

Competitive binding of the anticancer drug titanocene dichloride to *N,N'*-ethylenebis(*o*-hydroxyphenylglycine) and adenosine triphosphate: a model for Ti^{IV} uptake and release by transferrin

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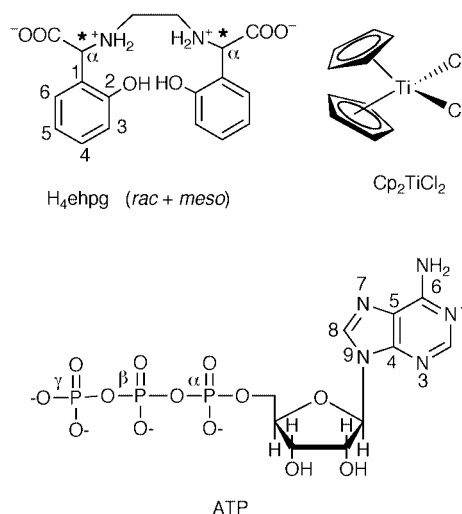
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^1H and ^{31}P NMR studies show that in aqueous solution the anticancer agent titanocene dichloride (Cp_2TiCl_2) binds selectively to *N,N'*-ethylenebis(*o*-hydroxyphenylglycine) (H_4ehpg) at neutral pH, but preferentially to adenosine triphosphate (ATP) at pH* values below 5.1; intermolecular Ti^{IV} transfer from $[\text{Ti}^{\text{IV}}(\text{ehpg})(\text{H}_2\text{O})]$ to ATP occurs at acidic pH values.

Ti^{IV} complexes are of current medicinal interest owing to the pronounced antitumour properties and low toxic side-effects of two Ti^{IV} complexes, titanocene dichloride (Cp_2TiCl_2) and Budotitan $[\text{Ti}(\text{bzac})_2(\text{OEt})_2]$ (Hbzac = 1-phenylbutane-1,3-dione), which are currently on phase II clinical trials.^{1,2} Cp_2TiCl_2 also exhibits pronounced antiviral, antiinflammatory and insecticidal activities.¹ Some Ti^{IV} complexes have recently been shown to exhibit antibacterial activity.^{3,4} The significant effectiveness of Cp_2TiCl_2 against cisplatin-resistant tumour cell lines indicates that it has a different mechanism of action to cisplatin.² However, in contrast to platinum-based anticancer drugs,⁵ very little is known about the biological chemistry of titanium and its mechanism of action as an anticancer agent is poorly understood.⁶ Attack on cellular nucleic acids is believed to be a key process for the antitumour activity of Cp_2TiCl_2 , which inhibits DNA synthesis rather than RNA and protein synthesis, and titanium accumulates in nucleic-acid-rich regions in tumour cells after *in vivo* or *in vitro* administration.^{7,8} However, unlike cisplatin, Ti^{IV} does not bind strongly to DNA bases at physiological pH, but forms strong complexes with nucleotides only at pH values below 5.⁹ Also, there is no evidence for stable complexes of the V or Mo analogues with nucleotides or DNA under physiological conditions.^{6,10} This raises doubts that nucleic acids are the major target.^{10,11} Efforts to identify the active Ti^{IV} species in biological media have been largely unsuccessful due to the rapid hydrolysis of Ti^{IV} complexes at neutral pH and precipitation of inactive polymeric hydrolysis products.¹²

Recently we found that Ti^{IV} -citrate and Cp_2TiCl_2 bind strongly to the specific Fe^{III} sites of human serum transferrin (hTF) and that Ti^{IV} is released from Ti_2 -hTF at low pH, which may provide a transport mechanism for Ti^{IV} .^{13,14} Transferrin is the 80 kDa iron-transport protein in the blood serum of vertebrates present at a concentration of about 35 μM .¹⁵ It takes up Fe^{III} from blood plasma at pH 7.4 and delivers it to cells *via* receptor-mediated endocytosis. Fe^{III} is released from transferrin in cell compartments called endosomes at pH *ca.* 5.5.¹⁵ The metal binding properties of transferrin have long been mimicked by using the chelating agent *N,N'*-ethylenebis(*o*-hydroxyphenylglycine) (H_4ehpg).¹⁶ This ligand contains donor groups similar to the metal binding sites of transferrin (2Tyr, His, Asp and CO_3^{2-}). In particular the two tyrosinate ligands are thought to play a dominant role in determining the strength of metal binding to transferrin.¹⁷ Recently we found that, in contrast to its rapid hydrolysis at neutral pH in the absence of chelator ligand, Cp_2TiCl_2 reacts readily with H_4ehpg (*rac*) at pH* 7 and forms a seven-coordinate Ti^{IV} complex $[\text{Ti}(\text{ehpg})(\text{H}_2\text{O})]$ (1).¹⁸ We have investigated the pH-dependent

competitive binding of Cp_2TiCl_2 to H_4ehpg and ATP and the intermolecular transfer of Ti^{IV} from the EHPG complex to ATP at low pH. ATP is a potential acceptor ligand for metals released from transferrin in cells and also a purine nucleotide present in DNA (as a phosphate monoester). Our results suggest that novel routes could exist for the transfer of Ti^{IV} onto DNA *in vivo*.



First we carried out solution ^1H and $^{31}\text{P}\{^1\text{H}\}$ NMR experiments to probe the competitive reaction of $\text{Cp}_2\text{TiCl}_2(\text{aq})$ with H_4ehpg (*rac* + *meso*, *ca.* 1:1) and ATP (1:1:1 mol ratio, 5 mM), at different pH*† values in D_2O . The ^1H NMR spectra (purine region and methylene region) and $^{31}\text{P}\{^1\text{H}\}$ NMR spectra recorded after 6.5 h of reaction at 298 K are shown in Fig. 1. At pH* 7.0, peaks due to free EHPG† [δ 3.12 and 3.03, methylene] and bound Cp ligand [δ 6.42] nearly disappeared, and new peaks characteristic of Ti^{IV} -EHPG complexes [δ 2.62 (d) and 2.97 (d), $-\text{CH}_2\text{CH}_2-$; δ 6.72 (d), 7.05 (t) and 7.40 (m), phenyl ring] appeared in the ^1H NMR spectrum. None of the ATP reacted, as indicated by both the ^1H and ^{31}P NMR spectra. At pH* = 5.1, peaks for both free H_4ehpg and free ATP decreased in intensity and new peaks characteristic of Ti^{IV} -EHPG and Ti^{IV} -ATP complexes [upfield shifted broad peaks for H8 and H2 at δ 8.43 and 8.20, respectively; broad $^{31}\text{P}\{^1\text{H}\}$ peak at δ -24.43 for β phosphate, broad shoulder at δ -10.85 for α and/or γ phosphate] emerged in both ^1H and $^{31}\text{P}\{^1\text{H}\}$ NMR spectra. Integration of ^1H NMR peaks indicated that *ca.* 30% of the $\text{Cp}_2\text{TiCl}_2(\text{aq})$ formed complexes with EHPG and about 36% of $\text{Cp}_2\text{TiCl}_2(\text{aq})$ formed complexes with ATP. However, at pH* \leq 4.6, the free peaks for EHPG remained unchanged and no peaks for Ti^{IV} -bound EHPG were detected, suggesting that Ti^{IV} -EHPG complexes were not formed. In contrast, new peaks assignable to Ti^{IV} -bound ATP dominated both the ^1H and ^{31}P NMR spectra, *e.g.* the broad upfield-shifted H8, H2 peaks for ATP, and new upfield-shifted broad peaks for the α , β and γ phosphates of ATP. This indicates that $\text{Cp}_2\text{TiCl}_2(\text{aq})$ forms

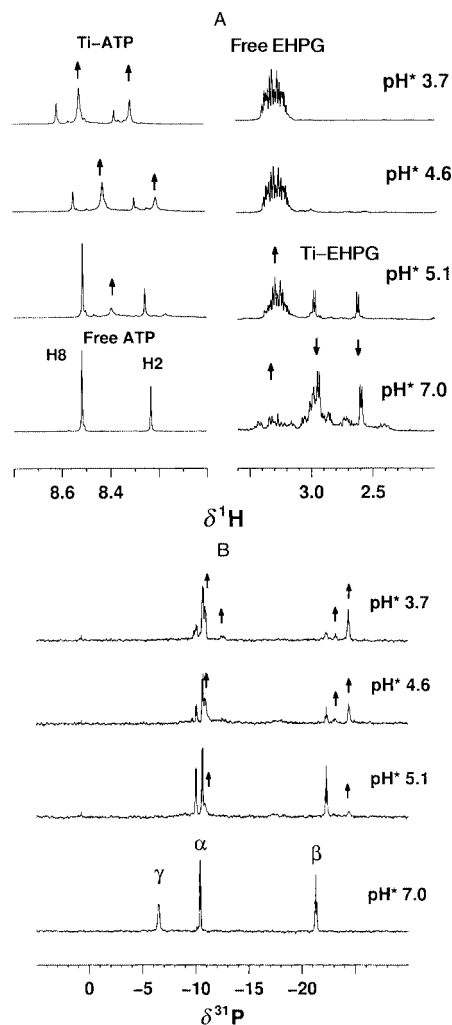


Fig. 1 (A) ^1H and (B) $^{31}\text{P}\{^1\text{H}\}$ NMR spectra of reactions of $\text{Cp}_2\text{TiCl}_2(\text{aq})$, EHPG and ATP (5 mM) in D_2O at different pH^* values, 298 K, after 6.5 h, showing that Ti-EHPG complexes are formed at $\text{pH}^* \geq 5.1$ while Ti-ATP complexes are formed at $\text{pH}^* \leq 5.1$.

complexes with ATP only at these lower pH^* values. The broad upfield shifts of H8 and H2 suggest coordination of Ti^{IV} to N7¹⁹ (downfield shifts of H8 and H2 are more common for Pt–N7 coordination, however, upfield shifts have been established for Cp_2Mo –N7 coordination¹⁹), and the upfield shifts of the new ^{31}P peaks indicate phosphate coordination.

The ^1H NMR assignments for free EHPG (*rac* and *meso*) ligands and Ti^{IV} –EHPG complexes have been established by 2D NMR techniques and their solid-state structures have been determined by X-ray crystallography.¹⁸ They are seven-coordinate with a hexadentate EHPG ligand and an additional water ligand. However, efforts to crystallise Ti^{IV} –ATP complexes were not successful and their structures cannot be established from the NMR data alone, although it is evident that both N7 and phosphate groups (β and γ) are involved in Ti^{IV} coordination.

At $\text{pH}^* 2.6$, new ^{31}P peaks emerged at δ ca. 0.70 and 0.81 (data not shown). These can be assigned to inorganic phosphate and AMP, respectively, indicating that the phospho-diester bonds of ATP are cleaved by reaction with titanocene dichloride.⁹ These results suggest that the affinity of $\text{Cp}_2\text{TiCl}_2(\text{aq})$ for EHPG or ATP is pH-dependent. At neutral pH, Ti^{IV} is more strongly bound to EHPG, while at acidic pH, it has a higher affinity for ATP. The cross-over point is at $\text{pH}^* \text{ ca. } 5.1$, which is comparable to the pH value inside the endosome (pH 5.0–5.5) where iron is released from ferritin.

The Ti-EHPG (*rac*) complex, $[\text{Ti}(\text{ehpg})(\text{H}_2\text{O})]$ (**1**), is very stable between $\text{pH}^* 7$ and 1,¹⁸ however, unexpectedly, in the presence of ATP, it becomes labile and Ti^{IV} transfers from the hexadentate ligand to ATP. Fig. 2 shows the time course of the

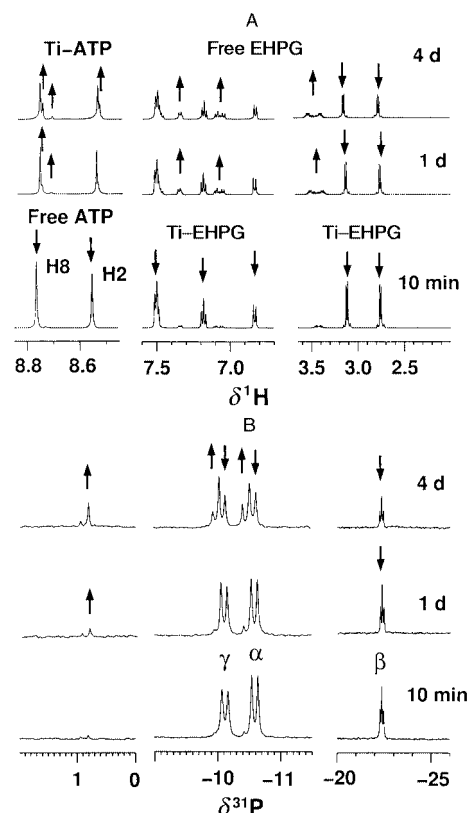


Fig. 2 Time course of the reaction between $\text{Ti}(\text{ehpg})(\text{H}_2\text{O})$ (**1**) and ATP (1:1) at 310 K, $\text{pH}^* 2.80$ followed by (A) ^1H and (B) $^{31}\text{P}\{^1\text{H}\}$ NMR, showing the gradual dissociation of complex **1** accompanied by formation of Ti-ATP complexes, indicating intermolecular Ti^{IV} transfer.

reaction between **1** and ATP monitored by ^1H and ^{31}P NMR at 310 K and $\text{pH}^* 2.80$. In the presence of 1 mol equiv. of ATP, ^1H NMR peaks for **1** [δ 2.76 (d) and 3.12 (d) (methylene), δ 6.82 (d), 7.19 (t) and 7.50 (m) (phenyl ring)] decreased in intensity, while, simultaneously, new peaks characteristic of free EHPG [δ 3.40 (m) and 3.56 (m) (methylene), δ 7.05 (d), 7.10 (t), 7.35 (d), 7.46 (t) (phenyl ring)] appeared and increased in intensity with time. This indicates that Ti^{IV} dissociates from the EHPG ligand. At the same time, the H8, H2 peaks for free ATP decreased in intensity, while new H8 and H2 peaks appeared upfield and continued to increase in intensity. Also new ^{31}P peaks emerged in the ^{31}P NMR spectrum (δ 0.95, 0.82, -10.00 , -10.47) and increased in intensity with time. These new peaks may be due to the species produced from the cleavage of ATP induced by Ti^{IV} (such as ADP, AMP and inorganic phosphate). Therefore Ti^{IV} is transferred from EHPG to ATP.

To investigate if intermolecular Ti^{IV} transfer can occur at physiologically relevant pH values, and the effect of ATP concentration, separate experiments were carried out in D_2O at 310 K, using **1**:ATP mol ratios of 1:1 or 1:10, at $\text{pH}^* 2.8$, 4.4 and 6.2. The simultaneous decrease in intensity of ^1H NMR resonances for **1** and free ATP, and increase in intensity of resonances for free EHPG and Ti-ATP adducts, confirm that Ti^{IV} transfer does occur under these conditions. Fig. 3 shows the time course of Ti^{IV} transfer, as determined by integration of ^1H NMR peaks. In the presence of 1 mol equiv. of ATP at $\text{pH}^* 2.8$, 38% of the Ti^{IV} was transferred in 2 d; at $\text{pH}^* 4.4$, 25% Ti^{IV} was transferred, while at $\text{pH}^* 6.2$, little Ti^{IV} transfer was detected over a 2 d period. In the presence of 10 mol equiv. of ATP, Ti^{IV} transfer was more complete and even occurred at $\text{pH}^* 6.2$. In 2 d, 83% Ti^{IV} was transferred at $\text{pH}^* 2.8$, 41% at $\text{pH}^* 4.4$, and 21% at $\text{pH}^* 6.2$. A similar reaction was also carried out using 5'-GMP instead of ATP at $\text{pH}^* 2.9$. Ti^{IV} transfer also occurred, though to a lower extent, in the presence of 5'-GMP (ca. 36% in the presence of 10 mol equiv. of 5'-GMP, data not shown).

Titanium transfer reactions to nucleotides mediated by EHPG, a chelator and amino acid derivative, may be relevant

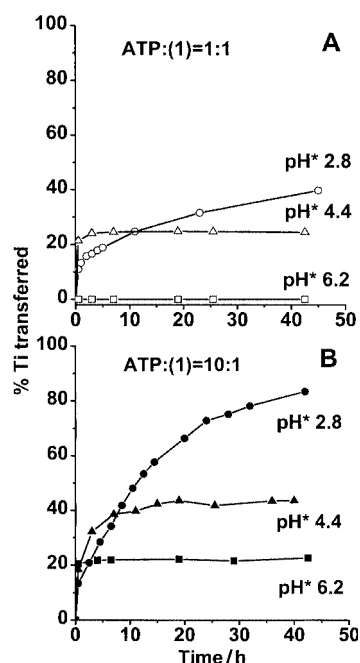


Fig. 3 Time course of intermolecular Ti^{IV} transfer from complex **1** to ATP in the presence of (A) 1 mol equiv. ATP or (B) 10 mol equiv. ATP at different pH^* values. The data show that both pH and ATP concentration affect the rate of Ti^{IV} transfer.

to the mechanism of action of titanium anticancer drugs. In terms of “HSAB” theory,²⁰ Ti^{IV} is a “hard” Lewis acid and readily hydrolyses to form insoluble polymeric species at neutral pH values. Both titanocene dichloride and Budotitan undergo rapid and complete hydrolysis to form anticancer-inactive insoluble polymers at physiological pH values.¹ However, biological experiments reveal that titanium is accumulated in the cellular nucleic-acid-rich regions (mainly the nucleus) after *in vivo* application of titanium drugs.^{7,8} Hence Ti^{IV} must be stabilised for transport by binding to biomolecules. Transferrin is a likely candidate for Ti^{IV} transport from blood plasma to cells.^{13,14} The current work shows that at neutral pH , Cp_2TiCl_2 has a higher affinity for the transferrin model ligand EHPG than for ATP while at pH^* values below 5.1 the affinity for the nucleotide chelator ATP is higher. Intermolecular Ti^{IV} transfer from Ti^{IV} -EHPG complexes to nucleotides occurs at low pH or high ATP concentrations. Ti^{IV} transfer from human transferrin (Ti_2 -hTF) to ATP also occurs at low pH .¹⁴ Extra-cellular ATP levels are low, but intracellular concentrations of ATP are as high as 3–5 mM.²¹ Inside cells, ATP is a major metal macro-chelator and intracellular iron carrier. It plays a major role in transport of Fe^{III} to the nucleus, and the γ -phosphate of ATP is hydrolysed during Fe^{III} transport.²² Therefore ATP could also facilitate the intracellular transport of Ti^{IV} and allow it to target polynucleotides which are condensed in the nucleus. DNA in the nucleus has a high negative charge and potentially a markedly lower pH value near its surface (up to 3 pH units lower than the bulk pH).²³ Cp_2Ti -DNA adducts have been detected *in vitro* at pH 5.3,²⁴ and Ti -DNA adducts in tumour cells treated with Cp_2TiCl_2 .²

Metal anticancer agents are often electrophilic and can react with many biomolecules, such as amino acids, polyphosphates, proteins and nucleic acids. However, these biomolecules are located in different extracellular and intracellular compartments, and there are carrier molecules (e.g. proteins such as albumin and transferrin, or small molecules such as ATP, citrate, and GSH) which communicate between them. The substrate binding properties (such as uptake and release) are finely controlled by natural gradients which exist in different tissues or cellular compartments (e.g. pH , ATP or ionic gradients). The

gradients could also alter the relative affinity of drug molecules for different cellular components and facilitate drug binding to its target. “Hard” Ti^{IV} may be transported into the cell by transferrin and subsequently bind to DNA at both the negatively-charged phosphates on the backbone and base N-donors.^{6b,25} The high DNA concentration in the cell nucleus and potentially low pH close to the surface of DNA may favour DNA as a target for Ti^{IV} binding under these conditions. Further work is needed to establish this.

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Notes and references

[†] pH^* is the pH meter reading in D_2O solution.

[‡] EHPG represents the H_4ehpg ligand without designation of the state of protonation.

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