Organometallic Anticancer Agents: Cellular Uptake and Cytotoxicity Studies on Thiol Derivatives of the Antitumor Agent Molybdocene Dichloride

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The effect of aqueous solubility, charge, and lability of four thiol derivatives of the antitumor metallocene molybdocene dichloride (Cp_2MoCl_2) on the cell uptake and cytotoxicity against V79 Chinese hamster lung cells has been determined. Addition of 4-thiol-2,3,5,6-tetrafluorobenzoic acid, 1-thio-β-D-glucose, and 1-thio-2,3,4,5-tetraacetyl-β-D-glucose to aqueous solutions of molybdocene dichloride afforded the corresponding metallocenes in which the deprotonated thiols are coordinated to the metal center. These metallocenes were studied, along with the previously reported glutathione derivative $Cp_2Mo(GS)_2$, which has been proposed to be formed from molybdocene dichloride in blood plasma. In contrast to Cp_2MoCl_2 which rapidly loses the chloride ligands to form a positively charged aquated complex at pH 7, the thiol derivatives are stable to ligand hydrolysis in 50 mM salt at 37 °C for 24 h. Cytotoxicity values determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay gave an IC_{50} value of 350 μM for molybdocene dichloride with V79 cells, with similar values obtained with human breast MCF-7 (620 μM) and ovarian 2008 (700 μM) cell lines. The water-soluble thiol derivatives were not cytotoxic, while the acetylated sugar derivative was insoluble in water or aqueous dimethyl sulfoxide. Cell uptake experiments in which the molybdenum content in cells treated with each metallocene for 24 h was measured by graphite furnace atomic absorption spectroscopy showed that the fluorinated aromatic derivative was most efficiently transported into cells, followed by molybdocene dichloride, with the lowest uptake observed for $Cp_2Mo(GS)_2$ and the glucose derivative. The cell uptake results do not correlate with overall charge of the complexes or the measured IC_{50} values. The distinct cytotoxicity and cell uptake profiles of Cp_2MoCl_2 compared with $Cp_2Mo(GS)_2$ 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.

Introduction

The design and synthesis of metal-based anticancer drugs has been motivated by the spectacular clinical success of cisplatin[1] for the treatment of testicular and other human cancers.[2,3] Metal complexes offer the opportunity to develop novel anticancer drugs that exert their activity via different mechanisms to organic cytostatic compounds. In particular, the rich coordination chemistry present in biological ligands, coupled with the diverse coordination geometries, redox chemistry, and the lability and stability afforded by metal−ligand interactions, provide access to a huge range of metal complexes that may be tailored for interaction with specific biomolecules. The stability, aqueous solubility, and slow kinetics of ligand substitution observed with cisplatin have resulted in extensive studies on new platinum(II) drugs that interact with DNA in a manner similar to cisplatin.[4,5] However, the emergence of resistance and limited progress in the development of successful platinum(II) complexes that offer significant benefits compared to cisplatin have led to the development of non-platinum(II) metal complexes[6−8] that have unique mechanisms of action compared with cisplatin. Promising preclinical results have been obtained with platinum(IV),[9,10] ruthenium(II),[11,12] and titanium(IV) complexes.[13,14]

Titanocene dichloride (Cp_2TiCl_2) is the first non-platinum metal complex to enter clinical trials.[13,14] The lack of cross-reactivity against cisplatin-resistant tumors and a pattern of reactivity and side effects that differs from standard organic chemotherapeutics are attractive properties that have resulted in significant interest in the development of titanium-based anticancer drugs. The tetrahedral geometry at the metal center and the pH-dependent rapid hydrolysis chemistry of both the cyclopentadienyl (Cp) and halide ligands in water contrast with the square planar geometry and slow hydrolysis kinetics that are characteristic of platinum(II) complexes. While interaction with DNA is implicated in the mechanism of action of Cp_2TiCl_2,[15−17] the poor aqueous solubility and stability of the complex at physiological pH[18] have hampered identification of the active species in vivo.[19] Current evidence is consistent with formation of a Ti(IV) species that lacks the Cp ligands which interact with DNA, possibly transported into the cell via the iron transporter protein transferrin.[20,21]

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In contrast to Cp_2TiCl_2, limited studies have been reported on the structurally related metallocenes Cp_2MCl_2 (M = Mo, V, Nb, Re).[17,19,22,23] Despite this fact, the metallocene dihalides have been frequently referred to as a general class of antitumor complexes. This single classification is misleading, as detailed antitumor testing against a range of tumor types is presently restricted to Cp_2TiCl_2 and Cp_2VCl_2[24] and no structure−activity results have been reported on any of these metallocenes except Cp_2TiCl_2. Furthermore, binding studies with nucleotides, oligonucleotides, amino acids, and proteins have clearly shown that each metallocene exhibits unique coordination chemistry and hence it is highly unlikely that formation of similar adducts, and hence similar mechanisms of antitumor action, would occur in vivo.[19]

Our recent studies have focused on molybdocene dichloride **1** (Cp_2MoCl_2).[25−28] Compared with Cp_2TiCl_2, this metallocene is an attractive candidate for drug development as the complex is water-soluble and stable to hydrolysis of the Cp ligands at physiological pH. Thus, detailed NMR studies with nucleotides,[29,30] oligonucleotides,[28,31] bulk DNA,[32] and amino acids[25,26] have been performed under biologically relevant conditions. Molybdocene dichloride coordinates to phosphate-centered (O) and heterocyclic (N) sites in nucleotides.[28,29,31] However, simultanous coordination to phosphate (O) and heterocyclic (N) binding sites in the DNA duplex would require significant distortion of the sugar−phosphate backbone. Hence, under physiological conditions, interaction with DNA is restricted to accessible terminal phosphate (O) groups in oligonucleotides.[28,31] In contrast, Cp_2MoCl_2 forms highly water-soluble, air-stable complexes with cysteine and glutathione, respectively, via coordination of the deprotonated thiol groups, and these thiols displace phosphate (O) or heterocyclic (N) coordinated groups.[25] These results show that thiol-containing amino acids, and thiols in proteins including serum albumin, are attractive targets for Cp_2MoCl_2 in plasma and may act as carriers for the active species into the cell and/or lead to inactivation by the formation of water-soluble derivatives that can be excreted.

In this paper we report the design and synthesis of a range of molybdocene derivatives that were designed to probe the importance of solubility, charge, and the lability of the Mo−X bond on cell uptake and cytotoxicity. The data obtained were compared with Cp_2MoCl_2 **1**, which has only been tested against one animal tumor model. These studies have provided fundamental data that are essential to assess whether Cp_2MoCl_2, or metal complexes based on this framework, are promising lead compounds for the development of molybdocene-based anticancer drugs.

Results

Design and Synthesis of Derivatives. Figure 1 shows the target metal complexes used in this study. Complexes **2**−**5** are all bisthiol derivatives of Cp_2MoCl_2 in which the coordinated thiols vary in size, net charge, lipophilicity, and aqueous solubility. The incorporation of thiol ligands was based on our previous studies that showed that Cp_2MoCl_2 forms stable complexes with cysteine and glutathione via coordination of the thiol side chains and that the thiols will displace phosphate (O) and/or heterocyclic (N) groups.[25]

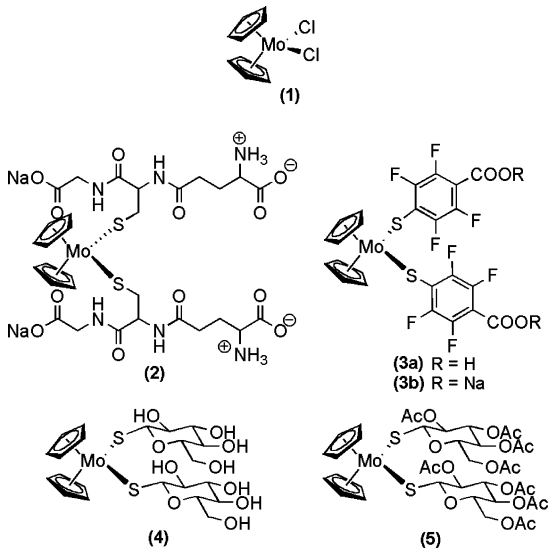


Figure 1. Summary of structures of molybdocene derivatives.

While the glutathione complex $Cp_2Mo(GS)_2$ (**2**) is highly water-soluble due to the presence of two zwitterionic side chains, the high charge is likely to have a significant impact on transport through cell membranes. Hence the thioglucose derivative **4** was designed as a neutral complex that would be expected to be transported into cells more readily than **2**. In addition, high aqueous solubility was expected to result from the presence of the two sugars. The acetylated derivative **5** was also studied as a lipophilic derivative with slow hydrolysis of the acetyl groups at physiological pH to form **4** expected to occur in vivo. Derivative **3** 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. The fluorine atoms in **3** also provide a convenient NMR handle for monitoring the interaction of this derivative with biomolecules.

The thiol derivative **3** was prepared in an analogous manner to that previously reported for the synthesis of the glutathione derivative **2**.[25] Thus, treatment of an aqueous solution of Cp_2MoCl_2 **1** with 2 equiv of 2,3,5,6-tetrafluoro-4-thiobenzoic acid at pH 6 over 24 h afforded the salt **3b**, which was most effectively purified by conversion to the insoluble bisacid **3a**. Recrystallization of **3a** from aqueous methanol afforded pure acid, which was converted back to the water-soluble salt **3b** by addition of 2 equiv of NaOH.

The direct formation of the glucose derivative **4** from **1** proved more difficult. Analysis of the NMR spectrum of the crude product in water showed the presence of a significant number of impurities in addition to signals arising from **4**. Purification of the crude product by reverse-phase high-performance liquid chromatography (HPLC) was problematic due to close elution of the major byproduct, assumed to be the monosubstituted metallocene containing a single glucose ligand, and isolation of pure complex **4** required multiple cycles of HPLC. In contrast, the acetylated derivative **5** was readily prepared in aqueous methanol from **1** and was easily purified by HPLC to remove a well-separated minor byproduct, similar to that seen in the formation of **4**. Conversion of the acetylated derivative **5** to **4** by

hydrolysis was therefore investigated. Under acidic conditions (aqueous acetonitrile, pH 2−3), partial cleavage of the thioglycosides in **5** occurred in addition to hydrolysis of the esters. In contrast, selective hydrolysis of the eight acetyl groups in **5** proceeded smoothly under basic conditions, to give pure **4**, which was desalted and purified by reverse-phase HPLC.

Stability and Solubility. To test the stability of the complexes under physiological conditions, samples of complexes **2**, **3**, and **4** were prepared in D_2O (3 mM, 50 mM NaCl, 37 °C, pD 7) and the solutions were monitored by NMR spectroscopy over 2 weeks. There was no evidence of any degradation or hydrolysis of either the Cp ligands or the glutathione ligands of **2** during this time.

The fluorinated complex **3** was designed as an analogue with a reduced Mo−X ligation to potentially undergo slow hydrolysis over time to form **1a**. After 2 weeks at 37 °C, ^{1}H and ^{19}F NMR spectroscopy showed almost complete dissociation of the thiol ligands. Similar loss of the thiophenol ligands was observed by vigorous stirring of a solution of **3** at room temperature in air for 2 weeks. While dissociation of the thiol ligands was observed, the rate of dissociation was slow, and after 24 h, integration of the signals in the ^{1}H NMR spectrum showed that <5% of the complex had hydrolyzed.

The sugar derivative **4** was quite stable under standard physiological conditions, and after 2 weeks, integration of the ^{1}H NMR spectrum showed that approximately 10% of the complex had degraded. The major degradation product gave the same signals in the ^{1}H NMR spectrum as the byproduct seen in the synthesis of **4**, which was tentatively assigned as the metallocene containing a single sugar ligand. The stability of the sugar derivative **4** in slightly acidic solution (pH 5) was also assessed to simulate conditions that are present in some tumor types. Glycosides are readily hydrolyzed under acidic conditions. However, after 24 h, no ligand dissociation or hydrolysis was observed by ^{1}H NMR spectroscopy.

The acetylated sugar derivative **5** is soluble in dimethyl sulfoxide (DMSO) and other organic solvents but insoluble in water. Aqueous DMSO is often used for the administration of poorly water-soluble complexes in in vitro cytotoxicity assays,[33,34] but the final concentration of DMSO needs to be very dilute (<5%). While complex **5** has excellent solubility in neat DMSO, dilution of a DMSO solution of **5** with even 50% water resulted in precipitation of the complex. Thus, the poor aqueous solubility in water or DMSO/water mixtures at concentrations applicable for in vitro and in vivo testing prevented biological studies on this complex.

Cytotoxicity Studies. Metallocenes **1**−**4** were assayed for cytotoxic activity against V79 Chinese hamster lung cells. These are standard mammalian noncancer cells and were used as a model to correlate cytotoxicity with cellular uptake of the thiol-coordinated derivatives relative to the parent metallocene. V79 cells provide a readily available, easy to handle model cell line, against which all the complexes could be tested and compared.

Cytotoxicity values were determined by a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay,[35,36] which measures cell viability in terms of metabolic turnover, as indicated by the oxidation of MTT to purple formazan by mitochondria. The cells were exposed to each of the metallocenes for a total of 24 h, to compare the results of the cell uptake experiments with the cytotoxicity. The concentrations of complexes ranged between 1 and 5000 μM, depending on the activity of the particular complex. Under these conditions, dose−response curves and IC_{50} values were generated for complexes **1**, **2**, and **3**. The glucose derivative **4** showed no significant activity and there was little decrease in cell viability even at a concentration of 5000 μM.

Table 1. Cytotoxicities of Metallocenes Studied as Measured by the MTT Assay in V79 Chinese Hamster Lung Cells

	IC_{50} (μM)	
complex	after 24 h	after 72 h
1	820	350
2	2230	
3	2900	
4	>5000	

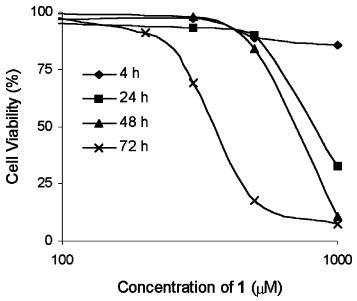


Figure 2. Treatment time dependence of the cytotoxicity of Cp_2MoCl_2 **1**.

The results of the cytotoxicity experiments are summarized in Table 1. The IC_{50} value represents the concentration of metallocene at which the cell viability is 50% of the control viability. These results show that Cp_2MoCl_2 **1** has the lowest IC_{50} of 820 μM with the three thiol derivatives **2**, **3**, and **4** exhibiting significantly higher IC_{50} values, following a 24 h exposure period. The derivatives **2** and **3** have comparable activity and achieved almost total inhibition (<10% cell viability) at the maximum concentration tested (5000 μM). The glucose derivative **4** showed almost no toxicity at the concentrations tested (<10% inhibition).

The effect of varying the total exposure time of the cells to Cp_2MoCl_2 **1** was also investigated. The dose−response curves for the treatment of V79 cells with **1** for 4, 24, 48, and 72 h are shown in Figure 2. It is clear that longer exposure times lead to increased cell death, with very little reduction in cell viability observed in the cells treated for just 4 h. The cells treated for 24 and 48 h exhibited similar levels of toxicity with IC_{50} values of 820 and 700 μM, respectively. The cells treated with Cp_2MoCl_2 **1** for 72 h displayed 50% viability at a concentration of 350 μM.

The ability of Cp_2MoCl_2 **1** to inhibit the growth of two human tumor cell lines was also assessed. An IC_{50} value of 620 μM was obtained against the human breast line MCF-7, and an IC_{50} value of 700 μM was obtained for the ovarian cell line 2008.

Cell Uptake Studies. The intracellular Mo content was determined after treatment of V79 cells with metallocenes **1**−**4** in order to determine the effect of

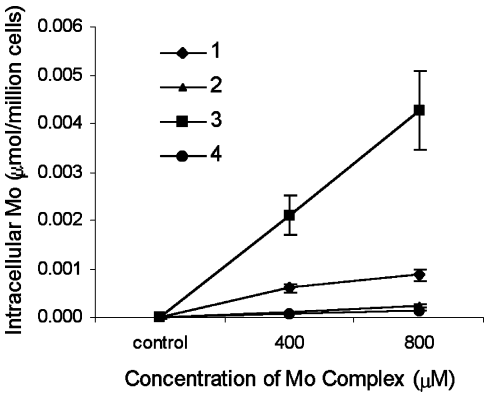


Figure 3. Comparison of the intracellular molybdenum in V79 cells after 24 h of exposure to metallocenes **1**−**4**.

altering the X-ligands on cell uptake. The correlation of the cell uptake with the cytotoxicity also provides information regarding the intracellular potency of the complexes. The Mo content of acid-digested whole cells was determined by graphite furnace atomic absorption spectroscopy (GF-AAS). Each metallocene complex was tested at 4 and 24 h exposure times, to provide information on the rate of uptake of the molybdocene complexes, and at two concentrations (400 and 800 μM Mo). These concentrations were chosen on the basis of the cytotoxicity data reported in the previous section, 400 μM being a subtoxic concentration and 800 μM producing approximately 50% toxicity for a 24 h treatment period with Cp_2MoCl_2 **1**.

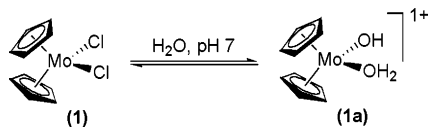
The intracellular Mo concentration for cells that had been treated with either 400 or 800 μM of each of the metallocenes **1**−**4** for a period of 24 h is shown in Figure 3. The thiol derivatives **2** and **4** displayed reduced cellular uptake relative to Cp_2MoCl_2 **1**. Slightly more of the charged glutathione derivative **2** enters the cell compared to the neutral sugar derivative **4**, but in each case, the relative amount of either **2** or **4** that enters the cell is only 15−20% of the amount of Cp_2MoCl_2 **1** at the maximum concentration tested. The derivative **3**, however, was able to enter the cell in significantly higher concentrations than Cp_2MoCl_2 **1**, at both concentrations tested (3.5 and 5 times greater for 400 and 800 μM, respectively). As expected, for all four metallocenes tested, the cells treated for 4 h contain less molybdenum than the cells treated for 24 h (data not shown).

These results show that after 24 h all four metallocenes are able to enter the cell and that the cellular uptake is dependent on the dose of each metallocene. However, the relative concentration of intracellular molybdenum is very low; in the case of Cp_2MoCl_2 **1**, there is less than 0.001 μmol of Mo/million cells. This corresponds to only 0.1% of the administered molybdenum.

Discussion

The potential for Cp_2MoCl_2 **1** as a new organometallic anticancer agent is based on in vitro studies with cultured Ehrlich ascites tumor cells[37] and cure rates for CF1 mice bearing fluid Ehrlich Ascites tumors, reported by Köpf and Köpf-Maier.[38] CF1 mice bearing fluid

Scheme 1



Ehrlich Ascites tumors were cured with an optimum dose range of 75−100 mg/kg and gave an LD_{50} of 175 mg/kg.[38,39]

While the exact species present in aqueous solutions of Cp_2MoCl_2 **1** are pH- and concentration-dependent,[40] at physiological pH the positively charged complex **1a** is present (Scheme 1).[29] The rapid hydrolysis of the halide ligands to form the aquated species **1a**, and the ability of this species to coordinate to nucleotides and amino acids,[25,26,28−31] strongly suggests that the mechanism of action is directly related to the interaction of **1a** with biological ligands. However, no structure−activity studies have been reported, and the active species that is transported into cells has not been established.

In this study, we have assessed the role of solubility, overall charge, and lability of the Mo−X bond on the cell uptake and cytotoxicity of molybdocenes **2**−**5** and compared these results with those obtained with the parent metallocene **1**. Conversion of **1a** to the bisglutathione complex $Cp_2Mo(GS)_2$ **2** has been proposed to occur in cell culture media and biological fluids.[25] Hence, comparison of the uptake of **1** versus **2** was performed to ascertain whether glutathione acts as a carrier for the active species into cells. The thiol derivatives **2**, **3**, and **4** were shown to be stable for 24 h under physiological conditions (50 mM NaCl, pH 7, 37 °C) and hence these complexes represent the first structure−activity studies of Cp_2MoCl_2 **1** that assess the affect of the lability of the Mo−X on activity. While the incorporation of electron-withdrawing aromatic rings in **3** increased the lability of the Mo−S bonds, the dissociation of the ligands was too slow to be biologically relevant, with minimal dissociation occurring in 24 h and full dissociation observed after 2 weeks.

Table 1 shows clearly that the thiol derivatives **2**, **3**, and **4** are not substantially cytotoxic to V79 cells and hence that a labile Mo−X bond is required for activity. The IC_{50} values measured after 24 h of exposure to each complex are in the millimolar range, with Cp_2MoCl_2 **1** being the only metallocene exhibiting a value less than 1 mM. Cytotoxicity studies of related metal complexes typically use longer exposure times of 48−72 h.[33,34,41−44] Longer exposure times were therefore assessed for **1** (Figure 2), and after 72 h of exposure a significantly lower IC_{50} value of 350 μM was obtained. Similar, modest IC_{50} values were obtained against human ovarian (700 μM) and breast (620 μM) cell lines. These values are high compared with cisplatin, which typically has IC_{50} values <10 μM with a range of cell lines.[34,44] In contrast, typical IC_{50} values in the 100−400 μM range have been noted with new platinum derivatives and ruthenium complexes.[34,42,44]

Comparison of the cytotoxicity data in Table 1 with other antitumor metallocenes is not straightforward. The metallocene dihalides Cp_2TiCl_2 and Cp_2VCl_2 have been extensively tested against animal tumor models. Patterns of activity in human xenografted tumors in

mice correlated well with subsequent human trials of titanocene but not with in vitro cell lines.[24,37,38] Thus, Cp_2VCl_2 was the most cytotoxic metallocene in vitro, but Cp_2TiCl_2 showed improved activity and selectivity in animal studies and was selected for human clinical trials.

Cell uptake studies were performed in order to ascertain whether the cytotoxicity results correlate to cell uptake. Measurement of the molybdenum content in cells after 24 h of exposure shows that all of the metallocenes **1**−**4**, including the highly charged glutathione derivative **2**, enter the cell but that there are significant differences in the final concentration of molybdenum with each complex. The uptake behavior does not correlate with overall charge: the negatively charged fluorinated derivative **3b** exhibited the highest uptake, followed by the parent complex **1**, and the neutral sugar derivative **4** and the highly charged glutathione derivative **2** showed relatively similar low uptake. While in general highly charged species do not readily penetrate cell membranes, there are clear exceptions to this rule, notably the highly charged bisplatinum(IV) complexes of Farrell.[9,10] The lipophilic character imparted by the two aromatic rings in the fluorinated complex **3**, coupled with the excellent solubility associated with the two carboxylates, appears to promote high cell uptake of this complex. In contrast, while the sugar derivative **4** was designed to promote high cell uptake of the neutral water-soluble complex, the high polarity and hydrophilic character due to eight hydroxyl groups does not facilitate this process.

Of particular interest is a comparison of the cell uptake of Cp_2MoCl_2 with $Cp_2Mo(GS)_2$ **2**. Rapid conversion of Cp_2MoCl_2 to **2** occurs with 2 equiv of glutathione under physiological conditions, and we have suggested that significant amounts of Cp_2MoCl_2 would be converted to $Cp_2Mo(GS)_2$ **2** upon administration.[25,26] Our results show that derivative **2** is inactive and that there is low cell uptake of this metallocene. Thus, any Cp_2MoCl_2 that is converted to **2** would be deactivated by formation of a species that never reaches the cell. In the case of cisplatin, slow hydrolysis of the chloride ligands facilitates transport into the cell, but competing coordination to thiols including glutathione is a major side reaction that results in deactivation and excretion of a significant amount of the administered complex.[45] Complete conversion of **1a** to **2** would result in similar cell uptake and cytotoxicities of **1** and **2**. The fact that there is higher uptake of **1** compared with **2** confirms that interaction of **1** with other thiols, including transport proteins such as human serum albumin, are probably involved in the cell transport process.

Finally, it is apparent that there is no clear relationship between cell uptake and cytotoxicity as the order of uptake was **3** ≫ **1** > **2** ∼ **4**, while the greatest cytotoxicity was observed for **1** (IC_{50} = **1** > **2** ∼ **3** ≫ **4**). There are clearly factors other than the ability of a complex to be transported into the cell affecting the ability of the complexes to kill the cells.

Conclusions

The results of this study imply that the labile Mo−Cl bonds in Cp_2MoCl_2 are a requirement for activity. Incorporation of thiols in place of the halogen ligands provides access to stable, water-soluble derivatives, some of which are able to enter cells, but which lack the cytotoxicity of the parent complex. Incorporation of the electron-withdrawing fluorinated aromatic rings in **3** resulted in more labile ligands, though not on a time scale to be relevant in this study. This complex also exhibited the highest cellular accumulation of the complexes tested, although it was not appreciably cytotoxic. Molybdocenes that incorporate more highly electron-withdrawing ligands that dissociate within several hours may show increased cytotoxicity. The distinct cytotoxicity and cell uptake profiles of Cp_2MoCl_2 compared with $Cp_2Mo(GS)_2$ show that while rapid coordination of Cp_2MoCl_2 to glutathione occurs in water at pH 7, this interaction is not significant in cell culture medium and that other transport mechanisms are involved in vitro and in vivo. Further testing of Cp_2MoCl_2 against human cell lines is required to establish whether cell line selectivity exists that warrants further study. However, the lack of a strong correlation between in vitro and in vivo results obtained with related metallocene dihalides is noted, and the use of xenografted human tumors that have been well-studied by Köpf and Köpf-Maier may be more informative in providing a critical assessment of the potential of Cp_2MoCl_2 for future development.

Experimental Section

General Synthesis Details. Reagents were used as provided from the Aldrich Chemical Company. UV−Visible spectra were recorded on a Cary 5E UV−vis spectrophotometer at 25 °C. NMR spectra were recorded on a Bruker WM AMX 400 (400 MHz, ^{1}H; 100.6 MHz, ^{13}C) or a Bruker Avance 300 (300 MHz, ^{1}H; 75 MHz, ^{13}C; 282 MHz, ^{19}F) spectrometer at 300 K in the solvent stated and were referenced to TSP at δ 0 ppm (^{1}H), to external $CDCl_3$ at δ 77 ppm (^{13}C), or to external hexafluorobenzene at δ −163 ppm (^{19}F). Spectral assignments were made with the aid of standard 2D NMR techniques, including correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments. pD values were measured by use of a Beckman Φ11 meter and a Mettler NMR tube pH probe and are related to the pH meter reading by the formula pD = pH (meter reading) + 0.4.[46] Measured pD values are ±0.3 due to fluctuations in sample pD that occurred over time. Electrospray ionization mass spectra were recorded on a Finnigan LCQ ion-trap mass spectrometer. High-resolution electrospray ionization mass spectra were recorded on a Bruker ApexII Fourier transform ion cyclotron resonance mass spectrometer, 7.0 T magnet, fitted with an off-axis Analytica electrospray source (University of New South Wales, Sydney). HPLC was performed on a Waters HPLC system, comprising a Waters 510EF pump, a Waters U6K injector, a Waters R403 refractive index detector, and an Isco Model 226 absorbance monitor fitted with a 254 nm filter, and equipped with a RTI ZorbaxSil 7 μm preparative column. Reverse-phase HPLC was performed on a Waters HPLC system, comprising a Waters 600E multisolvent delivery system, a Rheodyne 7125i injector, and a Waters 486 absorbance detector, and equipped with either an Alltech Alltima C18 5 μm analytical column or an Alltech Alltima C18 10 μm preparative column. Melting points were recorded on a Reichert melting point stage and are uncorrected.

Molybdocene bis(*S*-glutathione) **2** was prepared as reported previously.[25] 4-Thio-2,3,5,6-tetrafluorobenzoic acid was prepared from 4-thio-2,3,5,6-tetrafluorobenzene[47] and gave characterization data identical to previously reported data.[48] This, in turn, was prepared from commercially available pentafluorobenzene.[49]

Molybdocene Bis(*S*-4-thio-2,3,4,6-tetrafluorobenzoic acid) (3). Molybdocene dichloride **1** (9.7 mg, 33 μmol) was

sonicated in water (1.5 mL) until dissolution was complete (2−3 h) to form a deep maroon solution. The pH was adjusted to 6 with dilute sodium hydroxide. A solution of 4-thio-2,3,4,6-tetrafluorobenzoic acid (14.8 mg, 65 μmol, 2 equiv) in water (1.5 mL, pH 6) was added, and the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated to give the sodium salt **3b** as a brown solid, which was dissolved in water (1.5 mL) and acidified with concentrated hydrochloric acid. The resultant brown solid was washed with water (2 × 1 mL) and then recrystallized from methanol and water to give pure **3a** (6.5 mg, 29%) as brown needles, mp >230 °C. λ_{max} (methanol)/nm 373 (log ϵ 3.31), 539 (2.12). ^{1}H NMR (300 MHz, D_2O) δ 5.48 (s, CpH) ppm. ^{19}F NMR (282 MHz; D_2O) δ −132.57 (2 F, m), −145.31 (2 F, m) ppm. ^{13}C{^{1}H} NMR (100 MHz; D_2O) δ 98.9 (s, CpC), 116.8 (t, J = 22.4 Hz), 123.6 (t, J = 22.4 Hz), 142.5 (dddd, $^1J_{FC}$ = 250.6 Hz), 148.9 (dm, $^1J_{FC}$ = 237.7 Hz), 166.6 (s, COOH) ppm. ESI-MS (+ve ion) 701 ([M + Na]$^+$, 100%). HRMS (+ve ion ESI) $C_{24}H_{12}MoO_4S_2F_8Na^+$ calcd 700.8997, found 700.9026.

Molybdocene Bis(*S*-1-thio-β-D-glucose) (4): Method 1. Molybdocene dichloride **1** (10 mg, 34 μmol) was sonicated in water (3 mL) until dissolution was complete (2−3 h) to form a deep maroon solution. The pH was adjusted to 6 with dilute sodium hydroxide. A solution of 1-thio-β-D-glucose (14.7 mg, 67 μmol, 2 equiv) in water (1 mL, pH 6) was added, and the pH was readjusted to 6. The reaction mixture was stirred at room temperature for 24 h under an atmosphere of nitrogen, then filtered, and the solvent was removed by freeze-drying to give the crude product, which was purified by reverse-phase HPLC (acetonitrile/water) to give pure **4** (13 mg, 65%) as a red residue, mp (decomp) 186−188 °C. λ_{max} (H_2O)/nm 343 (log ϵ 3.08), 484 (2.11). ^{1}H NMR (400 MHz, D_2O) δ 3.02 (2 H, m, H2), 3.30−3.34 (6 H, m, H3, H4, and H5), 3.62 (2 H, dd, $J_{H6H6'}$ = 12.7 Hz, J_{H6H5} = 3.4 Hz, H6), 3.83 (2 H, br d, $J_{H6'H6}$ = 12.7 Hz, H6′), 3.93 (2 H, d, J = 9.3 Hz, H1), 5.44 (10 H, s, CpH). ^{13}C{^{1}H} NMR (100 MHz, D_2O) δ 61.1 (C6), 69.9 (C5), 76.3 (C2), 77.1 (C3), 79.7 (C4), 92.4 (C1), 97.1 (CpC) ppm. m/z (+ve ion ESI) 641 (M + Na$^+$, 35%). HRMS (+ve ion ESI) $C_{22}H_{32}MoO_{10}S_2Na^+$ calcd 641.0388, found 641.0400.

Method 2. Sodium hydroxide (0.6 mmol, 0.3 M, 2 mL) was added to a solution of metallocene **5** (57.7 mg, 60 μmol) in acetonitrile (10 mL), and the reaction was stirred for 1 h. The pH was decreased to 7 with dilute hydrochloric acid, and the solvent was removed by freeze-drying to give the crude product. Purification by reverse-phase HPLC afforded pure **4**, which gave identical spectroscopic data to the sample prepared by method 1.

Molybdocene Bis(*S*-1-thio-2,3,4,5-tetraacetate-β-D-glucose) (5). Molybdocene dichloride **1** (35.6 mg, 120 μmol) was sonicated in water (8 mL) until dissolution was complete (2−3 h) to form a deep maroon solution. The pH was adjusted to 6 with dilute sodium hydroxide. A solution of 1-thio-2,3,4,5-tetraacetate-β-D-glucose (96 mg, 264 μmol, 2.2 equiv) in methanol (2 mL) was added, and the reaction mixture was stirred at room temperature for 24 h. Over this time, a purple precipitate formed, which was filtered to give the crude product as a purple amorphous solid. Purification by HPLC (10% hexane/EtOAc) afforded pure **5** (62.1 mg, 54%) as a red solid, mp >230 °C. λ_{max} (acetonitrile)/nm 349 (log ϵ 3.19), 478 (2.18). ^{1}H NMR (400 MHz, acetone-d_6) δ 1.91 (6 H, s, OAc), 1.98 (6 H, s, OAc), 2.03 (6 H, s, OAc), 2.04 (6 H, s, OAc), 3.84 (2 H, ddd, $J_{H5,H4}$ = 10.0 Hz, $J_{H5,H6}$ = 6.9 Hz, $J_{H5,H6'}$ = 2.5 Hz, H5), 4.09 (2 H, dd, $J_{H6,H6'}$ = 12.0 Hz, $J_{H6,H5}$ = 6.9 Hz, H6), 4.17 (2 H, dd, $J_{H6',H6}$ = 12.0 Hz, $J_{H6',H5}$ = 2.5 Hz, H6′), 4.50 (2 H, d, $J_{H1,H2}$ = 10.0 Hz, H1), 4.71 (2 H, dd, $J_{H2,H1}$ = 10.0 Hz, $J_{H2,H3}$ = 9.3 Hz, H2), 4.88 (2 H, dd, $J_{H4,H3}$ = 9.5 Hz, $J_{H4,H5}$ = 10.0 Hz, H4), 5.11 (2 H, dd, $J_{H3,H2}$ = 9.3 Hz, $J_{H3,H4}$ = 9.5 Hz, H3), 5.55 (10 H, s, CpH) ppm. ^{13}C{^{1}H} NMR (100 MHz, acetone-d_6) δ 20.6 (2 C, 2× −CH_3), 20.9 (−CH_3), 21.1 (−CH_3), 64.0 (C6), 70.4 (C4), 74.9 (C3), 75.1 (C2), 76.0 (C5), 92.1 (C1), 97.8 (CpC), 169.4 (CO), 170.1 (CO), 170.4 (CO), 170.8 (CO) ppm. ESI-MS (+ve ion) 591 ([M − sugar]$^+$, 45%), 977 ([M + Na]$^+$, 100). HRMS (+ve ion ESI) $C_{38}H_{48}MoO_{18}S_2Na^+$ calcd 977.1236, found 977.1268.

General Cell Culture Details. All media and solutions either were purchased sterile or were sterilized by autoclaving or by filtration through 0.2 μm filters. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. V79 Chinese hamster lung cells, originally supplied by R. Newbold (Institute of Cancer Research, Sutton, Surrey, U.K.), were cultured from frozen stocks, and maintained in growth medium consisting of Eagle's minimum essential medium (9.76 g/L, Sigma) and sodium hydrogencarbonate (2.2 g/L), supplemented with fetal calf serum (10%, Trace), penicillin (100 IU/mL, Trace) and streptomycin (100 μg/mL, Trace), at 37 °C under an atmosphere of 95% air/5% CO_2. The cells were routinely subcultured biweekly with trypsin [0.25% in phosphate-buffered saline (PBS)]. All sterile work was carried out in a sterile laminar fume hood.

Cytotoxicity Experiments. Cytotoxicity against V79 Chinese hamster lung cells was determined via a modified MTT assay.[35,36] For each complex tested, a 96-well microtiter plate was prepared by adding 100 μL of cells suspended in growth medium at a concentration of 1 × 10^5 cells/mL to each experimental and control well. Growth medium (200 μL) was added to one column to serve as a blank. Sterile water (200 μL) was added to the perimeter wells. The plate was then incubated at 37 °C under an atmosphere of 95% air/5% CO_2 for 24 h. PBS solutions (100 μL) of the complex to be assayed at eight concentrations were added to the experimental wells, and PBS (100 μL) was added to the blank and control wells. The plate was then incubated for 24 h. The medium and drug were then removed, and fresh growth medium (200 μL) was added. After a further 48 h, the medium was removed, and fresh growth medium (100 μL) and MTT in PBS (20 μL, 15 mM) were added to each well, resulting in the precipitation of purple formazan crystals in certain wells. After 3 h, sodium dodecyl sulfate (10% in 0.01 M HCl) was added to each well, and the plate was incubated for a further 24 h, to solubilize the formazan crystals. The absorbance of each well at 540 nm was determined by analysis with a Labsystems Original Multiskan RC plate reader, and the percentage cell viability was determined by dividing the average absorbance for each column of Mo-treated wells by the average absorbance of the control wells. The IC_{50} was determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells were viable relative to the control.

Cytotoxicity values for Cp_2MoCl_2 **1** against 2008 ovarian and MCF-7 breast cell lines were determined by the Coulter counting assay at the Peter McCallum Cancer Institute, Melbourne, Australia.

Cell Uptake Studies. Cells were seeded at a density of 5 × 10^5 cells/dish and incubated at 37 °C under an atmosphere of 95% air/5% CO_2 for 24 h. Cells were washed with phosphate-buffered saline (PBS), and fresh growth medium (5 mL) was added to each dish, followed by freshly prepared Mo solution (20 mM; 4 μmol/dish, 200 μL or 2 μmol/dish, 100 μL) or sterile water (100 μL). Quadruplicate dishes were prepared for each experiment, and each experiment was repeated at least twice. The dishes were incubated at 37 °C under an atmosphere of 95% air/5% CO_2 for a period of 4 or 24 h. Following treatment, the growth medium was removed and each dish was washed with PBS. Trypsin (0.25% in PBS) was added, and the cells were harvested after 5 min. Dishes were washed with PBS, and this was added to the cell suspension to ensure that all the cells were collected. The resulting suspension was centrifuged, and the supernatant was discarded. The cell pellet was washed with PBS (2 mL) and saline (0.9% NaCl, 99.999% pure, 2 mL), and the supernatant was discarded each time. The number of cells per dish was determined by scoring one dish out of each series of four. The Mo content was determined by graphite furnace atomic absorption spectroscopy, after digestion of the cells in concentrated HNO_3 (Tracepur, Merck) and dilution to 5.0 or 10.0 mL.

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