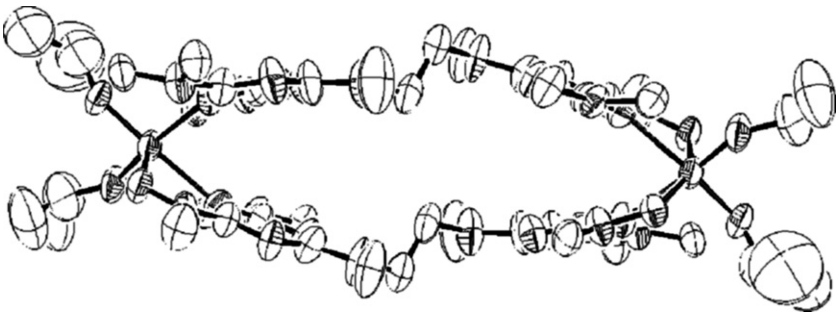


Fig. 18. ORTEP drawing of $L^{19}{}_2Ti_2(OiPr)_4$ [75].

The sp^2 character of the melamine nitrogen atoms leads to restriction to the rotation around the $N–C_{Ar}$ bond that is affected by steric bulk. Thus, all bis(triazine) compounds demonstrate restricted rotation of the $N(R^2)–C_{Ar}$ bond around room temperature, with H_2L^{19} also demonstrating restricted rotation of the $N(R^1)–C_{Ar}$ bond (Scheme 8). As the rotation around the $N(OH)–C_{Ar}$ is relatively free as also observed for the mono-triazine compounds $H_2L^{15,16}$ (Scheme 7), altogether three non-interconvertable isomers of the bis(triazine) compounds are present in solution at ambient temperature. This feature makes complexation rather difficult to fully analyze in solution. Fortunately, single crystals were obtained upon reaction of H_2L^{19} with $Ti(OiPr)_4$ and allowed analyses by X-ray crystallography. The structure of the complex formed (Fig. 18) features a dinuclear complex with two ligands bridging the two octahedral Ti(IV) centers donating a single covalent O-donor and a single coordinative N-donor to each, with two terminal isopropoxo ligands on each metal site. The high rigidity of the ligand does not allow its wrapping around a single Ti(IV) center to give a mononuclear complex, yet, altogether the complex exhibits a metal to L^{19} to OiPr ratio of 1:1:2, as required to establish the role of two monodentate ligands in activity. Nevertheless, some indications of high rigidity in the complex are apparent in the structure, as a 20° deviation from planarity is observed for the aromatic system, and more importantly, the Ti–N distances are back to the typical coordinative bond values of 2.3–2.4 Å, unlike the particularly short distance of 2.0 Å obtained for the analogous homoleptic complexes of tridentate ligands.

5.2.1. *Cytotoxicity and hydrolysis*

The dinuclear complex $L^{19}{}_2Ti_2(OiPr)_4$ demonstrates essentially no activity against the colon and ovarian cells tested. As high hydrolytic instability is observed for this complex in air only, we attribute the activity loss to the rigidity of the complex and its inability to place the ligand in a favored geometry to give the ideal short Ti–N bonds, and thus rapid ligand hydrolysis does not allow stabilization of an active species required to demonstrate cytotoxicity. Additional tetradentate bis(hydroxylamino-triazine) ligands designed to give more flexible Ti(IV) complexes are currently under construction. Overall, the results obtained with this family of complexes support the general conclusions made for the bis(phenolato) compounds.

6. Amine bis(carboxylato) Ti(IV) complexes

The ability of carboxylato ligands to bind in a bidentate mode and thus bridge two metal centers makes it difficult to isolate well-identified Ti(IV) complexes [85]. Several of the bis(carboxylato) ligands employed and the complexes obtained from their reaction with $Ti(OiPr)_4$ that were characterized by X-ray crystallography are shown in Fig. 19. A tridentate ligand leads to a complex where two ligand bind to the metal as in $TiL^{11}{}_2$ and $TiL^{13–16}{}_2$, only two additional DMF solvent molecule coordinate to the metal as well giving an octa-coordinate complex. A tetradentate ligand of branched donor connectivity featuring a pyridine side arm did not lead to a mononuclear complex, but rather different clusters were obtained depending on reaction conditions. When the reaction is performed at room temperature, a hexanuclear cluster is obtained which is held together by both bridging bis(carboxylato) ligands and bridging isopropoxo groups, with no oxo ligands. Increasing the temperature to 35 °C brought about an esterification reaction between the isopropanol released from the starting material and the carboxylic acid of the ligand precursor [86–89]. Thus, the traces of water released gave dinuclear and tetranuclear complexes, which are regarded as partial hydrolysis products of what in envisioned to be the mononuclear Ti(IV) complex of carboxylato ligands bound in a monodentate fashion, where either a single or both of the isopropoxo ligands on each Ti(IV) center were replaced with oxo groups. Interestingly, the two carboxylato groups of each ligand are in a *cis* configuration in both complexes unlike than the *trans* configuration of the phenolato ligands observed for the C_s-symmetrical complexes of branched ligands described above. It is certainly possible that the original complex exhibited a *trans* configuration, while ligand replacement by an associative mechanism forming the oxo bridge leads to the *cis* configuration observed in the final product, as occurs for the bis(phenolato) complexes. Another interesting feature of the dinuclear complex is the particularly wide Ti–O–Ti angle of 180.0°, which becomes narrower in the tetranuclear complex probably due to steric repulsion. We emphasize the simplicity upon which the original isopropoxo ligands are replaced with water to give oxo bridges, as traces of water that are in less than equimolar amounts are sufficient for this process to take place. This highlights once again the exceptionality of the C_2-symmetrical bis(phenolato) Ti(IV) complexes for which this process is particularly slow and only occurs with substantial amounts of water (*vide supra*).

A tetradentate bis(carboxylato) ligand of sequential donor connectivity allowed the isolation of a mononuclear Ti(IV) complex. The crystal structure of this compound points to another C_2-symmetrical complex, with highly similar coordination features to those observed for the analogous bis(phenolato) complexes as well as those of the aliphatic analogue $L^{12}Ti(OiPr)_2$ (Fig. 1). This compound is thus octahedral, with two covalent O-donors, two coordinative N-donors, two *trans* carboxylato ligands, and two *cis*-isopropoxo groups.

Cytotoxicity measured for the O-bridged dinuclear and tetranuclear bis(carboxylato) clusters as well as for the mononuclear bis(carboxylato) compound revealed no activity towards the colon and ovarian cells investigated. This is despite reports on budotitane derivatives which discussed the activity of O-bridged species, particularly those of wide Ti–O–Ti angles [29,40]. We suspect that the rapid hydrolysis in air observed for these compounds is again responsible for the lack of activity, particularly considering the ease of isopropoxo replacement with traces of water molecules. This also proves once again the importance of the particular chelating ligand

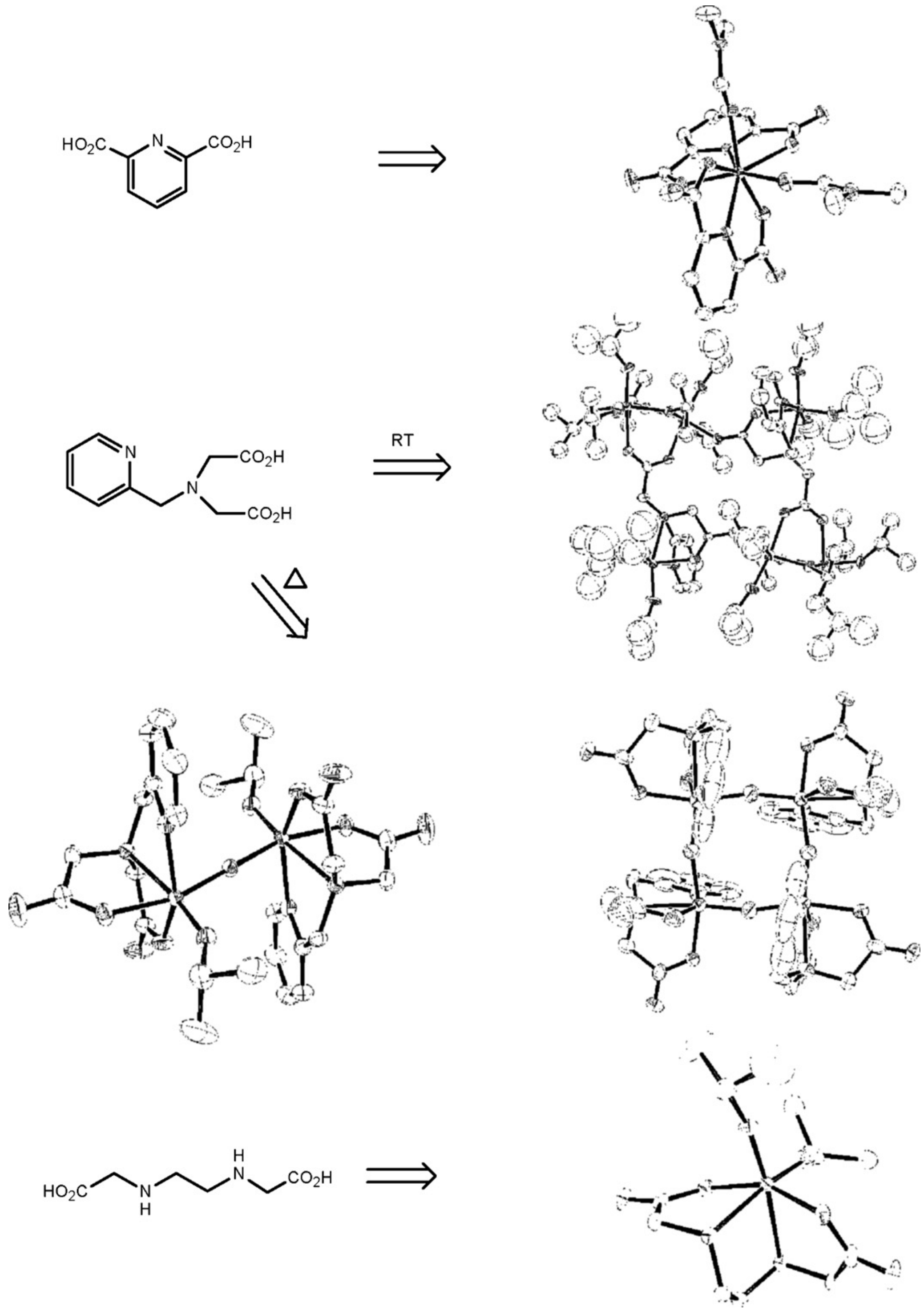


Fig. 19. Bis(carboxylato) ligands and complexes [85].

features in governing activity, rather than those of the Ti–O core obtained upon water addition.

7. Perspective

One of the main concerns regarding the application of transition metal complexes for chemotherapy is their potential toxicity. The advantage of Ti(IV) complexes is their relative biological compatibility, which mostly leads to mild and revisable side effects. However, the hydrolytic instability of known Ti(IV) complexes and formation of various different species upon water addition makes their therapeutic application problematic, and raises a strong interest in the development of relatively stable Ti(IV) complexes with well defined hydrolytic behavior that demonstrate appreciable cytotoxic activity. Strong ligand binding is also of interest to avoid complete ligand stripping by transferrin, so that the ligand may be used as a target for structure–activity relationship investigations.

Ti(IV) complexes of tetradentate diamine bis(phenolato) ligands of sequential connectivity of donor atoms show great promise as anti-tumor agents. They reveal cytotoxic activity which is higher than that we measured for the known Ti(IV) complexes $(bzac)_2Ti(OiPr)_2$ and Cp_2TiCl_2, as well as that we measured for cisplatin, towards colon and ovarian cells. Most importantly, a correlation is observed between defined hydrolytic behavior and cytotoxicity. Overall, we can say that an inert strongly bound chelating ligand is required in order to increase electron density on the Ti(IV) center and stabilize some sort of an active species, which is required either for cell penetration or for an interaction with the cellular target of reactivity. This is also supported by the lack of activity observed when the complexes are exposed to the biological medium for an extensive amount of time in the absence of cells. Thus, throughout a relatively slow hydrolysis in the presence of cells which allows for enough time prior to formation of inactive O-bridged species as the initial hydrolysis products, some active species with bound bis(phenolato) ligand can penetrate the cells in a non-transferrin dependent fashion, and demonstrate cytotoxic activity. The formation of such species presumably involves some hydrolysis of the most labile isopropoxo groups, a conclusion that is supported both by the reduced activity of the more inert catecholato complex, as well as by the strong correlation observed between the cytotoxicity and the eventual formation of a cluster throughout release of the isopropoxo groups to give isopropanol. Thus, an intermediate in the cluster formation is suspected to be the active species, or to the very least, its formation in the biological environment is governed by similar steric requirements as those governing the cluster formation in water. However, the lack of activity of the cluster itself does not rule out its biological participation in the cell, as perhaps its inactivity results from inability to penetrate the cell membrane. Thus, following cell entry of the original complex or a species obtained immediately following exposure to the biological medium, formation of the cluster in the cellular environment may also be of biological interest.

The investigations conducted with other ligand families and their Ti(IV) complexes generally supported these conclusions. Overall, we see that complexes of hydrolytic instability are not active biologically, probably due to rapid formation of inactive aggregates, similarly to the scenario proposed to occur with other labile complexes such as $Ti(OiPr)_4$ and $TiCl_4(THF)_2$. Thus, several requirements need to be met for obtaining appreciable cytotoxicity: (a) strong chelate ligand binding is required to stabilize the active species and delay hydrolysis; (b) only moderate lability of monodentate groups is required to form the active species throughout a reasonable period of time, which is not unrelated to the lability of the main chelating ligand; (c) small steric bulk all around the complex is favored; (d) planar moieties may be of interest; (e) decent water solubility is essential. Therefore, some of the Ti(IV) complexes presented herein follow these requirement to a certain extent, and we are currently working on the development of analogues of enhanced water solubility and general hydrophilicity. In addition, tetradentate ligands based on bis(triazine) moieties that can give mononuclear octahedral bis(isopropoxo) complexes with particularly short Ti–N coordinative bonds and thus particularly strong ligand binding and hydrolytic stability are suitable candidates to follow these requirements and are currently investigated. As also additional mechanistic investigations targeted at the characterization of the exact active species, the identification of the cellular target, and the evaluation of the general mode of operation of the Ti(IV) complexes are underway, we believe that the true merit of Ti(IV) complexes for pharmaceutical applications are yet to be discovered.

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