Notes

Organometallic Anticancer Agents. 2. Aqueous Chemistry and Interaction of Niobocene Dichloride with Nucleic Acid Constituents and Amino Acids

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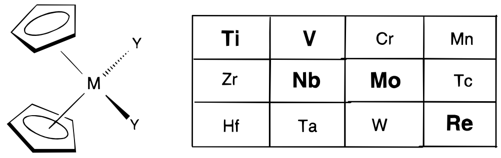
The solution chemistry and interactions of niobocene dichloride, Cp_2NbCl_2, with nucleosides, nucleotides, and amino acids has been studied by ^1H and ^{31}P NMR spectroscopy. The ^1H NMR spectrum of Cp_2NbCl_2 in 10% DMSO-d_6/D_2O contains two sharp singlets consistent with hydrolysis of the halide ligands and oxidation of the paramagnetic Nb(IV) complex to a diamagnetic Nb(V) complex. Cp_2NbCl_2 does not dissolve in the absence of oxygen, and $Cp_2NbCl_2(OH)$ and Cp_2NbCl_2 when dissolved in 10% DMSO/D_2O have identical ^1H NMR spectra, consistent with formation of a Nb(V) complex as the antitumor active species in solution. With all nucleosides and nucleotides, except adenosine-5′-monophosphate (dAMP), no evidence of binding was detected on addition of up to 2.0 equiv of niobocene dichloride. In the case of dAMP, a minor complex (10%) was detected with 0.5 equiv of Cp_2NbCl_2 (pH 5.2). No evidence for interaction of aqueous solutions of Cp_2NbCl_2 with glutamic acid, serine, or histidine was found. The lack of interaction of niobocene dichloride with nucleic acid constituents and amino acids is markedly different from the chemistry exhibited by antitumor active metallocenes Cp_2MCl_2 (M = Ti, Mo, V) and suggests that the mechanism of antitumor action of Cp_2NbCl_2 is significantly different from other metallocene dihalides.

Introduction

A variety of metallocene dihalides and pseudohalides, Cp_2MX_2 (M = Ti, V, Nb, Mo; X = F, Cl, Br, I, NCS, N_3, Figure 1), are known to be highly active anticancer agents against a number of tumor cell lines.[1−4] Most testing has focused on titanocene derivatives, and clinical trials of titanocene dichloride have commenced recently.[5] Initial results suggest a lack of experimental cross-reactivity with cisplatin and patterns of antitumor activity different from those produced by the platinum anticancer drugs. In common with cisplatin,[6] experimental results suggest that DNA is one of the intracellular targets of the metallocene dihalides.[7−9] However, the chemistry and hence mechanism of action of these organometallic anticancer agents is markedly different from that of cisplatin.

A number of studies on the interaction of metallocene dihalides with nucleobases, nucleosides, and nucleotides have been carried out in order to understand their mechanism of antitumor action at the molecular level.[10,11] Titanocene and molybdocene dichloride coordinate to both the nucleobase nitrogens and the phosphate oxygens of nucleotides, forming discrete complexes, while vanadocene dichloride shows only labile outer-sphere complexation to the phosphate groups.[10] Recently we reported a study of the interaction of both biologically active (Cp_2MX_2 M = Ti, Mo; X = Cl, Br, I) and biologically inactive metallocene dihalides (Cp_2MX_2 M = Zr, Hf; X = Cl) with nucleic acid constituents.[11] Taken with the data reported by other groups,[10] this study showed a correlation between the biological activity of these compounds and the strength and type of adducts formed with DNA constituents.



Ti	**V**	Cr	Mn
Zr	**Nb**	**Mo**	Tc
Hf	Ta	W	**Re**

Figure 1. Structure and position of central metals, indicated in bold, in antitumor metallocene complexes.

While titanocene and vanadocene derivatives have been extensively tested against a range of tumor cell lines,[1−4] there has only been limited testing of niobocene dichloride. This metallocene is an extremely potent cancerostatic agent against Ehrlich ascites tumor in CFI mice,[3] causing 100% cure rates but is accompanied by considerable side effects. Oxidation was reported to give a Nb(V) complex which had reduced toxic effects, but also diminished tumor-inhibiting properties.[3] Several ionic Nb(V) derivatives, $[Cp_2NbCl_2]X$ (X = BF_4, AsF_6, SbF_6, SO_3CF_3), have recently been studied and shown to have significantly reduced toxicity and an increased therapeutic range compared to the Nb(IV) complex Cp_2NbCl_2.[12]

This study extends our previous work on the effect of the central metal ion and the halide ligands of anticancer metallocene dihalides[11] to niobocene dichloride, and the aqueous chemistry and interaction of niobocene dichloride with nucleotides and amino acids are reported. The results obtained suggest that the mechanism of antitumor action of niobocene dichloride is vastly different from that of titanocene, molybdocene and vanadocene dichlorides.

Results

Solution Chemistry of Cp_2NbCl_2. Niobocene dichloride has very limited solubility in water, and extensive

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sonication and/or heating for extended periods of time is required to promote some dissolution. The complex has good solubility in DMSO, chloroform, methanol, and acetonitrile. In biological assays, niobocene dichloride was dissolved in 10% DMSO/saline with ultrasonic treatment for 5 min and injected immediately after dissolution.[3] Hence aqueous DMSO solutions were used for most of the studies in this work, in order to relate the chemical observations to the biological activity of the complex.[3]

The ^{1}H NMR spectrum of a freshly prepared solution of niobocene dichloride (40 mM) in 10% DMSO/D_2O (pH ~1.0) contained two sharp resonances (δ 6.65, 6.75 ppm) with a number of minor (<5%) resonances present. While some Nb(IV) halide complexes are known to be diamagnetic due to metal–metal bonding in the crystal lattice,[13a] niobium(IV) cyclopentadienyl complexes are paramagnetic[13] and hence would be expected to give rise to paramagnetic broadening in the ^{1}H NMR spectrum, in an analogous way to spectra obtained with vanadocene dichloride.[10b] The presence of two sharp peaks is consistent with metal being oxidized to a diamagnetic Nb(V) complex on dissolution. This interpretation was supported by sample preparation when oxygen was rigorously excluded. Under these conditions, Cp_2NbCl_2 did not dissolve, suggesting that dissolution is dependent on the oxidation of Nb(IV) to Nb(V) by dissolved oxygen. This oxidation/dissolution was accompanied by a change in color from dark green to yellow. A recent report showed that ultrasound treatment promotes the oxidation of Cp_2MoCl_2.[14] It is possible that Cp_2NbCl_2 ultrasound treatment, in addition to oxygen, promotes the oxidation and so the dissolution.

The presence of two singlets for the Cp resonances is consistent with hydrolysis of the halide ligand(s) in aqueous solution to give a Nb(V) complex in which the Cp rings are metal bound. A number of related Nb(V) complexes have been reported, including Cp_2NbCl_3, $Cp_2NbCl_2(OH)$, Cp_2NbOCl, and $(Cp_2NbCl)_2O$.[15] While these complexes are formed in organic solvents, it is unlikely that these species would exist in aqueous solution, and simple hydrolysis products in which both the halide ligands have been replaced by aquo or hydroxo ligands are most probably present.

For comparison, the model complex $Cp_2NbCl_2(OH)$ was prepared by the reaction of $NbCl_5$ with sodium cyclopentadiene in the presence of water.[16] The ^{1}H NMR spectrum in D_2O was identical to that of the hydrolyzed Cp_2NbCl_2 and confirmed the presence of a Nb(V) complex in aqueous solution. Mixtures of hydrolyzed Cp_2NbCl_2 and $Cp_2NbCl_2(OH)$ also gave rise to two sharp resonances in the ^{1}H NMR spectrum.

As in the case of Cp_2MCl_2 (M = Ti, Zr, Hf), the rate of hydrolysis of the metal-bound Cp rings of niobocene dichloride is highly pH dependent. The rate of hydrolysis was estimated at different pH values by integration of the metal-bound Cp resonances (δ 6.6–6.8 ppm) versus an internal standard (TSP). Initial experiments were carried out on 40 mM solutions in 10% DMSO/D_2O, but formation of significant amounts of precipitate with time made measurement of the NMR spectra difficult. The yellow insoluble precipitate was not further characterized but presumably is the binuclear oxo complex [Cp_2NbCl_2O] which has been previously reported.[15] Hence the hydrolysis experiments were carried out on less concentrated aqueous solutions (8 mM), which approximates the dose injection conditions used in recent studies.[12] At this concentration, addition of DMSO was not necessary to completely dissolve the complex, and sonication for 1 h gave a clear yellow solution.

The pH of a freshly prepared solution of Cp_2NbCl_2 (8 mM) is very acidic (pH ~1.6). The pH of these solutions was adjusted with NaOD to the desired pH value, and from integration of spectra, the half-lives for dissociation of the Cp rings were estimated to be 3 days (pH 1.8), 8–10 h (pH 4.0), 1–2 h (pH 4.8), and less than 15 min (pH 6.6). Small amounts of precipitate also formed in these experiments, and signals due to dicyclopentadiene were observed in solution, consistent with a decrease in the metal–Cp stability at high pH.

Interactions with Nucleic Acid Constituents. NMR spectra were recorded of titration experiments between Cp_2NbCl_2 with D-ribose-5′-phosphate, nucleosides, and nucleotides using procedures analogous to those previously reported. Freshly prepared solutions of Cp_2NbCl_2 in 10% DMSO-d_6/D_2O (pH 1.0) were used in these experiments, due to the problems of precipitation discussed in the previous section, that occur at higher pH values. These solutions were added to solutions of the nucleic constituents (10% DMSO/D_2O, pH 7–8), and the pH of the solution(s) was monitored throughout the experiments.

Addition of niobocene dichloride (1 equiv) to the sodium salt of D-ribose-5′-phosphate resulted in immediate formation of a precipitate. Precipitates were also produced when the metallocene solution was treated with perchlorate, iodide, or chloride salts, suggesting the formation of salts. Attempts to characterize these precipitates failed due to their poor solubility in a range of solvents. At low concentrations of niobocene relative to subunit some hydrolysis of the Nb–Cp bonds was observed, due to the change in solution pH. As more Cp_2NbCl_2 solution was added the pH lowered, and further hydrolysis of the Cp rings did not occur.

The interactions of Cp_2NbCl_2 with the four nucleosides and corresponding nucleotides (dAMP, dGMP, dCMP, dUMP) were examined in detail, with ^{1}H and ^{31}P NMR spectra and pH recorded after the addition of 0.25, 0.50, 1.00, and 2.00 equiv of Cp_2NbCl_2 to each solution. In all titrations, the solution pH dropped from an initial value of 7–8 in the absence of metallocene to <2.0 at the end of the titration. Blank experiments in which the pH of the nucleoside or nucleotide was varied were carried out in order to correct for changes in chemical shift that occur due to protonation of the nucleic bases. Precipitates also formed in the pH range 2–5. Samples were centrifuged and NMR spectra of the supernatant recorded.

For all four nucleosides, no evidence of any complexation was detected in the ^{1}H NMR spectra. A similar result was obtained with the nucleotides dGMP, dCMP, and dUMP. In the case of dAMP, a new minor complex (10% by integration) was observed after addition of 0.5 equiv of Cp_2NbCl_2(aq) (pH 5.2). This was evident from the appearance of two new signals for H3 and H8 which appeared in the spectra. Further addition of metallocene did not increase the intensities of the new signals due to the complex, and only signals due to dAMP were observed. Integration of the nucleotide signals versus an internal TSP standard showed that the precipitates that formed during the course of the titrations arose from hydrolysis of Cp_2NbCl_2 rather than from formation of insoluble nucleotide−metallocene complexes.

The ^{31}P NMR spectra were also recorded, but in contrast to studies with molybdocene dichloride where a downfield 40 ppm shift occurs on Mo−O−P coordination, no significant changes in the ^{31}P spectra were observed. However, it should be noted that this result does not entirely rule out any niobium phosphate(O) coordination. In the case of vanadocene dichloride and nucleotides, no change in the ^{31}P shift was observed, but broadening of the signal due to the paramagnetic center was consistent with phosphate coordination.

The interaction of the model complex $Cp_2NbCl_2(OH)$ with nucleotides was also studied. In contrast to Cp_2NbCl_2, $Cp_2NbCl_2(OH)$ is very soluble in aqueous solution. Identical spectra were obtained in titration experiments of $Cp_2NbCl_2(OH)$ with dAMP and dGMP to those obtained with Cp_2NbCl_2 and dGMP and dAMP. These results are consistent with formation of the same hydrolyzed Nb(V) complex in aqueous solutions of Cp_2NbCl_2 and $Cp_2NbCl_2(OH)$.

Interaction with Amino Acids. In view of the lack of interaction observed between niobocene dichloride and nucleotides, titration experiments were carried out also with serine, histidine, and glutamic acid as potential coordinating sites present in proteins. Recently, the characterization of several amino acid derivatives of titanocene dichloride have been reported,[17] albeit in organic solvents. Small, but significant, changes in the ^1H chemical shifts of the amino acid protons were observed on addition of Cp_2TiCl_2 and, along with an X-crystal structure, were taken as evidence for formation of amino acid adducts in which the carboxylate oxygens are coordinated to the metal center.[17]

In the case of niobocene dichloride, no evidence for the interaction of any of the amino acids with the metallocene was found. A DMSO/D_2O solution of Cp_2NbCl_2 was titrated into a solution of the amino acid (20 mM, pH 7.0), and NMR spectra were recorded after the addition of 1.0 and 2.0 equiv of the metallocene. For all the amino acids studied, the pH dropped as expected on addition of the metallocene and after 2.0 equiv the solution pH was ∼2.0. As in the case of the nucleotides, yellow precipitates formed during the titration. NMR samples were centrifuged and spectra of the supernatant analyzed. When compared to blank spectra of the amino aids at different pH values, there were no changes in the chemical shifts of any of the amino acid resonances, and the chemical shifts and relative intensities of the Cp resonances corresponded to spectra observed in hydrolysis experiments. Integration of the amino acid resonances versus an internal TSP standard were consistent with the yellow precipitate arising from hydrolysis of Cp_2NbCl_2 at different pH values.

Discussion

Studies of the solubility and aqueous chemistry of niobocene dichloride have shown that air oxidation of Nb(IV) to Nb(V) is required to dissolve samples of the complex in aqueous solution. Further oxidation of the complex occurs in solution over a period of days to give insoluble precipitates. In a report of the tumor inhibition studies of niobocene dichloride,[3] it was assumed that Nb(IV) was the active species present in solutions used for biological assays. The therapeutic efficacy and toxicity of Cp_2NbCl_2 solutions was also observed to diminish with time.[3] Samples administered after 6 days showed a decrease of the maximum cure rate to 67% and a reduction of the toxic side effects, especially intraocular haemorrhage, was observed. Oxidation over 6 days to give the biologically inactive complex $[Cp_2NbCl_2]_2O$ was proposed to account for these results.[3]

Our results are consistent with formation of a Nb(V) complex as the active species present in 10% DMSO/D_2O or D_2O. Over several days, slow hydrolysis of the Cp rings occurs (half-life 3 days). After 10 days a large amount of precipitate was present in the solution with no ^1H NMR resonances observable, suggesting further oxidation reactions generating complexes with low solubility. These results suggest that the reduction in both anticancer and toxicity effects[3] is due to degradation and/or further oxidation of the active niobium(V) species in solutions with time. The exact species formed with time could not be characterized due mainly to their poor solubilities.

Our studies with $Cp_2NbCl_2(OH)$ and nucleotides give results identical to studies with freshly prepared solutions of Cp_2NbCl_2. However, in both cases, no significant interaction of Cp_2NbCl_2 with nucleic acid constituents, under identical conditions to those previously reported,[11] was observed. This is in stark contrast to previous results which have shown a correlation between the interaction of antitumor active metallocene halides (M = Ti, Mo, V) and their complexation with DNA and nucleic acid constituents.[8−11] In particular, in the case of Cp_2TiCl_2 and Cp_2MoCl_2 (pH 2.0−5.0), formation of discrete adducts in which the metal is coordinated to both the phosphate oxygen and N7 of purines or N3 of thymidine was observed.[10] For Cp_2MoCl_2, these adducts, which were formed in titrations that resulted in acidic solutions, were also stable at pH 7.0 and in several cases were characterized in the solid state.[10c] Due to the greater lability of the Ti−Cp bonds, full characterization of the corresponding adducts formed with Cp_2TiCl_2 was not possible, but NMR data were consistent with the formation of adducts similar to those formed with Cp_2MoCl_2 at pH <5.0.[11] With these two metallocenes (M = Ti, Mo), the adducts were formed upon addition of less than 1.00 equiv of metallocene (i.e., initial pH 4−6)[10,11] and increased in concentration as further equivalents of metallocene were added.

The prime evidence that the metallocene dihalides interact with DNA as the cellular target comes from studies with Cp_2VCl_2 and Cp_2TiCl_2 which have shown that vanadium and titanium 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] The results for these two compounds appear to have been generalized in the literature to all metallocene dihalides. To our knowledge there is no independent biological data that has directly implicated molybdocene or niobocene dichloride in DNA interaction or damage. While there is strong chemical evidence that supports the interaction of molybdocene dichloride with DNA,[10a,c,11] the results of this study suggest that niobocene dichloride operates in an independent mechanism, and there is no chemical evidence for formation of stable DNA adducts under physiological conditions.

Given the very different aqueous and coordination chemistry that is exhibited by each member of the biologically active metallocenes (Figure 1), several authors have noted that it is not unreasonable to expect that the mechanism of antitumor action of each of the metallocene dihalides is different.[1,10] This is highlighted by the results of inductively coupled plasma spectroscopy experiments, which detected distinct adducts with the antitumor active metallocene dichlorides Cp_2TiCl_2 and Cp_2MoCl_2, but no adducts with Cp_2VCl_2, which is the most active metallocene *in vitro*.[18] DNA adducts were also detected with Cp_2ZrCl_2 and Cp_2HfCl_2, which show no anticancer activity. Our results with niobocene dichloride also reinforce this data and suggest that the mechanism of action of Cp_2NbCl_2 is quite different from that of other metallocene dihalides studied to date.

Finally, the complex $Cp_2NbCl_2(OH)$ has excellent aqueous solubility, and anticancer screening of this complex is of interest in view of the recent testing of four Nb(V) complexes $[Cp_2NbCl_2]X$, X = BF_4, AsF_6, SbF_6, CF_3SO_3, against Ehrlich Ascites tumors.[12] Especially in the case of the SbF_6 salt, the pharmacological and toxicological parameters were distinctly improved in comparison to Cp_2NbCl_2. It is unlikely that the anions directly contribute to antitumor activity, and variations in activity were attributed to differences in the physiochemical properties of the complexes. $Cp_2NbCl_2(OH)$ has excellent aqueous solubility, and the results of this study suggest that it will form the same active species as for the other Nb(V) complexes screened.

Experimental Section

The nucleosides, nucleotides, and D-ribose-5-phosphate were purchased from the Sigma Chemical Co. Niobocene dichloride was obtained from the Aldrich Chemical Co. and was used as provided. The complex $Cp_2NbCl_2(OH)$ was prepared according to the literature method.[14] ^1H NMR spectra were recorded on either a Bruker AC200 spectrometer (200.13 MHz) or a Bruker AMX400 spectrometer (400.13 MHz) and were referenced to TSP (0 ppm) as internal standard. ^{31}P spectra were recorded on a Bruker AMX400 spectrometer (161.98 MHz) and were referenced to external neat trimethyl phosphite (140.85 ppm).

Measurement of Cp Hydrolysis. Niobocene dichloride (1 mg, 0.04 mmol) was dissolved in D_2O (0.5 mL), 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 (6.6−6.8 ppm) or C_6H_6 (δ 2.9 ppm)[9b] versus the internal TSP standard. Some precipitation occurred with time, and for this reason TSP was used as the standard rather than the resonances due to the Cp signals.

NMR Titration Experiments. A typical experiment involved dissolving niobocene dichloride in 10% DMSO-d_6/D_2O (0.02 mmol, 0.5 mL) by sonication for 1−2 h. The solution was titrated into a solution of the DNA subunit in 10% DMSO-d_6/D_2O (0.01 mmol, 0.5 mL). The pD and ^1H and ^{31}P spectra were recorded after the addition of 0.25, 0.50, 1.00, and 2.00 equiv of Cp_2NbCl_2. The pD of the solutions were corrected to give the pH values by subtraction of 0.4.[18] Reference spectra of the relevant DNA subunit were recorded at the same pD values for comparison. Analogous procedures were used with solutions of amino acids (0.01 mmol, 0.5 mL), which were treated with 1.00 and 2.00 equiv of a 10% DMSO-d_6/D_2O solution of niobocene dichloride (0.02 mol, 0.5 mL).

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