Organometallic Anticancer Agents: The Effect of the Central Metal and Halide Ligands on the Interaction of Metallocene Dihalides Cp_2MX_2 with Nucleic Acid Constituents

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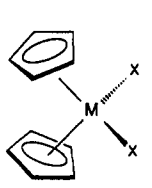
The interactions of the metallocene dihalides Cp_2MX_2 (M = Ti, Mo, Zr, Hf) and Cp_2TiX_2 (X = F, Cl, Br, I) and the nucleic acid building blocks D-ribose-5′-phosphate, nucleobases, nucleosides, and nucleotides have been studied by ^1H and ^{31}P NMR spectroscopy. In the series Cp_2TiX_2 (X = F, Cl, Br, I), similar ^1H NMR spectra were obtained in titrations of each metallocene with the four nucleotides. The spectra are consistent with dissociation of the halide ligands to give $Cp_2Ti^{2+}_{(aq)}$, which coordinates to nucleobase (N) and phosphate (O) binding sites. The metal center (Ti, Mo, Zr, Hf) strongly influences the nature and extent of interactions between metallocene dichlorides Cp_2MCl_2 and DNA subunits. Immediate complexation occurs between nucleotides and the antitumor active metallocenes Cp_2MX_2 (M = Ti and Mo, 0.25–1.00 equiv). In contrast, fomation of discrete complexes between nucleotides and the biologically inactive metallocenes Cp_2MCl_2 (M = Hf, Zr, 0.25–1.00) is not observed, and instead hydrolysis of the Cp rings to give free cyclopentadiene is the major reaction pathway. The complexes formed between titanocene dihalides and nucleotides are stable for hours at pH 2–5; at higher pH the binding is significantly weakened. These results are in agreement with the observed antitumor properties of the metallocene dihalides and provide support for the hypothesis that DNA–metallocene interactions are a major determinant in the antitumor properties of this class of compounds.

Introduction

The antitumor properties of a range of metallocene dihalide and pseudohalides Cp_2MX_2 (M = Ti, Mo, Nb, V; X = F, Cl, Br, I, NCS, N_3, Y) have been reported against a range of cell lines including leukemias P388 and L1210, colon 38 and Lewis lung carcinomas, B16 melanoma, solid and fluid Ehrlich ascites tumors, and several human colon and lung carcinomas transplanted into athymic mice.[1-5] Through a systematic study of metallocene dihalides derived from d block elements, it appears that the activity is directly related to the position of the central metal in a diagonal relationship in the Periodic Table (Figure 1).[3] Thus, metallocenes containing the central metals Ti, V, Nb, or Mo have equal cancerostatic potency with distinct dose–activity relationships and with cure rates of 100% at optimum doses. Within each group, the antitumor activity decreases with increasing atomic weight, and Ta or W metallocenes only sporadically increase the survival of treated tumor-bearing mice, whereas Zr and Hf metallocenes are inactive against Ehrlich ascites tumors.[1-5] The nature of the halide does not appear to affect significantly the antitumor properties, and within the series Cp_2TiX_2, equally potent tumor inhibition was demonstrated for a variety of halide and pseudohalide ligands.[1,2]

The mechanism of antitumor activity of the metallocene dihalides is believed to result from the interaction of a hydrolyzed metallocene species with DNA. Metals derived from metallocenes accumulate in nucleic acid-rich regions of cells, and nucleic acid synthesis, particularly DNA synthesis, is inhibited after *in vitro* and *in vivo* treatment with titanocene or vanadocene dichloride.[2,6] Direct support for the formation of metallocene–DNA adducts has been provided by a recent report where several adducts of metallocene dihalides with DNA were isolated and characterized by inductively coupled plasma (ICP) spectroscopy.[7] However, while distinct adducts were observed with the antitumor-active metallocene dichlorides Cp_2TiCl_2 and Cp_2MoCl_2, they were not detected with Cp_2VCl_2, which is the most active metallocene *in vitro*. The significance of these results is unclear, as DNA adducts were also detected with Cp_2ZrCl_2 and Cp_2HfCl_2, which show no anticancer activity. In an independent NMR spectroscopic study, we have shown that Cp_2MoCl_2 forms stable adducts with calf thymus DNA.[8]



Ti	V	
Zr	Nb	Mo
Hf	Ta	W

Key: M= maximum activity
M= sporadic activity
M= no activity

Figure 1. Relationship between the position of the central metal in metallocene dihalides and antitumor activity.[3]

The exact structures of metallocene–DNA adducts at the molecular level remain to be elucidated. A detailed study of the solution- and solid-state coordination chemistry of molybdocene dichloride with DNA constituents by Marks *et al.* has provided the first insight into metallocene–DNA coordination chemistry.[9] In the absence of competing ligands, Cp_2MoCl_2 coordinates to both nucleobase (N) and phosphate (O) in a nonlabile manner that affects major conformational changes, but does not appear to disrupt Watson–Crick hydrogen bonding.[9] These studies, along with the aqueous chemistry of Cp_2MCl_2 (M = Ti, V, Mo, Zr),[10,11] and a study of the interaction of Cp_2VCl_2 with nucleotides and phosphoesters,[11] have shown conclusively that the mechanism of interaction of metallocenes with DNA is completely different to that of cisplatin.[12]

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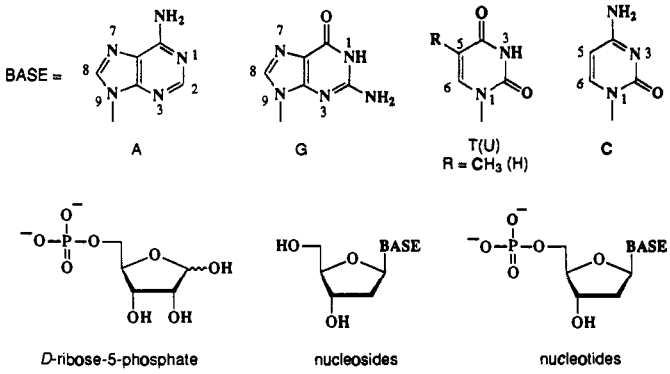

Figure 2. DNA constituents used in titrations with metallocene dihalides.

In order to further clarify the role of DNA adducts in the antitumor properties of metallocene dihalides, we have undertaken a study of the coordination chemistry of two series of metallocenes with a range of DNA constituents. Firstly, the central metal was varied in the series Cp_2MCl_2 (M = Ti, Mo, Hf, Zr) and included biologically active and inactive metallocenes. Secondly, the effect of the halide ligand in the series Cp_2TiX_2 (X = F, Cl, Br, I) was examined. Aqueous solutions of each metallocene were titrated into DNA subunits (Figure 2), and the products were analyzed by NMR spectroscopy. The data obtained show a clear correlation between the biological activity of these compounds and the strength and type of adducts formed with DNA constituents and provide further experimental evidence for the importance of metallocene–DNA interactions in the mode of antitumor action of this class of organometallic compounds.

Results

The four nucleotides found in DNA contain several potential coordination sites for metal ions. In this work a series of NMR experiments were undertaken with nucleotides, nucleosides, nucleobases and D-ribose-5′-phosphate (Figure 2). D-Ribose-5′-phosphate was used as a model system containing a single phosphate (O) coordination site, similar to that present along the DNA backbone, that would allow measurement of ^{31}P NMR chemical shifts in metallocene phosphate (O) complexes. The nucleobases and nucleosides contain identical coordination sites, with the exception of the sugar hydroxyls, which do not generally interact with transition metals.[13] The nucleotides contain all these potential binding sites, but the availability of these binding sites may be different to those present in double-stranded DNA.[8]

All experiments were carried out by titration of an aqueous solution of Cp_2MX_2 into samples of the appropriate DNA subunit (Figure 2), and the pD was recorded and adjusted as required throughout the experiment. Some of the metallocenes, notably Cp_2MoCl_2, have poor aqueous solubility, but extensive sonication gave solutions suitable for titration experiments. The experiments were carried out in water to avoid problems in interpretation of NMR spectra due to changes in chemical shift(s) that often result with mixed solvent systems. However, it is noted that metallocene dihalides have been administered in DMSO, DMSO/saline, and buffered solutions,[1-5] and for this reason selected experiments were also carried out in mixed and buffered solvent systems in order to compare the results to those in water.

Influence of the Metal Center. The aqueous chemistry of Cp_2MCl_2 (M = Ti, Mo, Zr, V) has been studied by Marks and co-workers.[9-11] Within this series, the rate of cyclopentadienyl (Cp) hydrolysis, as determined by integration of resonances due to liberated Cp in the ^1H NMR spectra, was determined to be Mo < V < Ti ≪ Zr.[9-11] As hafnocene dichloride was also studied in this work, initial experiments were carried out to determine the relative stability of the Hf–Cp bond. Integration of the liberated Cp resonances compared to an internal standard (TSP), in a freshly prepared sample of Cp_2HfCl_2 in D_2O, confirmed that the Hf–Cp bond is extremely labile, and hydrolysis occurs almost immediately and more rapidly than for Cp_2ZrCl_2.

The rate of Cp hydrolysis is sensitive to pH, and for Cp_2MX_2 (M = Ti, Zr, Hf) hydrolysis is rapid at physiological pH, with only V and Mo having appreciable stability at pH close to 7.[9-11] Freshly prepared solutions of Cp_2MCl_2 in water, which were used in the titration experiments, have pH values of 2.8 (M = Ti), 3.8 (M = Mo), 2.9 (M = Zr), and 3.1 (M = Hf). For Cp_2TiCl_2 or Cp_2MoCl_2, under these conditions, no resonances due to hydrolyzed Cp rings were detected by ^1H NMR in a 24-h time period, and hence in these metallocenes the Cp rings are >98% metal bound. In the case of Cp_2ZrCl_2, it was estimated that ~80–90% of the Cp rings were metal bound after 15 min, while for Cp_2HfCl_2, extensive hydrolysis was still observed at pH 3.1 and only 40–50% of the Cp rings were metal bound.

The interaction of the metallocene dichlorides Cp_2MCl_2 (M = Ti, Mo, Zr, Hf) with 1 equiv of D-ribose-5′-phosphate, nucleobases, and nucleosides were examined first. With D-ribose-phosphate, addition of Cp_2TiCl_2 and Cp_2MoCl_2 resulted in the appearance of new resonances in the ^{31}P NMR spectra, consistent with formation of phosphate (O)-centered complexes. While Cp_2MoCl_2 formed discrete complexes with nucleobases and nucleosides as evidenced by the appearance of new resonances in the ^1H NMR spectra, the interaction of Cp_2TiCl_2 with nucleobases and nucleosides was weak, and only ~10% complexation was observed. In contrast, no complexation was detected with Cp_2HfCl_2 and Cp_2ZrCl_2 with nucleosides or nucleobases, even after the addition of >5 equiv of metallocene. These spectra were dominated by resonances due to free cyclopentadiene (δ 2.9, 6.5, 6.6 ppm, 3 multiplets), and clearly hydrolysis of the Cp rings is favored over formation of N-substituted complexes. The spectra with Cp_2TiCl_2 also showed the presence of some free cyclopentadiene, after several hours, but this was a minor product (~5%) in the reaction.

The interactions of Cp_2MCl_2 with the nucleotides were examined in more detail, with ^1H and ^{31}P NMR spectra and pD recorded after the addition of 0.25, 0.50, 1.00, and 2.00 equiv of Cp_2MCl_2 to each nucleotide. Figure 3 shows a typical set of ^1H NMR spectra obtained on titration of 1 equiv of each metallocene with the four nucleotides.

For the metallocenes (M = Ti, Mo) after the addition of 0.25 equiv, for each nucleotide, a new set of signals were observed which increased in intensity according to the number of equivalents of metallocene added. After addition of 1.00 equiv, a mixture of nucleotide and complex(es) was observed (Figure 3a,b). The initial solution pH of the nucleotides (7–8) decreased with each successive addition of metallocene to give, after addition of 2.00 equiv, solutions of the complexes with pH 2–4. A

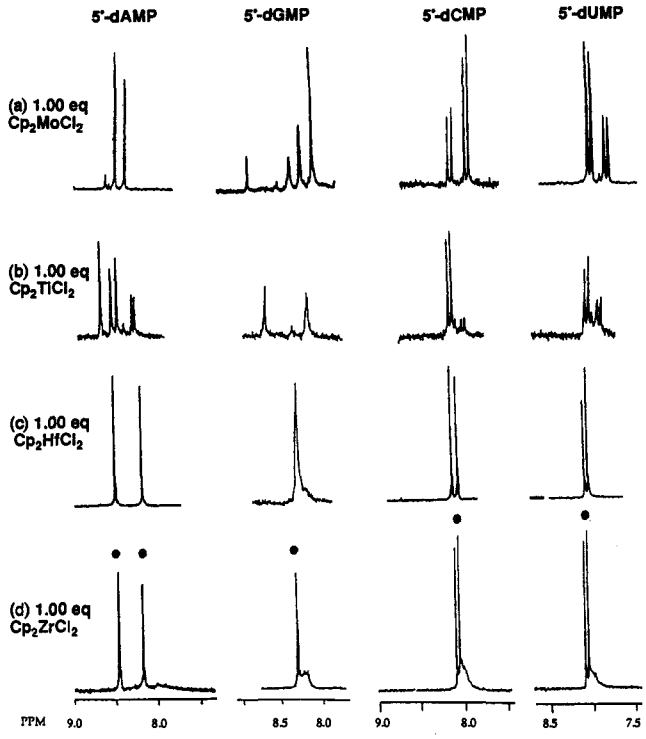

Figure 3. Aromatic region of ^1H NMR spectra (200 MHz, D_2O) obtained by titration of 1.00 equiv of Cp_2MCl_2 (20 mM, D_2O): (a) M = Mo, (b) M = Ti, (c) M = Hf, (d) M = Zr into nucleotides (20 mM); signals due to uncomplexed nucleotides are indicated (●) in spectrum d.

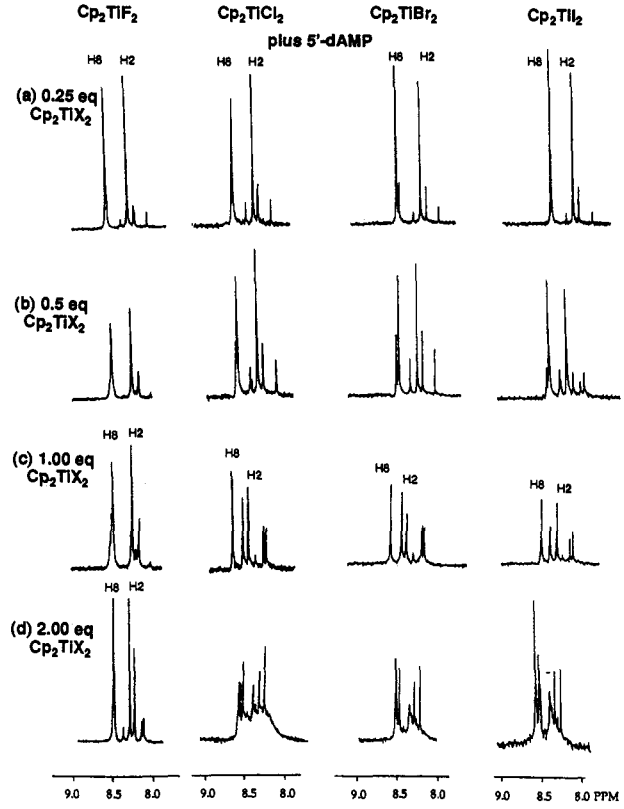

Figure 4. Aromatic region of ^1H NMR spectra (200 MHz, D_2O) obtained by titration of (a) 0.25, (b) 0.50, (c) 1.00, and (d) 2.00 equiv of Cp_2TiX_2 (20 mM, D_2O) into 5′-dAMP (20 mM). Assignments (H2, H8) refer to uncomplexed 5′-dAMP.

final spectrum was recorded after adjustment of the pH to ~7 with NaOD. Under these conditions, the complexes were still detected, but they were present at a lower concentration compared to at low pH; Cp_2TiCl_2 adducts were particularly sensitive to this final change in pH. Similar experiments using solid Cp_2MoCl_2 have been reported by Marks *et al.*,[9] and those results were confirmed in this study.

In contrast, no new aromatic signals were observed in the ^1H NMR spectra of the nucleotides and 0.25–1.00 equiv of Cp_2HfCl_2 (Figure 3c), and only minor broad new resonances (10%) were detected with 1.00 equiv of Cp_2ZrCl_2 (Figure 3d). After the addition of 2 equiv of these metallocenes to each nucleotide, multiple new signals were observed (spectra not shown), consistent with the formation of a complex mixture of species, which were not assigned (supplementary material). When the pD of the final solution was raised to ~7, only signals due to the free nucleotide were detected.

Effect of the Halide Ligand. The effect of the halide ligands on complexation of metallocene dihalides in the series Cp_2TiX_2 (X = F, Cl, Br, I) with DNA subunits was studied. Marks *et al.* have reported that for Cp_2TiCl_2 and Cp_2MoCl_2 in water, loss of the first chloride is extremely rapid and the second chloride is readily replaced, particularly if an appropriate ligand is present.[9,10] The pH of equimolar aqueous solutions of Cp_2TiX_2 have been reported to be 3.6 (F) and 2.7 (Cl, Br, I), respectively,[4] suggesting that the strength of the Ti–X bond is similar for X = Cl, Br, I but that F is more tightly bound. This data was confirmed in this work and the loss of the halide ligands was monitored by ^1H NMR spectroscopy. The Cp protons appear as a sharp singlet in Cp_2TiX_2. As each of the halide ligands is replaced [to give species such as $Cp_2Ti(OH)_x(H_2O)_y^{(2-x)+}$ or $Cp_2TiX_x(H_2O)_y^{(2-x)+}$ (x = 0, 1, 2)], new singlets at very similar chemical shift are observed. In none of the systems studied was any free Cp detected within a 12-h period. This data is consistent with suggestions that the active species in solution is the same for all titanocene dihalides ($Cp_2Ti^{2+}_{(aq)}$),[14] and hence similar coordination chemistry is expected within the series of metallocene dihalides Cp_2TiX_2.

Titrations of Cp_2TiX_2 with D-ribose-5′-phosphate showed, in each case, two new signals in the ^{31}P NMR spectrum (δ 1.1, 1.5 ppm) consistent with formation of phosphate (O) complex(es). In the ^1H NMR spectra, new sugar resonances and nonequivalent Cp resonances, with almost identical chemical shifts, were observed with each titanocene dihalide, suggesting that complexes of the same (or very similar) structures were formed in each case. In titrations with nucleobases and nucleobases, minor (5–10%) new peaks were observed in the spectra, suggesting that each of the titanocene dihalides coordinate to some extent to each of the bases, but complexation is comparatively weak.

Detailed experiments were undertaken with the four nucleotides, by titration of 0.25, 0.50, 1.00, and 2.00 equiv of an aqueous solution of Cp_2TiX_2 into each nucleotide. Figure 4 summarizes a typical series of ^1H NMR experiments with 5′-dAMP. Comparison of the spectra at each step during the titration for X = Cl, Br, I (columns 2–4, Figure 4) show that they are remarkably similar. Furthermore, integration of the spectra indicate that, within experimental error, the complexes were formed in approximately the same amounts during the titration for X = Cl, Br, I, and the similar chemical shifts and relative intensities of the new signals suggest that the complexes are structurally very similar. For each titanocene dihalide, very similar ^{31}P NMR spectra were also obtained with

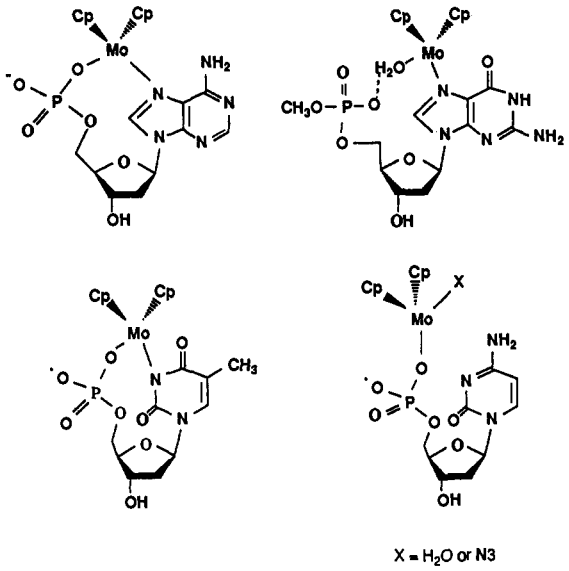

Figure 5. Complexes formed in aqueous solution between Cp_2MoCl_2 and nucleotides (taken from ref 9b).

each nucleotide. A similar result was obtained in titrations with each of the nucleotides (see the supplementary material). The combined ^{31}P and ^{1}H NMR spectra are consistent with rapid loss of the halide ligands from Cp_2TiX_2 (X = Cl, Br, I) to form $Cp_2Ti^{2+}_{(aq)}$, which coordinates to 5′-dAMP, giving rise to new signals in the NMR spectra.

The spectra obtained with Cp_2TiF_2 are noticeably different than those obtained with the other halide ligands. For example, after the addition of 1.0 equiv of Cp_2TiX_2 (Figure 4c), the spectra for X = Cl, Br, I (columns 2–4) are very similar and ~40% complexation has occurred. In contrast, for X = F (column 1, Figure 4c), the spectrum is different, and it is estimated much less complexation (~10–20%) has occurred. However, on addition of 2.00 equiv of Cp_2TiF_2 (column 1, Figure 4d), the new signals due to complex(es) are very similar to the signals due to the complex(es) in Figure 4c (columns 2–4, X = Cl, Br, I), obtained with only 1.00 equiv of Cp_2TiX_2. The spectra indicate that similar complex(es) are formed in each case, but in different amounts, for each of the titanocene dihalides which is consistent with the greater stability of the Ti–F bonds in Cp_2TiF_2 compared to the other halides X = Cl, Br, I.

The exact structures of the species formed in solution by Cp_2TiX_2 and nucleotides cannot be unambiguously assigned from the NMR data. However, the new signals in the ^{31}P NMR spectra, and the new aromatic resonances observed in the ^{1}H NMR spectra, are consistent with phosphate (O) complexation along with base (N) coordination at low pH (2–4). The complexes are most probably analogous to the well-characterized Mo adducts (Figure 5).[9] Comparison of the spectra obtained from addition of 1.00 equiv of Cp_2MoCl_2 and Cp_2TiCl_2 to each of the nucleotides (Figure 3a,b) shows that the chemical shifts of the aromatic protons in the newly formed complex(es) are very similar. However, the greater lability of the Cp–Ti bond most probably gives rise to complex(es) in which only one Cp ring is metal bound, unlike the Mo adducts in which both Cp rings remain metal bound in all the complexes (Figure 5). Due to the less stable nature of these titanocene derivatives, isolation and full characterization by X-ray crystallography will be difficult, and attempts to fully characterize these derivatives by us have been unsuccessful.

Effect of Buffers and Solvent. In order to assess the effect of other experimental conditions on the degree and mode of complexation, the interaction of Cp_2MoCl_2 with the four nucleotides was studied under a variety of conditions. The metallocene was dissolved to give the following solutions: aqueous solution (pH 3.7), aqueous solution (D_2O/NaOD, pH 6.5), DMSO solution (pH 4.6), HEPES buffer (pH 5.5), and Tris buffer (pH 5.7). Each of these solutions, as well as solid Cp_2MoCl_2, was titrated into 5′-dAMP, 5′-dGMP, 5′-dCMP, and 5′-dUMP, which resulted in the pH becoming lower, and the percentage complexation was estimated by integration of the aromatic resonances (full data in the supplementary material). These results showed that, within experimental error, approximately the same amount and type of complexes were formed regardless of the pH of the metallocene solution. In Tris buffer, ~20% less complexation was observed, and some coordination of the metallocene to the buffer is likely. Trial experiments carried out with phosphate buffer, monitored by ^{31}P NMR spectrsocopy, showed that all metallocenes bind to some extent to phosphate buffer in addition to the nucleotide phosphates.

The effect of solvent and buffers on the interaction of Cp_2TiCl_2 with 5′-dAMP was also examined. Within experimental error, titration of solutions with initial pH 1.5–5 gave similar complexation with 5′-dAMP. However, saturated saline resulted in a slight decrease in the amount of complex(es) formed, while in DMSO/saline mixtures, the amount of free Cp detected increased relative to the same experiment in water alone. Solutions with initial pH >6 gave no complexation, presumably due to the hydrolytic instability of the titanocene framework at this pH.[10]

Several studies of the interaction of titanocene dichloride with nucleosides and nucleobases in organic solvents (methanol, THF, toluene), in which the titanocenes are more stable, have been reported.[15] However, as noted by Marks and co-workers,[9b] extrapolation of such data to metallocene–nucleic acid interactions in water at physiological pH must be treated with caution. As discussed above, the stability and species present in aqueous solutions of the metallocene dihalides are highly sensitive to solution pH and solvent. While experiments were carried out in methanol and DMSO, and the NMR spectra showed similar trends to the results in aqueous solutions, this study focused on the aqueous chemistry of the metallocenes, in order to relate the results to the *in vitro* and *in vivo* antitumor properties.

Discussion

While the antitumor properties of the metallocenes are well-established,[1,2] several questions remain unanswered regarding their mechanism of antitumor action. This study addresses the importance of nucleic acid–metallocene interactions by a systematic study of the interaction of both antitumor active and inactive metallocenes with a range of DNA constituents. These results, taken together with the work of Marks *et al.*,[9–11] allow direct correlations between the antitumor activity of the metallocene dihalides and the adducts observed with nucleic acids to be made.

Two series of metallocenes were investigated. In the first series the central metal was varied in the metallocene dichlorides Cp_2MCl_2. As the loss of chloride by aqueous hydrolysis is expected to be rapid,[9,10] the major chemical

consequence of varying the central metal is rate of Cp hydrolysis. Within this series the stability of the Cp–M bond follows the trend Mo > V > Ti ≫ Zr > Hf, which can be monitored by observation of signals due to free cyclopentadiene.[9-11] In the pH range at which optimal cure rates are observed (pH 4–6),[1,2] the Cp rings in Cp_2MCl_2 (M = Ti, Mo, V) remain metal bound for at least 24 h. In contrast, significant amounts of free cyclopentadiene (~20%) are observed in freshly prepared solutions of Cp_2ZrCl_2 after 15 min, while extensive hydrolysis of the Cp rings occurs with Cp_2HfCl_2, and only ~40% of the Cp rings are metal bound after 15 min.

There are two biological aspects of this chemistry that need to be considered. Firstly, it is possible that the hydrolyzed Cp rings may have antitumor properties. However, while both cyclopentadiene and dicyclopentadiene effected cure rates against fluid Ehrlich ascites tumors comparable to the complexes, no significant growth inhibition of solid Ehrlich ascites tumors was observed by either hydrocarbon up to lethal doses.[2] Thus, the hydrocarbons do not exhibit systemic antitumor activity that is characteristic for the complexes and can be ruled out as the active species.[2] However, Döppert has suggested that slow Cp release may occur *in vivo* and that more experiments are needed to clarify the effect of Cp in these systems.[16] Secondly, it is noted that the species present in aqueous solutions of metallocene dihalides may not be the same as that in blood plasma as blood constituents may be able to stabilize transition metal complexes that would otherwise be hydrolyzed.[2] Indeed, titanocene dichloride, which undergoes rapid hydrolysis in aqueous solution at pH 7, is readily soluble in aqueous lipid emulsion, is not hydrolyzed, and retains its antitumor activity in this preparation.[2]

Both of the antitumor active metallocene dihalides containing the central metal M = Mo, Ti showed distinct phosphate (O)-centered complexes with nucleotides and also coordination to heterocyclic nitrogens. Formation of discrete phosphate-centered complexes between Cp_2VCl_2 and nucleotides has also been reported.[11] In contrast, no complexation between the biologically inactive metallocene dihalides (M = Zr, Hf) was observed with 1.00 equiv of each nucleotide, and the NMR spectra were dominated by signals due to Cp hydrolysis products. The lack of complexation of the metallocenes Cp_2ZrCl_2 and Cp_2HfCl_2 with nucleic acid constituents appears to be directly related to their hydrolytic instability; significant amounts of free Cp were detected for both these metallocene dihalides during the time-frame of a typical NMR experiment (5–60 min) and the initial aqueous solutions that were used in each titration contained significantly less $Cp_2M^{2+}{}_{(aq)}$ with the potential to coordinate to a nucleotide. Köpf and Köpf-Maier have also suggested that the inactivity of Cp_2ZrCl_2 is related to the enhanced hydrolytic lability of the Cp rings in this system.[2]

The second series of compounds studied varied the halide ligand in the series Cp_2TiX_2. The pH of aqueous solutions of Cp_2TiX_2 (2×10^{-3} M) have been reported to be 3.6 (X = F), 2.7 (X = Cl, Br, I),[2] and biological testing has shown that similar tumor-inhibiting properties occur for these compounds, suggesting that their mechanism of action is similar.[1,2] Our results also show a similar pattern, and by ^1H NMR spectroscopy, almost identical adducts were formed between nucleotides and Cp_2TiX_2 (Figure 4). The slight variations in the appearance of the spectra are most likely due to small differences in the solution pH. The combined chemical and biological results are totally consistent with formation of $Cp_2Ti^{2+}{}_{(aq)}$ as the active species in aqueous solution; this species, which is stabilized through coordination to DNA, will be formed by all titanocene dihalides. We also note that optimal cure rates are observed in the pH range 4–6,[4] and within this range the titanocene dihalides formed stable complexes with nucleotides. However, at higher pH, biological activity is diminished. The complexes formed at low pH (2–5) were also much less stable at pH values close to 7, and addition of NaOD to samples of Cp_2Ti^{2+}–nucleotide complexes resulted in almost complete disappearance of the signals due to the complexes, accompanied by hydrolysis of the metal-bound Cp rings. At pH 7, Cp_2TiCl_2 undergoes rapid hydrolysis of the Cp rings,[10] and as a result, the complexes formed at lower pH are disrupted. The effect of pH on complexation, and the similar adducts formed between Cp_2TiX_2 and nucleotides, suggests that Cp_2Ti^{2+}–nucleotide interactions are directly related to the antineoplastic properties of titanocene dihalides and that these properties arise via their interactions with DNA.

Finally the effect of buffers and pH on the degree of complexation was studied in view of biological tests that have been carried out under different conditions.[1,2] Our results showed only minor differences in the degree of binding of metallocene dihalides to nucleotides based on the pH or solvent of the injected solution, provided the pH <6. This agrees with experiments carried out on titanocene halides against solid Ehrlich ascites tumors. Both nonbuffered (DMSO/saline, pH 1.4–3.9) and buffered (DMSO/saline, $NaHCO_3$, 4.2–5.9) solutions achieved optimal dose–cure rates within the same time period.[4] The major influence of the initial solution pH was that drug-induced side effects were strikingly reduced by pH elevation in the buffered injected drug solutions.[4]

In summary, our results imply that metallocene–nucleotide complexation is directly related to the cancerostatic potency of the metallocene dihalides and suggest that provided the solution pH is such that Cp hydrolysis is minimal, complexation via Cp_2M^{2+} coordination will occur with DNA constituents. The biological tests with titanocene dihalides[4] indicate that in order to minimize side effects associated with administration of solutions of low pH, the solution pH should be increased to values approaching physiological. Our data suggests that the pH value must be such that metal–Cp ligation is maintained and DNA–metallocene adducts are stable.

Conclusions

The results of this work provide experimental data in support of the role of metallocene–DNA interactions in the antitumor activity of metallocene dihalides. All metallocenes in the series Cp_2TiX_2 formed similar adducts with DNA subunits. These adducts are stable in the pH range at which optimal cure rates are observed. This result is in good agreement with the similar biological activity of all titanocene dihalides and correlates well with $Cp_2Ti^{2+}{}_{(aq)}$ as the active species. The central metal has a major influence on the anticancer activity of the metallocenes, with a diagonal activity relationship within the early transition metals of the Periodic Table. This effect appears to have a sound chemical basis with the biologically active metallocenes observed to interact with DNA subunits to a far greater degree than the biologically inactive metal-

locenes. These results suggest that a valid approach to the rational design of metallocenes with improved biological activity must include consideration of metallocene–DNA interactions.

Experimental Section

The nucleosides, nucleotides, D-ribose-5'-phosphate, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) buffer and *N*-tris(hydroxymethyl)methylglycine (Tris) buffer were purchased from the Sigma Chemical Co. and were used as provided. Titanocene, molybdocene, zirconocene, and hafnium dichlorides were obtained from the Aldrich Chemical Co. Titanocene dibromide, difluoride, and diiodide were prepared from titanocene dichloride according to the literature procedures.[17,18] ^{1}H NMR spectra were recorded on a Bruker AC200 spectrometer (200.13 MHz) and were referenced to TSP (0.00 ppm). ^{31}P spectra were recorded on a Bruker AMX400 spectrometer (161.98 MHz) and were referenced to external neat trimethyl phosphite (140.85 ppm).

NMR Titration Experiments. A typical experiment involved dissolving the metallocene dihalide in D_2O (0.02 mmol, 0.5 mL) by sonication where necessary. The solution was titrated into a solution of the DNA subunit in D_2O (0.01 mmol, 0.5 mL). The pD and ^{1}H and ^{31}P spectra were recorded where applicable, after the addition of 0.25, 0.50, 1.00, and 2.00 equiv of Cp_2MX_2. The pD was adjusted to 6.5–7.5 after the addition of 2.00 equiv by addition of NaOD or DCl. The pD of the solutions were corrected to give the pH values by subtraction of 0.4.[18] In cases where precipitates formed, the samples were centrifuged prior to acquisition of the NMR spectra. Attempts to characterize the precipitates, which were insoluble in most solvents, were unsuccessful.

Measurement of Cp Hydrolysis. Each of the metallocene dichlorides Cp_2MCl_2 (M = Mo, Ti, Hf, Zr) was dissolved in D_2O, and NMR spectra were recorded immediately and at regular time intervals. The rate of protonolysis of the cyclopentadiene rings was estimated by integration of the signals due to C_5H_5D or C_5H_6 (δ 2.9 ppm)[9b] versus the metal-bound C_5H_5 signals. For Cp_2MoCl_2 and Cp_2TiCl_2, no free cyclopentadiene was detected in a 24-h period. The relative amount of free cyclopentadiene measured by integration (±10%) was Cp_2ZrCl_2, 5 min (<1%), 20 min (20%), 60 min (35%); Cp_2HfCl_2, 5 min (35%), 15 min (60%), 48 h (90%).

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Supplementary Material Available: Tables showing the variation of pH in titrations of metallocene dihalides with nucleotides and the extent of complexation of Cp_2MoCl_2 with nucleotides administered in different solvents and buffered solutions; NMR spectra showing the titration of Cp_2TiX_2 with all four nucleotides (6 pages). Ordering information is given on any current masthead page.

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