agreement with data reported for its benzoannulated analogue.[25c] **8,11-Dichloro[11-^{13}C][5]metacyclophane ([11-^{13}C]1b)**: ^{13}C NMR (62.89 MHz, $CDCl_3$). An enhancement of the signal assigned to C(11) (δ = 141.4 ppm) was observed.[15] Compound [11-^{13}C]**1b** rearranged either by acid or by irradiation into [^{13}C]**3b** as major product. **1,3-Dichloro-6,7,8,9-tetrahydro-5*H*-benzocycloheptene (3b)**: ^{13}C NMR (62.89 MHz, $CDCl_3$) δ 147 (s, C(5a)), 139.3 (s, C(9a)), 133.8 (s, C(1)), 128.8 (s, C(3)), 127.5 (d, J(CH) = 164.2 Hz, C(2)), 126.6 (d, J(CH) = 170.3 Hz, C(4)), 36.6 (t, $-CH_2-$), 32.7 (t, $-CH_2-$), 32.1 (t, $-CH_2-$), 30.2 (t, $-CH_2-$), 27.8 (t, $-CH_2-$), 26.8 (t, $-CH_2-$). The coupling constants of the oligomethylene bridge (J(CH)) could not be assigned unambiguously due to overlap of the signals. The signals of the aromatic part of **3b** were assigned on the basis of coupling patterns, intensity considerations, and additivity rules.[39] The ^{13}C-NMR spectrum of [^{13}C]**3b** was identical with that of **3b** except that, in the case of the acid-catalyzed rearrangement C(9a) (δ 139.3 ppm) and in the case of the irradiation, C(1) (δ 133.8 ppm) was enhanced.

Acknowledgment. We thank Dr. F. J. J. de Kanter for help with the measurement and interpretation of the NMR spectra. This investigation was supported (L.W.J.) by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

Appendix. ^{1}H-NMR Spectroscopy of 18

The easy access to **18** from **1b** allowed us to reinvestigate its spectral properties more thoroughly. In Table IV, the ^{1}H-NMR spectrum of **18** and assignments based on spectrum simulations are presented. The ^{1}H-NMR spectrum of the aliphatic protons could only be simulated by invoking additional long-range couplings and by assigning some vicinal coupling constants a value of 0 Hz, implying a dihedral angle between those protons close to 90°. This is supported by a MNDO calculation on **18** (see Supplementary Material); a satisfactory agreement is found between the MNDO structure of **18** and the X-ray crystal structure of its 5-carboxylic acid derivative.[28b] In line with the spectral analysis, both structures have dihedral angles between H(12)/H(22) and H(52)/H(42) close to 90°.

Supplementary Material Available: MNDO structure and a table of bond lengths and valence angles for **18** (1 page). Ordering information is given on any current masthead page.

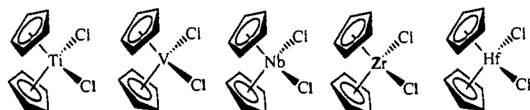
DNA-Metal Binding by Antitumor-Active Metallocene Dichlorides from Inductively Coupled Plasma Spectroscopy Analysis: Titanocene Dichloride Forms DNA-Cp_2Ti or DNA-CpTi Adducts Depending on pH

Mark L. McLaughlin,*,† John M. Cronan, Jr.,† Tamara R. Schaller,† and Ronald D. Snelling‡

Contribution from the Department of Chemistry and Basin Research Institute, Louisiana State University, Baton Rouge, Louisiana 70803-1804. *Received November 6, 1989*

Abstract: Inductively coupled plasma (ICP) spectroscopy is used to measure the DNA-metal binding of the antitumor agents Cp_2TiCl_2, Cp_2VCl_2, Cp_2NbCl_2, and *cis*-$(H_3N)_2PtCl_2$ and of Cp_2ZrCl_2 and Cp_2HfCl_2 in 10 or 110 mM sodium perchlorate with an initial phosphorus to metal ratio of 10:1. All the metals of these complexes bind DNA except vanadium from vanadocene dichloride. There is no release of metal when isolated DNA-metal adduct is dissolved in fresh 10 mM sodium perchlorate for up to 48 h. DNA binding studies using ^{3}H-labeled titanocene dichloride are consistent with a DNA-Cp_2Ti adduct at pH 5.3 and a DNA-CpTi adduct at pH 7.0. A DNA-titanium adduct is also formed from $CpTiCl_3$ and DNA in 10 mM sodium perchlorate with an initial phosphorus to metal ratio of 20:1.

The metallocene dichlorides, Cp_2TiCl_2, Cp_2VCl_2, Cp_2NbCl_2, and Cp_2MoCl_2, exhibit antitumor activity for a wide spectrum of murine and human tumors.[1,2] Similar screening tests reveal



sporadic antitumor activity for $CpTiCl_3$, Cp_2TaCl_2, and Cp_2WCl_2 and no antitumor activity for Cp_2ZrCl_2 and Cp_2HfCl_2.[3] Administration of titanocene dichloride or vanadocene dichloride causes cell gigantism and inhibits DNA synthesis more than protein synthesis; thus, it is reasonable that inhibition of replication is responsible for the antitumor activity of these compounds.[1e] The first hypothesis proposed to explain the antitumor activity of the metallocene dichlorides assumed the cytotoxicity resulted from the metallocene dichlorides binding with DNA via DNA-cisplatin-like adducts.[1e,4,5] This postulate was based on structural similarities noted for the most active metallocene dichloride antitumor agents and cisplatin. The pseudotetrahedral ligand geometries of these metallocene dichlorides and the square-planar geometry of cisplatin are very different, but the complexes have similar Cl-M-Cl bond angles. However, the aqueous chemistry of cisplatin and the metallocene dichlorides differ substantially. The first and second chloride hydrolysis rates for cisplatin are slower than those of Cp_2TiCl_2, Cp_2VCl_2, Cp_2ZrCl_2, and Cp_2MoCl_2.[6] Also, the ammine ligands of cisplatin are essentially inert to hydrolysis,[5] whereas the η^5-cyclopentadienyl ligands of the metallocene dichlorides hydrolyze with rates that depend on the central metal atom and pH. The relative η^5-cyclopentadienyl

† Department of Chemistry.
‡ Basin Research Institute.

(1) (a) Köpf, H.; Köpf-Maier, P. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 477-478. (b) Köpf-Maier, P.; Köpf, H. *Naturforsch., B: Anorg. Chem., Org. Chem.* **1979**, *34B*, 805-807. (c) Köpf-Maier, P.; Leitner, M.; Voigtländer, R.; Köpf, H. *Naturforsch., C: Biosci.* **1979**, *34*, 1174-1176. (d) Köpf-Maier, P.; Leitner, M.; Köpf, H. *J. Inorg. Nucl. Chem.* **1980**, *42*, 1789-1791. (e) Köpf, H.; Köpf-Maier, P. *ACS Symp. Ser.* **1983**, *No. 209*, 315-333. (f) Köpf-Maier, P.; Köpf, H. *Drugs Future* **1986**, *11*, 297-319.
(2) (a) Toney, J. H.; Rao, L. N.; Murthy, M. S.; Marks, T. J. *Breast Cancer Res. Treat.* **1985**, *6*, 185. Murthy, M. S.; Toney, J. H.; Rao, L. N.; Kuo, L. Y.; Marks, T. J. *Proc. Am. Assoc. Cancer Res.* **1986**, *27*, 279. (c) Murthy, M. S.; Rao, L. N.; Kuo, L. Y.; Toney, J. H.; Marks, T. J. *Inorg. Chim. Acta* **1988**, *152*, 117-124.
(3) (a) Köpf-Maier, P. Hesse, B.; Köpf, H. *J. Cancer Res. Clin. Oncol.* **1980**, *96*, 43. (b) Köpf-Maier, P.; Köpf, H. *Chem. Rev.* **1987**, *87*, 1137-1152.
(4) Rosenberg, B. *Cancer* **1985**, *55*, 2303.
(5) Sherman, S. E.; Lippard, S. J. *Chem. Rev.* **1987**, *87*, 1153-1181.
(6) Toney, J. H.; Marks, T. J. *J. Am. Chem. Soc.* **1985**, *107*, 947-953.

0002-7863/90/1512-8949$02.50/0 © 1990 American Chemical Society

ligand hydrolysis rates at neutral pH for some Cp_2M moieties are $Cp_2Zr > Cp_2Ti \gg Cp_2V > Cp_2Mo$.[6] The $Cp_2Ti(OH_2)_2^{2+}$ moiety is quite stable at pH <3, but at neutral pH, a Cp_2Ti species is expected to be short-lived. Döppert suggested that the antitumor activity of the metallocenes and particularly titanocene dichloride results from delivery of 1,3-cyclopentadiene.[7] However, this hypothesis appears to be incorrect since 1,3-cyclopentadiene and dicyclopentadiene, the Diels–Alder dimer of 1,3-cyclopentadiene, exhibit only unspecific cytotoxicity, do not effect the growth of solid tumors, and are much less toxic than titanocene dichloride.[8] The other η^5-cyclopentadienyl ligand hydrolysis product from the Cp_2Ti moiety, a CpTi moiety, oligomerizes in water at higher than dose level concentrations. Similar CpTi species form rapidly upon hydrolysis of $CpTiCl_3$, but the antitumor activity of $CpTiCl_3$ is less than that of titanocene dichloride.[9] The η^5-cyclopentadienyl ligand hydrolysis rates of vanadocene dichloride and molybdocene dichloride at neutral pH exhibit half-lives of 10 and 30 days, so delivery of 1,3-cyclopentadiene, DNA–CpV, or DNA–CpMo adducts are probably unimportant for these metallocenes.[6]

Model studies have failed to find that the Cp_2M moiety, where M = Ti, V, or Mo, chelates adjacent N7-guanine sites like cisplatin.[10-13] Mononucleotides complex the Cp_2Ti moiety in methanol via a N–Ti ligation,[11] but there was *no* evidence that analogous Cp_2Ti–nucleotide complexes form in water, although this binding has been shown with the hydrolytically more stable Cp_2Mo moiety.[13] Electron energy loss spectroscopy (EELS) of cell cross sections revealed an accumulation of titanium in cell areas containing DNA after administration of titanocene dichloride; thus, it was reasonable that a Ti species interacts with DNA.[14] But, a DNA–metal adduct had not been shown for any of the metallocene dichlorides.

This paper shows that DNA–metal adducts form upon treatment of unbuffered DNA solutions with titanocene, zirconocene, hafnocene, and niobocene dichlorides. DNA–Ti binding is measured as salt concentration and pH (5–7) are varied, but for the other metallocenes, only salt concentration is varied. We use inductively coupled plasma (ICP) spectroscopy to measure the DNA–metal binding, which simultaneously determines the phosphorus and metal concentrations. This technique could have broad application to measuring the nonlabile DNA binding of drugs, carcinogens, or other compounds that contain elements that do not occur in DNA. The detection of mononucleotides via ICP monitoring for phosphorus had been reported previously.[15]

We also report the results of tritium-labeled titanocene dichloride–DNA binding studies that are consistent with a DNA–Cp_2Ti adduct at pH 5.3 and DNA binding of the partially hydrolyzed CpTi moiety at pH 7.0.

Experimental Section

Materials and Methods. Salmon testes DNA (Sigma, sodium salt, type III) was dissolved in 10 or 110 mM sodium perchlorate by gentle swirling overnight and for some experiments was purified by ethanol precipitation before use. Similar metal binding results were found for either DNA sample. Concentrations of phosphorus from ICP analysis agreed within experimental error (3–6%) with the nucleotide concentration determined by UV absorbance using $\epsilon_{260} = 6600\ M^{-1}\ cm^{-1}$. This indicated that these DNA samples do not contain significant concentrations of inorganic phosphate as an impurity. ICP analysis showed that the DNA samples contain no measurable levels of the metals used in this study. Calf thymus DNA (Sigma, sodium salt, type I) was phenol extracted and dialyzed four times with 10 mM sodium perchlorate before use.

Titanocene dichloride (Alfa), zirconocene dichloride, hafnocene dichloride, niobocene dichloride (Aldrich), *cis*-diamminedichloroplatinum (Johnson Mathey), cacodylic acid, sodium cacodylate, MES, MES sodium salt, (Sigma), sodium perchlorate (Fischer), and 0.91 mCi/10 μL tritiated water (NEN) were used as received. Vanadocene dichloride and cyclopentadienyltitanium trichloride were synthesized and freshly recrystallized according to published methods.[16] Molecular sieves were activated by heating to 200 °C at 0.4 mmHg for several hours. The synthesis of tritiated titanocene dichloride was performed in a hood. The digestions of tritiated titanocene dichloride treated DNA were conducted in sealed vessels with a minimum of headspace and worked up in a hood.

ICP measurements were made on a Perkin-Elmer ICP-6500 spectrometer using a rf power of 1400 W, reflected power of <25 W, and gas flow rates of 14 L/min plasma, 1.1 L/min nebulizer, and 2.0 L/min auxiliary. Sample uptake rate was 0.8 mL/min and viewing height was 15 mm above the load coil. Wavelengths (nm) used were P, 213.618; Ti, 334.941; V, 290.882; Pt, 214.423; Zr, 343,823; Hf, 277.336; Nb, 309.418. Standard solutions (Spex) of the elements were diluted to prepare calibration curves for each element. UV measurements were made on a Cary 219 UV/vis spectrophotometer. Homogeneous solutions were prepared in Packard Ultima Gold scintillation fluid and were counted on a Packard A300C liquid scintillation counter.

DNA Binding Assay. The experiments were run in 10 or 110 mM sodium perchlorate except as noted below. The final pH of DNA plus the metal complex solutions ranged between 5.0 and 6.0. DNA solutions of 0.80–1.60 mM nucleotide, measured by ICP for phosphorus and by UV for nucleotide concentration, were mixed with the solid metal complexes of Cp_2TiCl_2, Cp_2VCl_2, Cp_2ZrCl_2, Cp_2HfCl_2, or Cp_2NbCl_2 or standard solutions of cisplatin to prepare solutions that contained approximately 10:1 phosphorus to metal ratios. Solid sample dissolution was facilitated by sonication in an ultrasonic bath at room temperature for the first 2–15 min. Aliquots (1 mL) of the resulting solution were transferred to 5-mL centrifuge tubes. The phosphorus and metal concentrations of the initial solution were determined. After incubation from 5 to 10 000 min, the DNA was precipitated at 0 °C with 2 mL of 95% ethanol saturated with sodium acetate. The suspensions were cooled at −20 °C for 20 min and pelleted by centrifugation for 5 min, and the supernatant was decanted. The pellets and the aliquots of the initial solutions were digested in 2–4 mL of 5% ammonium hydroxide for at least 4 h and the phosphorus and metal concentrations were determined by ICP. Concentrations were determined at least in triplicate for each aliquot, and the values for three identically treated aliquots were averaged. The relative metal to phosphorus ratios of the aliquots were related to the initial metal to phosphorus ratios. This data treatment eliminated variation from DNA loss due to incomplete precipitation of DNA samples and during the supernatant removal.

Control Experiments for DNA Precipitation. No metals are detected by ICP analysis of identically treated aliquots that are void of DNA. Thus, the metallocenes or the partial hydrolysis products of metallocenes probably do not coprecipitate during the ethanol DNA precipitation. Additional washing of the metal-complexed DNA pellets with the ethanol solution or redissolution of the metal-complexed DNA pellets in more 10 mM sodium perchlorate and reprecipitation up to 48 h later gives the same metal binding values. The redissolved DNA–metal adduct solutions were pH 6.5–7.5. The DNA binding titanocene dichloride in 10 and 110 mM sodium chloride gave similar results. Highly purified calf thymus DNA gave the same DNA–Ti binding results.

Tritiated Titanocene Dichloride. Freshly cracked 1,3-cyclopentadiene was collected and briefly stored at −78 °C with the exclusion of atmospheric moisture. A 100-mL dry argon filled Schlenk flask was charged with 20 mL of ethyl ether, a small stir bar, and 0.10 mL (1.2 mmol) of 1,3-cyclopentadiene. The stirred solution was cooled to 0 °C and 0.49 mL (1.2 mmol) of 2.5 M *n*-butyllithium in hexanes was added dropwise by syringe. The ice bath was removed and a white precipitate formed. After 2 h, a solution of 10 μL of tritiated water with a specific activity of 0.91 mCi/10 μL in 0.1 mL of distilled water was added, and the mixture was stirred for 5 minutes. The Schlenk flask was opened, and an additional 5 mL of water was added. Stirring was stopped, and the ether layer was separated and sequentially dried over magnesium sulfate and activated 4-Å molecular sieves. The ether solution was cooled to 0 °C, treated with 0.39 mL (0.97 mmol) of 2.5 M *n*-butyllithium in hexanes, and stirred for 2 h at room temperature. The suspension was cooled to −78 °C, 0.212 g (0.970 mmol) of $CpTiCl_3$ dissolved in a minimum

(7) Döppert, K. *J. Organomet. Chem.* **1987**, *319*, 351–354.
(8) Köpf-Maier, P.; Köpf, H. *J. Organomet. Chem.* **1988**, *342*, 167–176.
(9) Köpf-Maier, P.; Grabowski, S.; Köpf, H. *Eur. J. Med. Chem.* **1984**, *19*, 347.
(10) (a) Beauchamp, A. L.; Cozak, D.; Mardhy, A. *Inorg. Chim. Acta* **1984**, *92*, 191. (b) Cozak, D.; Mardhy, A.; Olivier, M. J.; Beauchamp, A. L. *Inorg. Chem.* **1986**, *25*, 2600. (c) Beauchamp, A. L.; Bélanger-Gariépy, F.; Mardhy, A.; Cozak, D. *Inorg. Chim. Acta* **1986**, *124*, L23.
(11) Pneumatikakis, G.; Yannopoulos, A.; Markopoulos, J. *Inorg. Chim. Acta* **1988**, *151*, 112–128.
(12) Toney, J. H.; Brock, C. P.; Marks, T. J. *J. Am. Chem. Soc.* **1986**, *108*, 7263–7274.
(13) Kuo, L. Y.; Kanatzidis, M. G.; Marks, T. J. *J. Am. Chem. Soc.* **1987**, *109*, 7202–7209.
(14) Köpf-Maier, P.; Krahl, D. *Naturwissenschaften* **1981**, *68*, 273–274.
(15) Heine, D. R.; Denton, M. B.; Schlabach, T. D. *Anal. Chem.* **1982**, *54*, 81–84.
(16) (a) Wilkinson, G.; Birmingham, J. M. *J. Am. Chem. Soc.* **1954**, *76*, 4281. (b) Gorsich, R. D. *J. Am. Chem. Soc.* **1960**, *82*, 4211–4214.

volume of dry THF (~20 mL) was added via cannula, and the cooling bath was removed. After being stirred for 12 h, the dark red solution with a brown solid suspension was cooled to 0 °C, and 1 mL of 12 M hydrochloric acid was added. The organic phase was diluted with methylene chloride to dissolve the remaining brown solid, and calcium chloride was added to dry the solution. Solvent removal gave a red solid that was recrystallized twice from hydrogen chloride saturated refluxing chloroform. The ^{1}H NMR and the melting point are the same as those of authentic titanocene dichloride. The isolated yield was 0.048 g (20%).

Specific Activity of Tritiated Titanocene Dichloride. A 1-mL volumetric flask was charged with 0.82 mg of tritiated titanocene dichloride and dissolved in dry hydrogen chloride saturated chloroform. Aliquots (10 μL) were placed in scintillation vials and solvent was evaporated with a slow argon flow. The red solid samples were dissolved in 20 mL of scintillation fluid and counted. A specific activity of 0.020 ± 0.003 mCi/g is found for the titanocene dichloride.

Tritiated Titanocene Dichloride Reaction with DNA. The complexation and precipitation steps were done as described above except using purified salmon testes DNA and washing the pellets once with 1 mL of ethanol-sodium acetate solution. Also, the DNA-metal pellet digestion procedure was modified because a precipitate formed when the scintillation fluid was added. The pellets instead were dissolved in 0.25 mL of concentrated ammonium hydroxide for 1 h and then diluted to 2 mL with water. Aliquots (0.25 mL) of these solutions and (1.00 mL) of the supernatant solutions were made to approximately 20-mL total volume with scintillation fluid and counted. Background counts from the scintillation fluid were subtracted. The remainder of the DNA digest was used to determine the DNA concentrations and the DNA-metal binding by the ICP methods described above.

Cyclopentadienyltitanium Trichloride Reaction with DNA. The general method described above applies to these experiments except that solid $CpTiCl_3$ or a freshly prepared (5-10 min from start of sonication to complete dissolution) solution of $CpTiCl_3$ in 10 mM sodium perchlorate was added to prepare a solution with 20:1 phosphorus to titanium ratio. These solutions also remain homogeneous until ethanol addition.

Results and Discussion

ICP Measurements of Metal-DNA Binding. The ICP analysis described in the Experimental Section allows direct measurement of both phosphorus and metal concentrations. The phosphorus concentration is proportional to the DNA nucleotide concentration in the absence of inorganic phosphate and the metal concentration is proportional to the metallocene concentrations if the η^5-cyclopentadienyl ligands are bound to the metals. UV measurements of DNA concentration after metal binding are not reliable for these experiments because the absorption maximum of the diaquo complex of titanocene, $Cp_2Ti(OH_2)_2^{2+}$, overlaps with the DNA absorption maximum at 260 nm.[17]

In the beginning, we used DNA binding conditions that favor the DNA-Cp_2M adducts, even for the hydrolytically unstable Cp_2Ti moiety. The pH of the unbuffered DNA solutions during metal complexation varied from 5.0 to 6.0. The pH-dependent rate of depurination of DNA calculated from an Arrhenius plot of measurements from 45 to 70 °C gives a rate constant of 2×10^{-6} min^{-1} at pH 5 and 25 °C, so depurination is very slow compared to DNA-metal binding.[18] However, it is possible that metal binding can facilitate depurination. We could not monitor depurination because the purines, some aquated metallocenes, and unprecipitated DNA fragments absorb in the same region of the UV.

The metal to phosphorus ratios are normalized by dividing the metal to phosphorus ratio by the initial metal to phosphorus ratio of the unprecipitated solution to give a percentage of the total metal bound to DNA. We use the relative metal to phosphorus concentrations to eliminate errors due to product loss. This percentage is a useful way to present the data for the time studies since many of these metals bind DNA quantitatively. Because the initial phosphorus to metal ratio was held at 10:1, the fraction of nucleotides bound (FNB) can be calculated by multiplying the percentages by 1.0×10^{-3} FNB/%.

Figures 1 and 2 show titanium binding from titanocene dichloride as salt concentration and pH are varied and cisplatin binding determined by ICP analysis. Figure 3 shows similar

(17) Cronan, J. M., Jr., unpublished findings.
(18) Lindahl, T.; Nyberg, B. *Biochemistry* **1972**, *11*, 3610-3618.

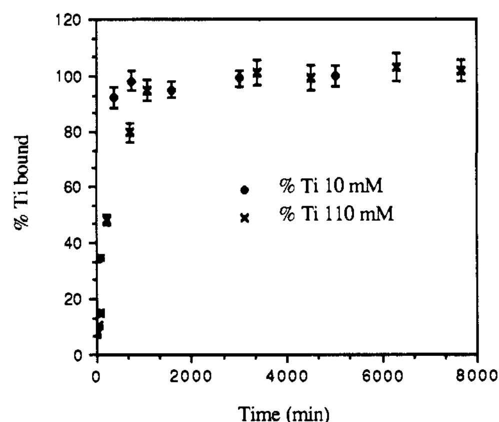


Figure 1. DNA-Ti binding in 10 and 110 mM sodium perchlorate with 10:1 phosphorus to titanium ratios.

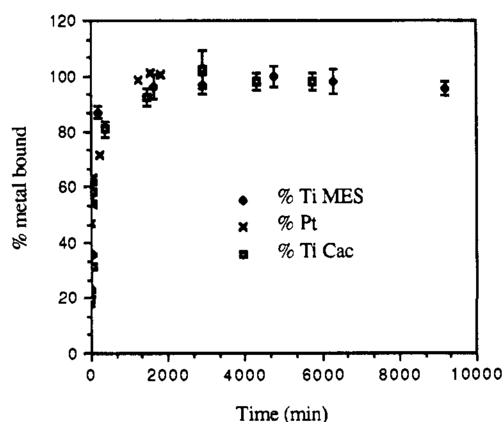


Figure 2. DNA-Ti binding in 20 mM MES buffer (pH 6.0), DNA-Ti binding in 20 mM cacodylate buffer (pH 7.0), and DNA-Pt binding in 10 mM sodium perchlorate with 10:1 phosphorus to metal ratios.

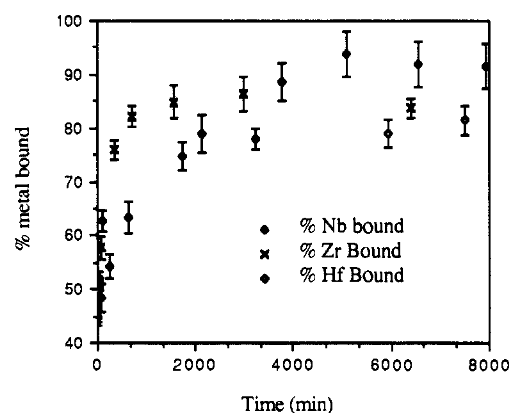


Figure 3. DNA-Nb binding in 110 mM sodium perchlorate, DNA-Zr binding in 10 mM sodium perchlorate, and DNA-Hf binding in 10 mM sodium perchlorate with 10:1 phosphorus to metal ratios.

DNA-metal binding experiments for zirconocene, hafnocene, and niobocene dichlorides.

Our results show that DNA-metal adducts form when ~1 mM DNA (nucleotide concentration) is treated with titanocene, zirconocene, hafnocene, or niobocene dichloride in either 10 or 110 mM sodium perchlorate with an initial phosphorus to metal ratio of 10:1. We have found no evidence that vanadium from Cp_2VCl_2 covalently binds with DNA using this protocol. The experiments with vanadocene dichloride were done without inert gas purging of the aqueous solutions. Vanadocene dichloride is somewhat susceptible to air oxidation; however, it seems unlikely that this accounts for the lack of binding because niobium derived from niobocene dichloride is also susceptible to air oxidation[1d] and binds under these conditions. Our results showing no vanadium binding are consistent with the findings of Toney and Marks. They

Table I. Tritium-Labeled Titanocene-DNA Binding at pH 5.3

aliquot	counts in digest[a]	counts in supernatant[a]	% ^3H in digest[b]
1	3133	241	92.8
2	1789	128	93.3
3	1147	117	90.7
4	1773	113	94.0
5	1648	109	93.8

[a] These are cpm above the background. [b] The digest should contain ~95% of the counts for the DNA-Cp_2Ti adduct, ~47.5% for the DNA-CpTi adduct, and ~0% for a DNA-Cp_0Ti adduct.

Table II. Tritium-Labeled Titanocene-DNA Binding at pH 7.0

aliquot	counts in digest[a]	counts in supernatant[a]	% ^3H in digest[b]
1	133	181	42.4
2	101	121	45.5
3	141	141	50.0
4	85	157	35.1

[a] These are cpm above the background. [b] The digest should contain ~100% of the counts for the DNA-Cp_2Ti adduct, ~50% for the DNA-CpTi adduct, and ~0% for a DNA-Cp_0Ti adduct.

reported only a labile interaction of $Cp_2V(OH)_2$ with mononucleotides, possibly a hydrogen-bonding interaction with a phosphate.[12] If the $Cp_2V(OH)_2$ is hydrogen bound to a DNA phosphate, it would probably dissociate during ethanol precipitation.[19]

The rate of DNA-metal binding was slowed somewhat upon increasing the salt concentration from 10 to 110 mM sodium perchlorate. The DNA-cisplatin binding results are similar to those determined by other methods, providing a check of our technique.[20] The DNA-metal adducts from zirconocene, hafnocene, and niobocene dichlorides bind less than titanium, with 80-95% of the total metal bound at a 10:1 phosphorus to metal ratio. It is clear from preliminary dose experiments, there are additional DNA binding sites available for DNA-Zr and DNA-Hf binding. DNA binding that is competitive with η^5-cyclopentadienyl ligand hydrolysis could account for the lower metal binding for zirconocene dichloride and hafnocene dichloride. The η^5-cyclopentadienyl ligand hydrolysis rates for hafnocene dichloride and niobocene dichloride have not been measured but it is reasonable that $Cp_2ZrCl_2 \approx Cp_2HfCl_2$ and that $Cp_2VCl_2 \approx Cp_2NbCl_2$.[6]

The DNA-metal binding is not readily reversible. The DNA-metal binding ratios do not change when the DNA pellet is redissolved in fresh 10 mM sodium perchlorate solution and reprecipitated, although the pH of these solutions were typically 7. Thus, the DNA-metal binding derived from the hydrolytically unstable Cp_2Ti and Cp_2Zr moieties appears to be stable once the adduct forms.

The DNA-Ti adduct from titanocene dichloride forms quantitatively in 20 mM MES buffer at pH 6.0 and in 20 mM cacodylate buffer at pH 7.0.

DNA-Cp_2Ti and DNA-CpTi Adducts. ICP analysis only measures the metal concentration; therefore, it provides information on molecular binding only by inference. This uncertainty is a particularly acute problem for titanocene dichloride, since η^5-cyclopentadienyl ligand hydrolysis could compete with DNA binding for titanocene dichloride. The titanium-labeled titanocene dichloride-DNA binding experiments determine the average number of η^5-cyclopentadienyl ligands that remain bound to the Ti after DNA complexation. Table I shows the radioactive counts for the precipitated DNA-Ti adduct digest and the supernatant after DNA binding of tritiated titanocene dichloride for 48 h at pH 5.3. The average ratio of the cpm for the digest to the total counts gives 0.93 ± 0.01. The DNA-Ti binding is complete ($98 \pm 6\%$) and DNA concentration in the digest is $95 \pm 2\%$ of the initial DNA concentration according to ICP measurements. The digest should contain ~95% of the tritium if both of the η^5-cyclopentadienyl ligands remain bound to titanium, ~47.5% is one of the η^5-cyclopentadienyl ligands remains bound, and ~0% is no η^5-cyclopentadienyl ligands remain bound to the metal. Thus, 90-100% of the titanium binding is the result of DNA-Cp_2Ti binding at pH 5.3.

Table II shows the radioactive counts for the precipitated DNA-Ti adduct digest and the supernatant after DNA binding of tritiated titanocene dichloride for 48 h at pH 7.0. The DNA-Ti binding is complete ($101.9 \pm 0.9\%$) and the DNA concentration in the digest is $101 \pm 3\%$ of the initial DNA concentration according to ICP measurements. Therefore, the ratio of counts in the digest to the total counts in the digest and supernatant should be 0.5 for the DNA-CpTi adduct. The average ratio of the cpm for the digest to the total counts is 0.43 ± 0.06. The value lower than 0.5 indicates a small amount of DNA-Cp_0Ti binding. However, slightly greater scintillation quenching in the digest probably accounts for the lower than ideal experimentally determined value and the data support the premise that the dominant species binding DNA at neutral pH is the CpTi moiety. 1,3-Cyclopentadiene is soluble to at least 4.0 mM under similar conditions,[6] and the possible DNA intercalation by the hydrocarbon is too labile to withstand DNA precipitation with ethanol-sodium acetate.[19]

ICP analyses provides independent although less direct evidence that a DNA-CpTi adduct can form. Cyclopentadienyltitanium trichloride as it dissolves in aqueous media rapidly dissociates chlorides to presumably form the same species expected from titanocene dichloride η^5-cyclopentadienyl ligand hydrolysis. DNA treatment with solid $CpTiCl_3$ gives $60 \pm 7\%$ DNA-Ti binding of the total titanium present after 5 min and for up to 5 days later. However, if the $CpTiCl_3$ is dissolved in 10 mM sodium perchlorate and within 5-10 min mixed with DNA, then there is *no* DNA-Ti binding. This could mean that complete hydrolysis to a CpTi trioxide oligomer inhibits DNA complexation.

Conclusions

Covalent DNA-metal binding by the antitumor-active metallocene dichlorides has been speculated for many years. The DNA-metal binding with titanocene, zirconocene, hafnocene, and niobocene dichlorides and cyclopentadienyltitanium trichloride has been measured by using ICP analysis in unbuffered solutions and at pH 5-7 for Cp_2TiCl_2. The DNA-metal adducts once formed are stable at neutral pH for up to 48 h. Tritium-labeled titanocene dichloride-DNA binding studies are consistent with a DNA-Cp_2Ti at pH 5.3 and a CpTi-DNA adduct at pH 7.0. On the basis of these results, the DNA-Cp_2Ti adduct cannot be ruled out as the molecular interaction that is responsible for the antitumor activity of titanocene dichloride, since serum components could stabilize the Cp_2Ti moiety against hydrolysis at physiological pH.[1e,6] However, it is clear that further studies that probe the DNA-CpTi adduct are warranted. It is possible that the nonlabile DNA-metal adduct derived from Cp_2TiCl_2, Cp_2ZrCl_2, Cp_2HfCl_2, or Cp_2NbCl_2 has no correlation with antitumor activity since vanadium derived from the active vanadocene dichloride fails to covalently bind DNA, whereas zirconocene dichloride and hafnocene dichloride do bind DNA and are inactive as antitumor agents. However, there is no reason to assume all the metallocene dichlorides should have similar modes of antitumor activity, and the absence of antitumor activity for zirconocene dichloride and hafnocene dichloride correlates with poorer DNA-metal binding derived from those metallocene dichlorides in spite of conditions designed to optimize DNA binding.

Acknowledgment. We thank the Research Corporation, the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the LSU Council on Research, for support of this research. Thanks to Prof. Mary D. Barkley for many helpful discussions and to Mr. Steven M. Pomarico of Prof. Sue G. Bartlett's laboratory for the tritiated water.

(19) Barton, J. K.; Lolis, E. *J. Am. Chem. Soc.* **1985**, *107*, 708-709.
(20) (a) Ushay, M. H.; Tullius, T. D.; Lippard, S. J. *Biochemistry* **1981**, *20*, 3744-3758. (b) Ciccarelli, R. B.; Solomon, M. J.; Varshavsky, A.; Lippard, S. J. *Biochemistry* **1985**, *24*, 7533-7540.