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Review

Bioorganometallic chemistry of molybdocene dichloride

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Abstract

The study of molybdocene dichloride and related metallocenes has been dominated by their remarkable catalytic properties in organic synthesis and polymer chemistry. Interest in the aqueous, bioorganometallic chemistry of metallocene dihalides has stemmed from the potent antitumor properties of titanocene dichloride, including results from human clinical trials. This review will summarize key results reported in the last decade on the biological chemistry of molybdocene dichloride. The effect of concentration, pH and ionic strength on the rates of hydrolysis of both the cyclopentadienyl and halide ligands have established that the positively charged monoaquated species $Cp_2Mo(OH)(OH_2)^+$, in equilibrium with the dipositively charged dimer $Cp_2Mo(\mu\text{-}OH)_2MoCp_2$, is present under physiological conditions. Systematic studies of the coordination chemistry of Cp_2MoCl_2 with nucleobases, nucleotides, single-stranded and double-stranded oligonucleotides, and calf-thymus DNA have shown that while simultaneous phosphate(O) and heterocyclic(N) adducts are formed with nucleotides, negligible interaction with DNA occurs under physiological conditions. In contrast, Cp_2MoCl_2 forms strong, non-labile complexes with deprotonated thiols in amino acids. Molybdocene dichloride is able to catalyse the hydrolysis of activated phosphate esters under physiological conditions, but hydrolysis of unactivated phosphodiesters is only significant at pH 4. Limited antitumor activity results, inhibition studies with protein kinase C and topoisomerase II, structure–activity and cell-uptake studies have provided some insight into possible mechanisms of antitumor action.

Keywords: Molybdocene; Metallocene; Antitumor; Metal-based drug; Nucleotides; Thiol-complexes; DNA

1. Introduction

Molybdocene dichloride (Cp_2MoCl_2) (**1**) contains two η^5-coordinated cyclopentadienyl (Cp) ligands and two chloride ligands bound to molybdenum in the (IV) oxidation state in a pseudo tetrahedral environment. The preparation of this metallocene was first reported by Cooper and Green in 1964 [1,2] and X-ray analysis confirmed the structure of the complex [3]. The unit cell contains two molecules in which the Cp ligands are alternatively eclipsed and staggered and other key structural features are highlighted in Fig. 1. The interest in metallocenes in general has stemmed from their remarkable catalytic properties in organic synthesis and as polymerization catalysts [4–6]. As a result, the coordination chemistry and properties of these complexes has centred on their reactivity in inert, organic solvents.

In contrast, the aqueous, biological chemistry of metallocene dihalides has only been a subject of interest in the last 20 years. The potential application of metallocene dihalides as novel anticancer agents was first recognized in 1979 with reports that Cp_2TiCl_2 exhibited broad spectrum activity against a range of animal tumors [7]. Subsequent testing of a range of structurally related metallocene dihalides identified Cp_2MCl_2 (M = Ti, V, Nb and Mo) as cytotoxic agents with potential as anticancer agents [8–10]. Titanocene dichloride entered clinical trials in 1991, and phase I and phase II clinical

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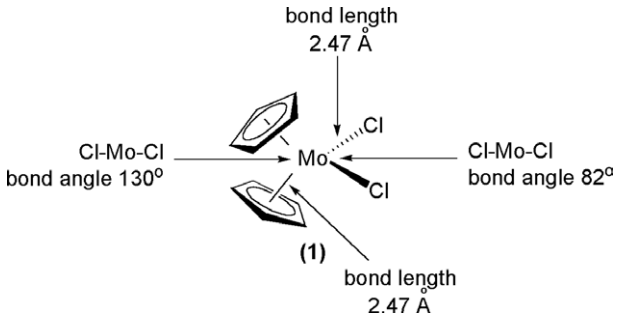

Fig. 1. The molecular structure of molybdocene dichloride, showing key features.

trials have been reported [11–15]. These studies have resulted in extensive structure–activity and mechanistic studies on Cp_2TiCl_2 in order to understand the mechanism of antitumor action, particularly as this metallocene is the first non-platinum metal complex to undergo trials [16]. Side-effects and profiles of activity that are distinct from organic drugs suggest that there is significant potential for the use of Cp_2TiCl_2 in combination therapy with other drugs [11–15,17]. These promising results have increased the interest in the potential of structurally related metallocenes as a new class of anticancer agents that exert their mechanism of action via distinct pathways to platinum drugs [11–16].

This review will focus on the bioorganometallic chemistry of Cp_2MoCl_2. It is now well-established that despite the structural similarity of the antitumor metallocenes (Cp_2MCl_2, M = Ti, V, Nb, Mo), the individual complexes exhibit unique biological chemistry, and antitumor properties are a result of distinct mechanisms of action [18]. In contrast to Cp_2TiCl_2, detailed studies on Cp_2MoCl_2 have emerged only in the last decade. Key results include detailed studies of the hydrolysis chemistry, interactions with nucleic acids and proteins, novel catalytic properties that may be relevant for cleavage of biomolecules, and structure–activity and mechanistic studies.

2. Hydrolysis chemistry

Molybdocene dichloride has poor solubility in water and organic solvents. Dissolution of the dark green solid in water results in an acidic solution (pH ~ 2), which is initially green, but rapidly changes to a deep maroon-red color. The rate of hydrolysis of both the Cp and chloride ligands has been studied. Of particular relevance to the bioorganometallic chemistry of Cp_2MoCl_2 are the effect of solution pH, concentration, and the presence of salt on the species present in solution.

The aqueous chemistry of Cp_2MoCl_2 has been well-characterized [19] and the key equilibria, and species that are formed are summarized in Fig. 2. The Cp ligands of Cp_2MoCl_2 were reported to be very stable to protonolysis and no evidence for formation of cyclopentadiene or dicyclopentadiene was observed in unbuffered solutions (10 mM, 37 °C) for up to 4 weeks [19]. At physiological pH (pD 7.4) the Mo–Cp bond was also stable for up to 2 weeks and high ionic strength conditions (100 mM NaCl) had no effect on Cp hydrolysis. This stability is an attractive feature of Cp_2MoCl_2 which has allowed experiments to be performed in aqueous solutions under approximately physiological conditions and contrasts sharply with the rapid hydrolysis of the Cp ligands in Cp_2TiCl_2, Cp_2ZrCl_2 and Cp_2HfCl_2 [20]. In the case of titanocene dichloride, this instability has hampered efforts to identify the active species in vivo, and has required the use of special formulations for administration.

In contrast, hydrolysis of the chloride ligands was measured to be very rapid, with more than half the chloride displaced by the time of dissolution of Cp_2MoCl_2 (Fig. 2(a)). The ratio of free chloride to Mo approached 1.95 within 60 min, in unbuffered solution [19]. The rate of chloride hydrolysis was unaffected by increasing the ionic strength with 0.318 mM KNO_3. At pH 7.4 the chloride hydrolysis was too rapid to measure. This contrasts with the chloride hydrolysis of both Cp_2VCl_2 [20] and cisplatin [22], which undergo much slower, incom-

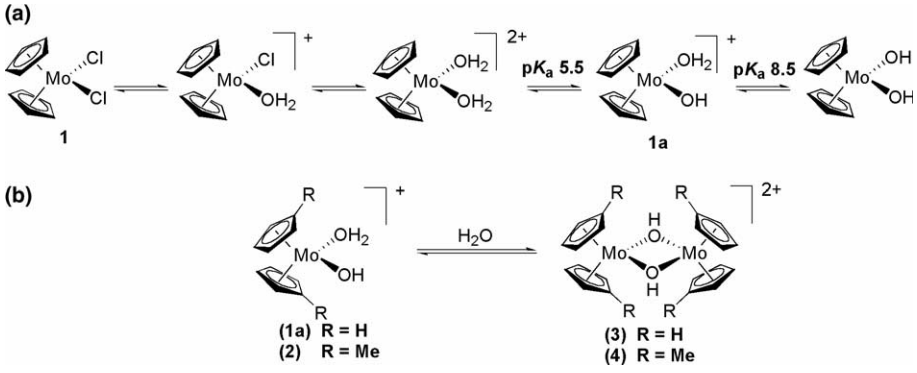

Fig. 2. Hydrolysis chemistry of Cp_2MoCl_2: (a) halide hydrolysis; (b) pH and concentration dependent equilibrium between aquated monomer and dimer.

plete hydrolysis under similar conditions. It has been suggested that **1** gives rise to the initial green color and that the maroon color is due to the aquated species **1a** [21].

Potentiomeric titrations of aqueous solutions of Cp_2MoCl_2 showed two deprotonations with pK_a (1) = 5.5 and pK_a (2) = 8.5 (Fig. 2(a)) [19], and therefore under physiological conditions, it was concluded that the monocation **1a** is the predominant species present. Independent support for the presence of **1a** at pH 7.4 was provided by crystallization of the BPh_4 salt of **1a** in high yield (77%) from an aqueous solution at this pH. Although crystals suitable for X-ray diffraction were not obtained, the isolated salt gave appropriate elemental analysis data in support of this structure.

Harding et al. [23] performed studies of the hydrolysis chemistry of Cp_2MoCl_2 in 50 mM salt, i.e., conditions required to stabilize DNA duplexes. Not surprisingly, the presence of high chloride concentrations affected the equilibria shown in Fig. 2(a), and on the basis of ^1H NMR spectra, it was proposed that an increased amount of species such as $Cp_2Mo(D_2O)Cl^+$, and $Cp_2Mo(OD)Cl$ were present. In addition, sharp downfield signals suggested that some Cp hydrolysis had occurred to give a species in which only one Cp ring is bound to the metal centre.

Later, independent studies have shown that the aqueous chemistry of Cp_2MoCl_2 is complicated by the reversible formation of dimeric species (Fig. 2(b)) [24–26]. Investigations on the catalytic properties of Cp_2MoCl_2, and results regarding the rate constants of catalysis, suggested that there may be a dimer–monomer equilibrium in solution [24]. Furthermore, molybdocene μ-hydroxo dimers were prepared from molybdocene dihydride and molybdocene ditosylate, in aqueous acetone [27,28], and hence formation of analogous dimers in water was considered. A study on the methylated derivate of Cp_2MoCl_2 confirmed the presence of a concentration-dependent equilibrium between two species. The methylated dimer $(MeCp)_2Mo(\mu\text{-}OH)_2Mo(MeCp)_2$ (**4**) was prepared from $(MeCp)_2MoH_2$ in aqueous acetone, and gave an identical NMR spectrum to a D_2O solution of $(MeCp)_2MoCl_2$ (**2**) [25]. Thus, regardless of the precursor used, two sets of identical Cp and Me peaks were detected, consistent with the formation of the same species in solution. Based on the hydrolysis chemistry put forward by Kuo et al. [19], an equilibrium between the monomer **2** and the dimer **4**, as shown in Fig. 2(b) was proposed [25].

Based on the results reported for the methyl derivative **2** (Fig. 2(b)), the presence of analogous species in aqueous solutions of Cp_2MoCl_2 (**1**) was re-examined [26]. This system was more complicated than the methylated system, due to the appearance of three Cp resonances in the ^1H NMR spectrum at neutral pD in D_2O. However, on the basis of changes in the ^1H NMR spectra with varying concentration and pH, the presence of the analogous equilibrium between **1a** and **3** (Fig. 2(b)) to that present in the methylated derivative was proposed.

The aqueous chemistry of Cp_2MoCl_2 is summarized in Fig. 2. Under physiological conditions, there is an equilibrium between the monomeric cationic species **1a**, and the dimeric μ-hydroxo species, **3**. The relative amounts of these two species are concentration, salt concentration, and pH dependent and can be detected by ^1H NMR spectroscopy; the cation **1a** gives rise to a singlet at δ 5.97 ppm while the signal arising from the dimer appears slightly upfield at δ 5.85 ppm. While Cp_2MoCl_2 has been reported to be air-sensitive and prone to oxidation to Mo(V) complexes, studies in our group have been performed without exclusion of oxygen [23,29–33], in order to mimic conditions that have been used for biological testing. These studies have shown that oxidation of Mo is not significant in aqueous conditions, and identical results have been obtained in solutions exposed to air and solutions in which air was rigorously excluded.

3. Coordination chemistry with nucleic acids

The antitumor activity of almost all clinically used chemotherapuetic drugs is related to interaction with DNA and/or DNA processing enzymes. Based on the fact that titanium and vanadium derived from Cp_2TiCl_2 and Cp_2VCl_2 accumulate in nucleic acid rich regions of tumor cells, and that these metallocenes inhibit nucleic acid synthesis, interaction with DNA was considered to be directly related to the antitumor properties of these metallocenes [34,35]. No corresponding cellular studies have been performed to establish where molybdenum derived from Cp_2MoCl_2 concentrates in cells and hence whether interaction of Cp_2MoCl_2 with DNA is related to activity. However, the potential for Cp_2MoCl_2 to form stable adducts with nucleic acids in vivo has been assessed by solution studies with nucleic acid building blocks (sugars, nucleic bases, nucleosides and nucleotides), complemented by NMR studies with oligonucleotides, and studies with bulk DNA.

3.1. Nucleobases, nucleosides and nucleotides

Kuo et al. [19] reported a comprehensive solution and solid-state study of the aqueous nucleobase and nucleotide coordination chemistry of Cp_2MoCl_2. In the case of the nucleobases, *N*-methyladenine and *N*-methylcytosine (*N*-methylated nucleobases were used to mimic the *N*-functionalized coordination environment in DNA), strained four-membered Mo(IV) cyclic chelates were formed by deprotonation of one amino proton and simultaneous coordination to both the amine (exo-)

and heterocyclic (endocyclic) nitrogen atoms of adenine **(5a)** and cytosine **(6)** (Fig. 3). The four-membered adenine chelate **5a** isomerized to the five-membered, thermodynamically favored product **5b**, after prolonged heating. The structures of the four-membered chelates were confirmed by X-ray crystallography. This chelation mode caused significant distortion in the bond angles of the complexes, including a 20° decrease in the L–Mo–L bond angle upon substitution of chloride for the nucleobase, and an 11° decrease in the N_{endo}–C–N_{exo} bond angle of the nucleobase.

In the case of the purine 2′-deoxynucleotides, 5′-dAMP and 5′-dGMP, changes in the ^{1}H and ^{31}P NMR resonances arising from the phosphate phosphorous and H8 of the nucleobases was consistent with coordination to the phosphate and N7 of the heterocycle [19]. This coordination mode was confirmed by X-ray crystal structure analysis of $[Cp_2Mo(5'dGMP)]_2$ (**8**) (Fig. 3). While Cp_2Mo^{2+} coordinated to the phosphate(O) and N7 in both purine nucleotides, the 5′-dGMP adduct **8** crystallized as a dimer (Fig. 3). The crystal structure showed significant changes in the puckering of the ribose sugar in $[Cp_2Mo(5'\text{-dGMP})]_2$ (**8**), believed to be due to steric constraint of the Cp ligands, or to geometrical constraints imposed by the dimeric structure. In the case of the pyrimidine nucleotides, 5′-dCMP and 5′-TMP, similar phosphate(O) coordination and heterocyclic N coordination was observed. Two complexes **10a** and **10b** were formed in a ratio of 2:1 with 5′-dCMP. These complexes were unable to be isolated and purified and in the case of **10b**, weak coordination to N3 cannot be ruled out. In contrast, a single adduct **9** was formed with 5′-dTMP. Competition experiments between nucleotides showed little or no selectivity of Cp_2MoCl_2 for any of the nucleotides and when an equivalent of a second nucleotide was added to a preformed Cp_2Mo(nucleotide) solution, a rapid equilibrium between the two species occurred.

These results were confirmed by Harding et al., who also showed that monodentate coordination between Cp_2MoCl_2 and ribose monophosphate occurred, to form Cp_2Mo(ribose monophosphate) **11** (Fig. 3), via the phosphate(O), as evidenced by the characteristic downfield shift of the phosphate in the ^{31}P NMR

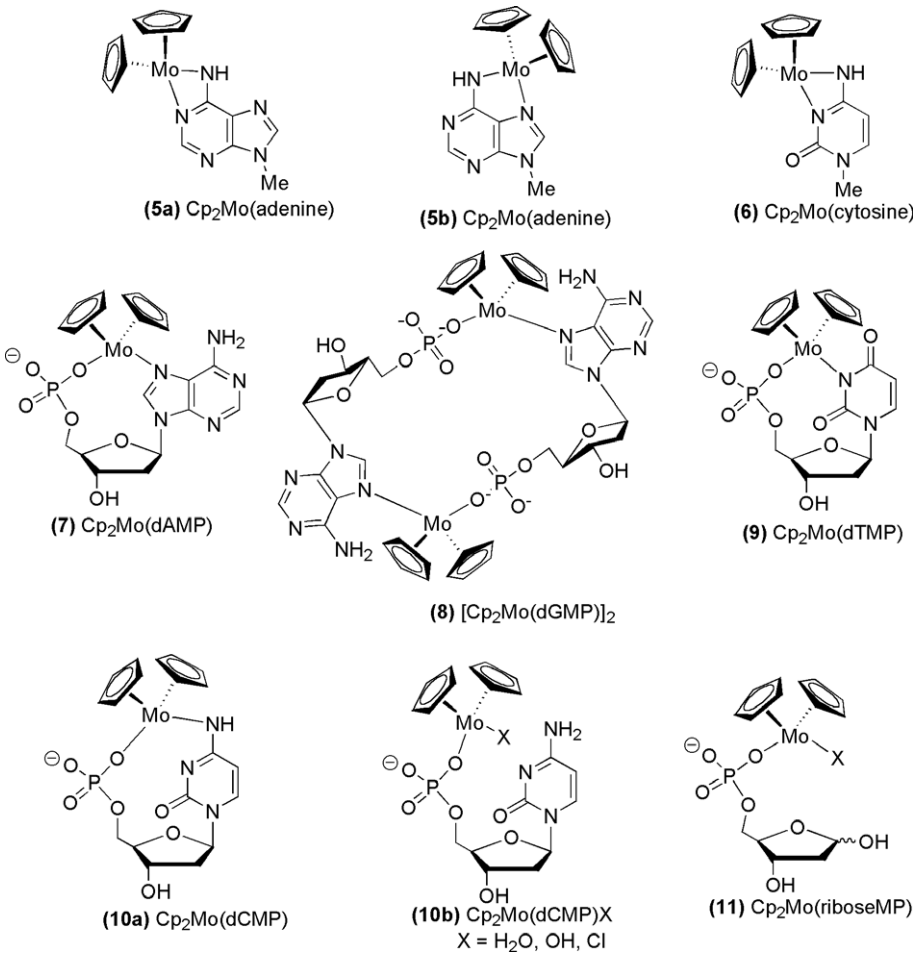

Fig. 3. Key coordination modes observed between Cp_2MoCl_2 and nucleic acid constituents.

spectrum [29,31]. Monodentate coordination was also seen between Cp_2MoCl_2 and nucleosides [29], although pure complexes were not isolated or fully characterized.

3.2. Oligonucleotides

While the complexes in Fig. 3 show that phosphate(O) and heterocyclic(N) atoms are potential coordination sites for Cp_2MoCl_2, on the basis of these results alone it cannot be concluded that analogous complexes will form with DNA. The steric accessibility of phosphate(O) coordination sites in a DNA duplex is significantly different to these sites in isolated nucleotides, and the heterocyclic(N) sites are located within the major and minor grooves. Simultaneous coordination of tetrahedral Cp_2Mo^{2+} to phosphate(O) and nucleic base(N) sites is not possible, as shown in Fig. 4(a), as it would require significant distortion of the DNA backbone to accommodate this binding mode. These steric effects are a direct consequence of the tetrahedral coordination geometry in Cp_2MoCl_2 and contrasts to square planar platinum(II) complexes which can easily slot into the major groove. For example, aquated cisplatin binds strongly to adjacent N7 atoms in guanine bases in the major groove [22].

Two independent NMR studies with oligonucleotides have confirmed that the coordination modes observed with nucleotides are not paralleled in oligonucleotides [23,36]. It is also significant that Cp_2MoCl_2 is the only antitumor metallocene that is hydrolytically stable in 50–100 mM NaCl at pH 6–7, i.e., conditions required to stabilize short duplex DNA. Thus, the molecular level nature of the interaction of Cp_2MCl_2 (M = Ti, V, Nb) with DNA has not been able to be studied by NMR analysis of oligonucleotides as these metallocenes hydrolyze and precipitate under these conditions [20,29,30,37].

Titration of Cp_2MoCl_2 (**1**) with the sequence d(pApGpGpCpCpT) which contains a 5′-phosphate in water (pH 7.0, no salt), showed that formation of phosphate centred complexes on the termini of the duplexes is possible [36]. More detailed studies by Harding et al. with oligonucleotide sequences that lack terminal phosphates were carried out in 50 mM salt [23]. Titration experiments were performed with the self-complementary 10-base pair sequence $d(CGCATATGCG)_2$ as a model of double-stranded DNA, and dATGGTA as a model for single-stranded DNA. The absence of any changes in the ^{31}P NMR spectra showed clearly that Cp_2MoCl_2 is unable to coordinate to the phosphate backbone, presumably as the salt effectively competes for these binding sites via electrostatic interactions. At pH < 4.0, conditions that favor single stranded oligonucleotides, new signals in the ^{1}H NMR spectra were consistent with formation of adducts between Cp_2MoCl_2 and the DNA bases (Fig. 4(b)). These adducts were unable to be isolated or characterized, and were only formed at low pH. It was concluded that the formation of stable metallocene-DNA adducts in vivo at pH > 6.0 is unlikely; however, the possibility that plasma constituents could stabilize complexes with DNA could not be ruled out [23].

3.3. Bulk DNA

The formation of stable molybdocene adducts with calf-thymus DNA has been detected using inductively coupled plasma (ICP) spectroscopy [39] and ^{31}P NMR spectroscopy [38]. However in each case different experimental conditions were used so direct comparison of the results and conclusions of each study needs to be treated with caution.

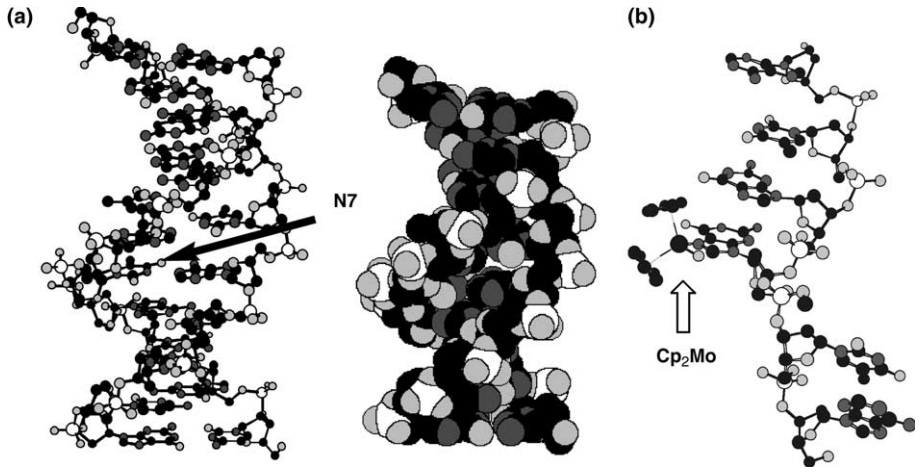

Fig. 4. (a) Computer generated view of duplex DNA $d(CGCATATGCG)_2$ highlighting the steric accessibility of heterocyclic(N) coordination sites in the major groove; and (b) proposed adduct formed between Cp_2MoCl_2 and single-stranded (ATGGTA) at low pH; only one conformation of the oligonucleotide is shown.

Direct support for the formation of molybdocene-DNA adducts has been provided using inductively coupled plasma (ICP) mass spectrometry in which the molybdenum content in DNA samples was measured. Calf-thymus DNA was incubated with a 100 mol equivalent of Cp_2MoCl_2 for 48 h. Dialysis through a 30,000 MW membrane allowed removal of unbound and hydrolyzed metallocene, and the DNA was digested and analyzed for Mo content by ICP [39]. While the results were not quantitated, in all experiments there was molybdenum associated with the DNA, relative to controls.

Treatment of sonicated calf-thymus DNA (~200 base pairs) with Cp_2MoCl_2 afforded a metallocene-DNA complex which was characterized by ^{31}P NMR spectroscopy [38]. In addition to the resonance for the phosphate backbone (δ–1.6 ppm), the spectrum contained 2 signals assigned to a phosphate bound Mo-DNA complex(es) (δ 37.2, 36.5 ppm) and a broad signal at δ 6.2 ppm. On the basis of these chemical shift changes, covalent attachment of Cp_2MoCl_2 to DNA via phosphate(O) coordination was suggested, with this binding accompanied by local distortion of the DNA backbone. However, in light of later studies with oligonucleotides, which show that binding to accessible phosphates at the end of the sequences [39], the observed chemical shift changes could also be explained by binding of Cp_2MoCl_2 to the terminal phosphates at the sheared ends of the sonicated DNA.

The above results appear to contradict the results from studies with oligonucleotides (Section 3.2) and highlight the danger in extrapolation of studies with nucleic acid constituents and short DNA fragments to bulk DNA. While studies with nucleotides and short oligonucleotides are extremely informative in providing a molecular level picture of drug–DNA interactions, and in many cases, results do correlate well with studies with bulk DNA, this is not always so. In the case of molybdocene (and titanocene) dichloride, stabilization of the small hydrophobic metallocene by bulk, folded conformations of DNA appears to occur. This is apparent from qualitative comparison of the solubility in the presence and absence of DNA and proteins (see Section 4). In the presence of the biomolecule more metallocene dissolves and remains soluble.

4. Coordination chemistry with proteins

4.1. Amino acids and peptides

A range of amino acid and related derivatives of Cp_2MoCl_2 have been reported that illustrate that diverse coordination modes involving the amino, carboxylate and side-chains are possible, and that the complex formed is highly dependent on the reaction conditions. Bidentate thiolate, phenolate, and amino complexes of Cp_2MoCl_2 formed in organic solvents [40,41], as well as metal–sulfur bond enthalpy calculations [42], support the possible formation of complexes with amino acids and proteins involving oxygen, nitrogen and sulfur donor ligands.

Gore and Green [43] reported the first syntheses of amino acid derivatives of molybdocene complexes (Fig. 5(a)). In general, bidentate coordination of non-polar amino acids to Cp_2Mo^{2+} occured, via the amine nitrogen and the carboxylate oxygen, forming a five-membered ring with a pendant side-chain (**12**). Of significance was the fact that in the case of cysteine, a 1:1 complex in which the deprotonated thiol and amino group coordinated to the metal was observed (**13**). The structures of the 1:1 complexes shown in Fig. 5(a) were proposed on the basis of NMR and other spectroscopic data [43], and were subsequently confirmed by

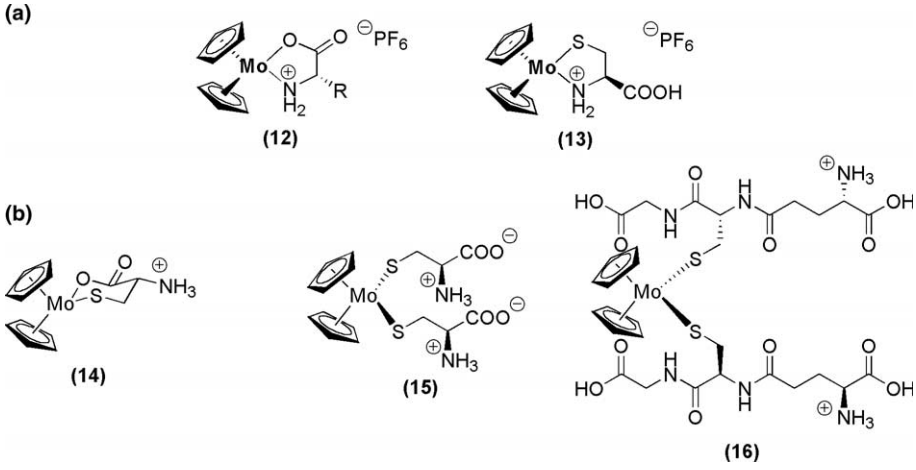

Fig. 5. Key coordination modes observed between Cp_2MoCl_2 and amino acids: (a) 1:1 complexes with Gly, Ala, Val, Leu, Phe, Met and Cys; (b) 1:1 and 2:1 complexes formed with Cys, and 2:1 complex formed with glutathione.

independent single crystal X-ray diffraction studies [21,44,45]. These complexes were prepared under basic conditions, usually with triethylamine as the base, at reflux, with the complexes extracted into methanol or liquid SO_2 and precipitated as hexafluorophosphate salts. These complexes are therefore not indicative of biologically relevant species that may form in vivo.

Smets et al. have reported the interaction of Cp_2MoCl_2 with glycine and glycine methyl ester at pH 10, with a view to developing transition metal mediated hydrolysis chemistry [46,47]. An equilibrium between the monodentate glycine chelate, with coordination via the amine nitrogen, and the bidentate five-membered ring was proposed. However limited characterization data or evidence for the complexes was presented.

While the studies above are important in establishing the different binding modes that are possible with Cp_2MoCl_2, the conditions used are not biologically relevant. Our group has reported the first systematic study to establish the relative affinity of Cp_2MoCl_2 for amino acids in water at physiological pH, and hence to predict the complexes that would most likely form in biological media, including blood plasma [31]. No evidence for coordination of the carboxylate or amino groups in Lys, His, Ala or Cys to Cp_2MoCl_2 was detected using ^1H NMR spectroscopy in the pD range 2–7. Weak coordination to the imidazole group of His was detected, but isolation of a stable complex was not possible and the coordination heterocycle is readily displaced (discussed below).

In contrast, non-labile thiol coordination of the deprotonated thiol sidechain in Cys allowed isolation and characterization of $Cp_2Mo(Cys)_2$ (**15**) (Fig. 5(b)); this complex is highly water soluble and stable to oxygen in the pH range 2–7 for several weeks. NMR titration experiments showed that the bisamino acid complex $Cp_2Mo(Cys)_2$ **(15)** was formed via the 1:1 complex Cp_2MoCys (**14**). While this complex was unable to be isolated and fully characterized, NMR data, and comparative experiments with *N*-acetylCys were consistent with bidentate coordination involving the carboxylate(O) and deprotonated thiol (Fig. 5(b)). This structure of complex **(14)** contrasts with the structure of the 1:1 complex (**13**) reported by Gore and Green [43] (Fig. 5(a)) in which the amino group rather than the carboxylate is coordinated to the metal. The different coordination complexes further emphasize that the complexes formed with amino acids, particularly those with acidic and basic sidechains are strongly dependent on conditions. The non-ambient conditions and isolation techniques employed by Gore and Green [43] are significantly different to those of Waern and Harding [31].

Analogous strong coordination to the deprotonated thiols in the tripeptide glutathione (GSH) to form $Cp_2Mo(GS)_2$ (**16**) was also observed [31]. This highly charged derivative is very water soluble and stable under physiological conditions. GSH is a major source of biological thiols, and the interaction of GSH with transition metal ions is fundamental to a number of cellular processes [48], as well as to the detoxification and excretion of exogenous metal ions, including platinum upon administration of the antitumor drug cisplatin [22,49]. Given the high concentration of this thiol in most cells, these results suggest that significant amounts of Cp_2MoCl_2 will be converted to the thiol derivative $Cp_2Mo(GS)_2$ (**16**) in vivo and that coordination to other accessible thiols present in blood plasma will also occur [31].

4.2. Proteins

Normal protein function is absolutely essential to maintain viability of organisms, as well as individual cells. Proteins have two major roles; as enzymes, where they catalyse chemical reactions and cellular processes, and as transporter molecules, whereby they are used as simple shuttles, either to transport molecules that cannot alone surpass the cell membrane, or to bind and carry exogenous molecules away from the body. Disruption of either type of process will often be lethal to the organism, and hence proteins are an important target for drugs.

4.2.1. Serum proteins

The most abundant protein in blood plasma is human serum albumin (HSA) which is present at approximately 0.63 mM [50]. This protein serves a number of important functions including the transport of drugs, metals and hormones [51]. Albumin has a free cysteine residue, located in a crevice close to the surface of the protein, and interaction of metal complexes with this thiol has been implicated in the mechanism of Pt(II) [22,52], Pt(IV) [53,54] and Ru(II) [55] anticancer drugs, and gold from the antiarthritic drug, auranofin [56], as well as other transition metal ions. While the single available thiol in HSA is buried below the surface of the protein and hence is less accessible than the thiol in Cys and GSH [50], we have proposed that formation of Cp_2Mo-HSA adducts must be considered in the mechanism of action given the high affinity of Cp_2MoCl_2 for thiols under biological conditions [31].

In addition to HSA, there are a number of other serum proteins that are important metal regulators, notably transferrin. Transferrin has been shown to uptake titanium from Cp_2TiCl_2 into the vacant Fe binding sites and thus act as a delivery agent for "Ti(IV)" to the cell via receptor-mediated endocytosis [57,58]. Uptake into the Fe binding sites requires hydrolysis of the Cp ligands. While no studies of the interaction of Cp_2MoCl_2 with transferrin have been reported, the hydrolytic stability of Cp_2MoCl_2 means that similar coordination is unlikely to occur.

Given the high affinity of Cp_2MoCl_2 for thiols, studies with other cysteine-rich proteins and metalloenzymes may also be of interest. For example, metallothionen is a cysteine rich, low molecular weight protein that binds a wide range of metals, including Zn(II), Cd(III), Cu(I), Ag(I), and Au(I) [59], and is known as a cytoprotectant protein, due to the ability to bind and remove heavy metals, usually Zn(II) and Cd(III), from the cell [60]. Pt(II) from cisplatin has been shown to displace Zn(II), to form a complex with up to 7 Pt(II) ions bound to one protein [61]. This protein has been implicated in the detoxification of metal containing drugs [61]. Given the high affinity of Cp_2Mo^{2+} for thiols, the interaction of Cp_2MoCl_2 to accessible cysteine residues in metallothionen is likely and may play a role in detoxification of the complex.

4.2.2. DNA processing proteins

As noted in Section 3, most clinically used drugs disrupt normal cellular function by interaction with either DNA and/or DNA processing enzymes. Given that there is no strong evidence to support formation of Cp_2Mo-DNA adducts in vivo, antitumor activity via inhibition of the function of DNA processing enzymes has been considered.

Protein kinase C (PKC) refers to a family of enzymes involved in cell growth and differentiation, and signal transduction [62]. Among a number of roles, PKC has been shown to bind to metal ions, such as Zn(II) and Ca(II) [63], and the enzyme function is inhibited by heavy metals, including Hg(I) and Pb(II) [64]. Preliminary studies reported that PKC activity was inhibited by Cp_2MoCl_2 and Cp_2VCl_2 [36], but detailed experimental details have not been published. Copper coordinated anthracycline complexes have also been shown to inhibit PKC, as has Cu(II) alone, however, no suggestions have been put forward regarding the binding site [65].

Topoisomerases, which are further classified as type I and II, are enzymes responsible for maintaining the topology of DNA. Inhibition or poisoning of human topoisomerases has been implicated in the mechanism of activity of several antitumor drugs [66–70] and since tumors utilize an increase in topoisomerase activity, the inhibition of topoisomerases becomes an important target in achieving antitumor activity [67,70]. There is some precedent for small metal complexes, including metallocenes, interacting with topoisomerases; diacetyl and dicarboxaldoxime derivatives of ferrocene and several cobalt salicylaldoxime complexes poison topoisomerase II and have shown that the mechanism of action depends critically on substituents present in the metal complexes [71].

The inhibition of bacterial topoisomerase II by Cp_2MoCl_2 (**1**) has been reported by Marks et al. [36]. Topoisomerase II was investigated as a potential target since antitumor metallocenes were observed to inhibit cellular DNA synthesis in vitro by arresting cells preferentially at the G2 or at the G1/G2 phases of the cell cycle [72], a phenomenon observed for other antitumor drugs that target topoisomerase II [73,74]. Concentration-dependent inhibition of bacterial DNA topoisomerase II (winding) activity on relaxed DNA plasmid treated with Cp_2MoCl_2 **1** was reported [36], but no further details or experimental data have been published.

The ability of both antitumor active and inactive metallocene dihalides to inhibit the relaxation of supercoiled plasmid DNA pBR322 by human topoisomerase II was reported by Mokdsi and Harding [33]. In the case of Cp_2MoCl_2 maximum inhibition was observed at 3.0 mM. Thus, inhibition of topoisomerase II is a possible mechanism that may be related to anticancer activity, but the precise process whereby this occurs requires further studies to establish the exact biologically active species that is formed for each of the metallocenes in vivo. The mechanism of topoisomerase II inhibition on a molecular level is unknown. Recent studies have established that thiol alkylating agents (e.g., maleimide [75]) and organic disulfides (e.g., 2,2′-dithiobis(5-nitropyridine) [76]) inhibit the catalytic activity of topoisomerase II through covalent modification of thiol groups on the enzyme. Given the high affinity of Mo for thiols, similar modification of the thiol in topoisomerase II may occur with Cp_2MoCl_2.

4.3. Competition between nucleotides and amino acids

Figs. 3 and 5 show that Cp_2MoCl_2 is able to coordinate to available sites in nucleotides, amino acids and proteins. Clear evidence for the preferential strong coordination to thiols over all other potential coordination sites in biological ligands has been demonstrated in a recent ^1H and ^{31}P NMR spectroscopic study [31].

Competition experiments in which Cp_2MoCl_2 (**1**) was treated with 5′-dAMP, ribose 5-monophosphate, and cysteine showed preferential coordination to the cysteine thiol over the phosphate(O) and heterocyclic(N) groups (Fig. 6). Furthermore, the amino acid derivative $Cp_2Mo(Cys)_2$ (**15**) was shown to be stable in the presence of excess dAMP or ribose monophosphate, and Cys displaced coordinated histidine, dAMP (**7**) or ribose

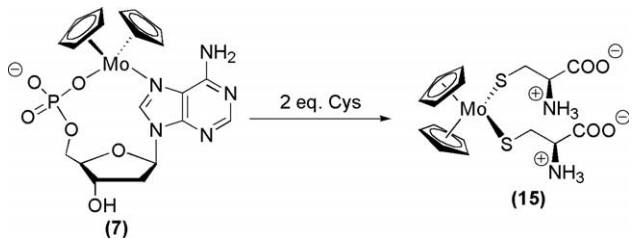

Fig. 6. Competition experiment illustrating the strong preferential coordination of molybdocene to Cys thiol group over dAMP.

monophosphate to give $Cp_2Mo(Cys)_2$ (**15**). Thus, these results strongly suggest that Cp_2MoCl_2 will preferentially coordinate to thiols in vivo.

5. Catalytic properties

5.1. Phosphate ester hydrolysis

Molybdocene dichloride has been shown to catalyse the hydrolytic cleavage of both activated and unactivated phosphate esters in aqueous solutions [24,77]. The hydrolysis of unactivated diesters which mimic the stable phosphate diester backbone of DNA is of particular importance as a possible reaction that may be related to antitumor activity [24].

The catalytic hydrolysis of *p*-nitrophenyl phosphate by Cp_2MoCl_2, releasing *p*-nitrophenol and inorganic phosphate, was first reported by Kuo and co-workers in 1995 [77]. The production of *p*-nitrophenolate was readily detected spectrophotometrically at 400 nm, and there was a remarkable 10^5 hydrolysis rate enhancement. The mechanism was proposed to involve initial monodentate coordination of the phosphate ester group to give **17** which undergoes intramolecular nucleophilic attack by the metal-bound hydroxide ion, to liberate one equivalent of the *p*-nitrophenolate and the bischelated molybdocene phosphate **19** (Fig. 7(a)). Dimerization to form **21**, with a second equivalent of molybdocene was proposed on the basis of the ^{31}P NMR signal at δ 77 ppm, followed by conversion to monomeric **19** by the addition of base. In the case of the diester, bis(*p*-nitrophenyl) phosphate, Cp_2MoCl_2 catalysed the cleavage of only one ester, with a rate enhancement of 10^7. In this case, formation of bischelated intermediate **20** after the hydrolysis of one *p*-nitrophenolate group was proposed to prevent further hydrolysis (Fig. 7(a)).

While the hydrolysis chemistry shown in Fig. 7(a) is interesting, the requirement for an activated ester means that this chemistry is unlikely to be relevant to cleavage of nucleic acids. Hence the hydrolysis of dimethyl phosphate, as a mimic of the phosphodiester DNA backbone was studied [24]. In aqueous solutions at pH 7.0 and 100 °C, the rate of hydrolysis of dimethyl phosphate was measured to be 3.4×10^{-14} s^{-1} while a rate acceleration of 10^4 at pH 4.0 was achieved by the addition of Cp_2MoCl_2 to the reaction. No catalytic hydrolysis was observed at pH > 6. On the basis of ^{31}P NMR spectroscopy mechanism shown in Fig. 7(b) was proposed. P–O bond cleavage, rather than C–O bond cleavage was demonstrated by labelling of the solvent with $^{18}OH_2$ which showed that no oxygen from the solvent was incorporated into the methanol produced in the reaction. As for the hydrolysis of the activated *p*-nitrophenyl phosphate, the mechanism appears to be proceeding by coordination of the phosphate to the metal centre, followed by intramolecular nucleophilic attack of a metal-bound hydroxide species.

The absence of significant catalytic activity above pH > 4 shows that potential hydrolysis and cleavage of

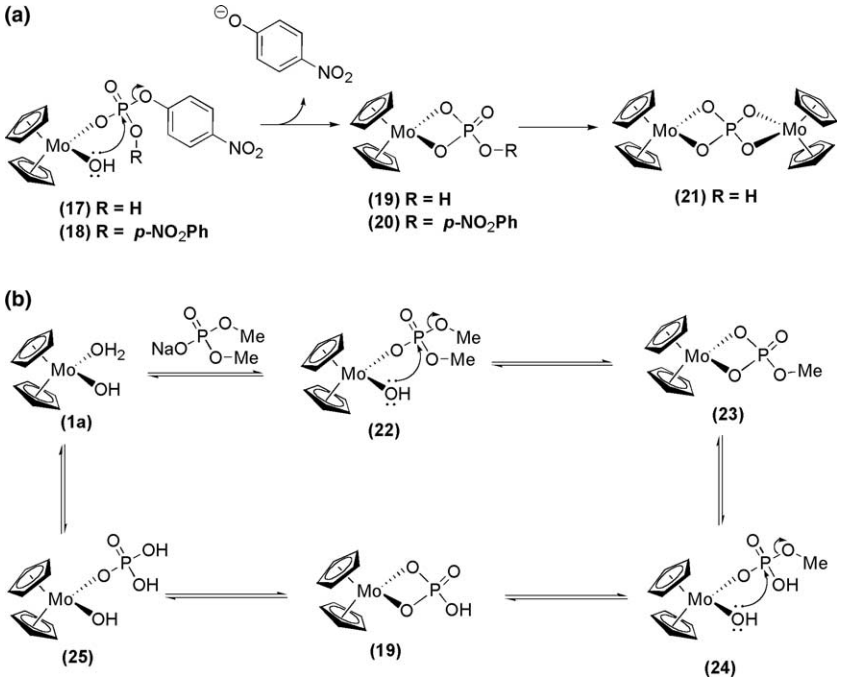

Fig. 7. Catalysis of ester hydrolysis by Cp_2MoCl_2: (a) activated mono and diphosphates; (b) diphosphate methyl esters are models for the phosphate esters present in DNA.

DNA phosphate esters by Cp_2MoCl_2 is unlikely to occur in vivo. It is noted, however, that acidic environments characterize some tumor types, and under these conditions DNA cleavage cannot be ruled out. The only current application of this hydrolysis chemistry in biology is in the hydrolysis of the organophosphate pesticides parathion and paraoxon [78]. These pesticides both contain activated ester groups and hence hydrolysis was promoted at pH 7. However, the potential (if any) advantages of accelerating cleavage of these esters to pesticide action or soil chemistry is unclear.

5.2. Other catalysis reactions

In addition to phosphate ester hydrolysis, molybdocenes have also been shown to catalyse a number of other reactions in water. Molybdocene hydrides reduced carbonyls in aqueous solutions at neutral pH [79]. Tyler and co-workers have reported that $(MeCp)_2MoCl_2$ catalysed the hydrolysis of esters and difunctional ethers [80], and the conversion of nitriles to amides [81]. In each case the proposed mechanism involved the intramolecular nucleophilic attack of a hydroxide onto the metal coordinated substrate, similar to the phosphate ester hydrolysis discussed in the previous section. The reactions were carried out in neutral water, at 80 °C. The activation of C–H bonds in aqueous solution by Cp_2MoCl_2, leading to H/D exchange of the α-hydrogens in primary alcohols in D_2O has also been reported [25,82].

While the above examples are not directly relevant to the bioorganometallic chemistry of Cp_2MoCl_2, they serve to illustrate that the metallocene is able to catalyse a number of different reactions and hence further investigations of the ability of Cp_2MoCl_2 to hydrolyze amides, thioesters and other biologically relevant functional groups under physiological conditions is warranted.

6. Biological activity

6.1. Antitumor properties

While many prior articles assumed that structurally related metallocenes exert their activity via similar mechanisms and hence referred to these complexes as a single class of antitumor agents, there is currently only limited biological data for Cp_2MoCl_2. Biological testing has been restricted to in vitro studies with cultured Ehrlich ascites tumor cells, and CF1 mice bearing fluid Ehrlich Ascites tumors. CF1 mice bearing fluid Ehrlich Ascites tumors were cured with an optimum dose range of 75–100 mg/kg and gave a an LD_{50} of 175 mg/kg. Both the optimum dose and LD_{50} are higher than the corresponding values achieved with titanocene dichloride [10,83].

We have recently determined cytotoxicity values for Cp_2MoCl_2 against human breast MCF-7 (620 μM) and ovarian 2008 (700 μM) cell lines [31]. These values are comparable to some metal complexes but are significantly higher than IC_{50} values for cisplatin. In addition, as noted previously [31], the merit of in vitro assays to identify metallocenes for animal and human trials is not clear [83]. In the case of titanocene dichloride, patterns of activity in human xenografted tumors in mice correlated well with subsequent human trials of titanocene, but not with in vitro cell lines [84].

6.2. Structure–activity studies

The effect of structural modification of Cp and halide ligands on antitumor activity are key results that are required to understand the biological chemistry of Cp_2MoCl_2. There are no reports of the effect of modification of the Cp ligands on activity and limited data on the effect of modification of the chloride ligands.

Our group has designed and synthesized of a range of molybdocene derivatives to probe the importance of solubility, charge and the lability of the Mo–X bond on cell uptake and cytotoxicity [85]. The thioglucose derivative **26** was designed as a neutral complex which would be expected to be transported into cells more readily than the glutathione complex **16** (shown in Fig. 5). The acetylated derivative **27** was also studied as a lipophilic derivative with slow hydrolysis of the acetyl groups at physiological pH to form **26** expected to occur in vivo. Derivative **28** contains two electron-withdrawing fluorinated aromatic rings, which were designed to enhance the lability of the Mo–S bonds, while the carboxylate group was incorporated in the aromatic ring to confer aqueous solubility (see Fig. 8).

The results of these structural modifications on the cellular uptake and cytotoxicity against V79 Chinese hamster lung cells are summarized in Table 1. The IC_{50} values for all 3 thiol derivatives, which are stable to hydrolysis in water (50 mM, 37 °C, pH 7), demonstrate that a labile Mo–X bond is required for activity. The cell uptake experiments show that the lack of activity is not due to insufficient metallocene entering the cell, and there is no correlation between the overall charge, cell uptake and cytotoxicity values. The high cell uptake results obtained with the fluorinated derivative **28** show that the increased lipophilicity of this derivative facilitates cell uptake. This result, taken with slow hydrolysis to liberate Cp_2Mo^{2+} [31], suggests that further tailoring of the pseudohalide ligands to increase the rate of hydrolysis, may allow the design of new molybdocene prodrugs that are efficiently taken up in the cells and then liberate the active species.

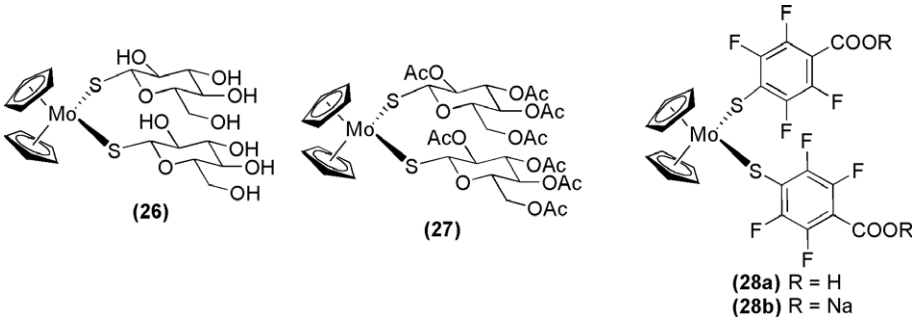

Fig. 8. Summary of structure–activity and cell uptake studies on selected thiol derivatives.

Table 1

Complex	IC_{50} (24 h; μM)	Intracellular Mo ($\times 10^{-4}$ μmol/million cells)
1	820	8.7
16	2230	2.3
26	>500	1.5
27	Not tested	Not tested
28	2900	42.7

6.3. Mechanism of antitumor action

While the chemistry described in the previous sections suggests that proteins are implicated in the mechanism of action of Cp_2MoCl_2 there is insufficient data available to ascertain the likely target in vivo that is related to anticancer activity in the human and animal tumors that have been screened to date. The structure activity studies (Table 1) demonstrate the requirement for a labile Mo–X bond activity. The high donor affinity of Cp_2Mo^{2+} for biological ligands (Section 4) clearly support the formation of complexes in biological media. The distinct cytotoxicity and cell uptake profiles of Cp_2MoCl_2 (**1**) compared with $Cp_2Mo(GS)_2$ (**16**) show that while rapid coordination of Cp_2MoCl_2 to glutathione occurs in water at pH 7, significant deactivation of molybdocene dichloride by conversion to $Cp_2Mo(GS)_2$ does not occur in cells.

Detailed cellular studies that ascertain where molybdenum concentrates in cells after administration (nucleus, mitochondria, membranes, etc.), are essential to understanding the biological fate of Cp_2MoCl_2 and in identification of the biologically active species. While in vitro studies show that Cp_2MoCl_2 is capable of inhibiting topoisomerase II and protein kinase II activity, in vivo this can only occur if the active species is transported and delivered close to DNA.

6.4. Anti-viral activity

Many antitumor active compounds also display other anti-biological properties, such as antibiotic, antifungal, anti-HIV or antiviral properties. To the best of our knowledge, there has been only one report of the antiviral behavior of metallocenes. Although Cp_2TiCl_2 was found to exhibit in vitro antiviral activity against orthopoxvirus, herpes virus, orthomyxoviruses, paramyxovirus, and rhabdovirus, Cp_2MoCl_2 failed to show any antiviral activity against these strains [86].

7. Bis(cyclopentadienyl) molybdenum(VI) complexes

In addition to Cp_2MoCl_2 there has been limited studies on the corresponding Mo(VI) complex. The ionic complex $[Cp_2Mo(VI)Cl_2](SbF_6)_2$ was designed as a charged metallocene that would have improved aqueous solubility compared with Cp_2MoCl_2 [87]. This ionic complex was reported to be air-stable with a similar pseudotetrahedral arrangement of the ligands about the Mo atom, as in the neutral Mo(IV) complex, as determined by X-ray crystallography [88]. The complex was screened for activity against EAT tumors in CF1 mice, the standard tumor type that has been used extensively in screening all of the antitumor metallocenes [83]. The ionic Mo(VI) complex gave an optimum cure rate of 100%, with an optimal dose range 20–80 mg/kg, compared to an optimal dose range 75–100 mg/kg for Cp_2MoCl_2 [10]. Although this optimal dose range is slightly lower than that for Cp_2MoCl_2, the LD_{50} is also lower (105 mg/kg vs. 175 mg/kg), as is the therapeutic index. No other molybdenum(VI) derivatives were investigated.

8. Mono(cyclopentadienyl) molybdenum complexes

While the major focus of this review has centred on Cp_2MoCl_2, the bioorganometallic chemistry of molybdenum mono(cyclopentadienyl) half-sandwich complexes is also mentioned. As for the parent metallocenes, there has been wide-spread use of these metallocenes in catalysis. However there are also several reports of biological applications, notably mono(cyclopentadienyl) molybdenum carbonyl complexes (Fig. 9(a)), which have attracted interest for their potential use as biological tracer agents [89–91]. The infrared

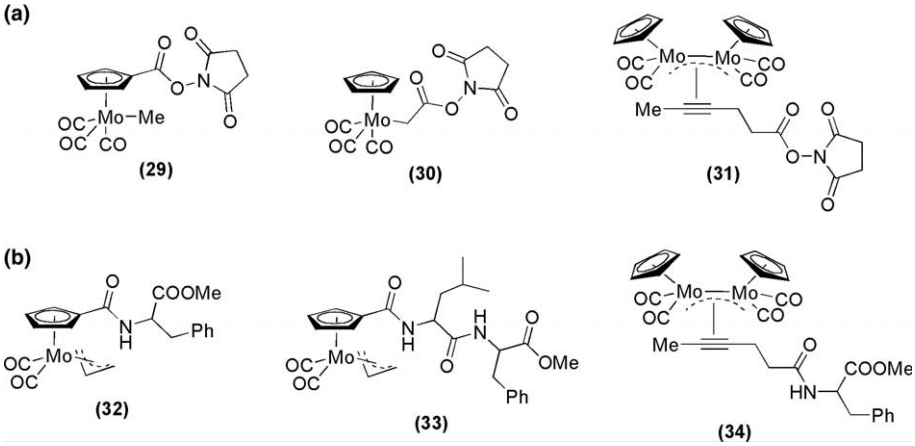

Fig. 9. Key monocyclopentadienyl derivatives: (a) selected NHS esters suitable for functionalization with amines; (b) examples of peptide conjugates that have been prepared as potential biological tracer agents.

stretching vibrations of the carbonyl groups in these complexes provide characteristic spectroscopic handles which permit detection of the complex when tagged to a biomolecule. The sensitivity of these vibrations are comparable to radioactive tracers, and clearly have many advantages over the use of traditional radiotracers.

Most research has centred on efficient synthetic methods to attach the molybdenum tracer molecule onto biomolecules. Thus activated succinimide esters, for example **29**–**31**, that can be readily derivatized with amino acids or peptides have been reported (Fig. 9(a)) [90]. While simple peptide conjugates, illustrated by **32**–**34** in Fig. 9(b), have been prepared there are no examples showing applications as radiotracers. Jaouen and coworkers also reported the use of cyclopentadienyl molybdenum dimer complexes as analogous bioconjugate tracer agents [89]. In this case, the succinimide ester is bound to the molybdenum carbonyl dimer via metal–alkyne coordination, which is reported to give stable complexes, with easily detectable molybdenum carbonyl stretching vibrations. Similar functionalized mono-(cyclopentadienyl) complexes that incorporate a η-allyl ligand, and maintain the use of organometallic carbonyl infrared stretching vibrations for tracer activity, have been reported [91].

The ability of the monocyclopentadienyl molybdenum carbonyl complex **35** (Fig. 10) to cleave DNA photo-oxidatively has been studied [92]. While analogous Fe and W complexes cleaved DNA reproducibly by production of carbon-centred radicals, the Mo derivative was inactive, and no DNA cleavage was observed.

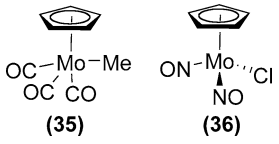

Fig. 10. Examples of biologically active monocyclopentadienyl derivatives: complex **35** was designed to cleave DNA photo-oxidatively and complex **36** releases nitric oxide to exert a vasodilatory effect.

Another reported application of biologically active molybdenum cyclopentadienyl complexes is as nitric oxide donors, exerting a vasodilatory effect in aortic rings and in rats [93]. The molybdenum complex **36** (Fig. 10) was found to be an efficacious nitrovasodilator in vitro and n vivo, through the release of nitric oxide. However, in this example it is clearly not the cyclopentadienyl molybdenum species exerting the biological effect; it is merely a release agent for nitric oxide.

9. Conclusions

The hydrolytic stability of molybdocene dichloride to Cp hydrolysis under physiological conditions is an attractive feature that has allowed potential bioorganometallic applications to be investigated. Detailed studies of the coordination chemistry with models for DNA and proteins have provided important molecular level data regarding the kinetics and thermodynamics of coordination to biological ligands. However, there is presently insufficient biological data to complement these chemical studies and establish the mechanism of antitumor action. Further screening against a range of tumor types, cellular studies that establish the principle cellular target in vivo, and structure–activity studies are required to evaluate whether molybdocene dichloride has potential as an anticancer drug. The catalysis of hydrolysis of phosphate esters by molybdocene dichloride is currently restricted to acidic pH or the use of activated esters. Further studies involving amides and esters under physiological conditions may provide insight into potential catalytic roles for molybdocene dichloride in vivo.

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