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A new series of titanocene dichloride derivatives bearing cyclic alkylammonium groups: Assessment of their cytotoxic properties

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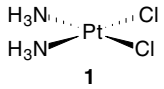
Abstract

Ten new water soluble titanocene dichloride derivatives have been synthesized and characterized and their cytotoxicities against the human lung cancer cell line A549 have been assessed. The potencies of the compounds vary greatly, but dicationic 3-picolylium and 4-picolylium compounds exhibit IC_{50} values that are unusually low for this class of compounds. In view of their potency against A549 cells, three of the new complexes were tested further on additional human cell lines including the small cell lung cancer cell line H69, the widely used cervical carcinoma cell line HeLa, the ovarian carcinoma cell line A2780 and its cisplatin resistant derivative A2780/CP. All three compounds exhibited potencies in all cell lines comparable to or better than those observed with the A549 cells, while one complex is actually more potent than cisplatin for HeLa cells.

Keywords: Anticancer drugs; Titanocene dichloride; Metallocenes

1. Introduction

The discovery of the anticancer activity of *cis*-diaminodichloroplatinum (II) (cisplatin, **1**) marked the beginning of a rich field of transition metal based medicinal chemistry.



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Currently cisplatin is still one of the three most widely used chemotherapeutic agents in the world and is prescribed in the treatment of a wide variety of tumors, notably ovarian, testicular, bladder, head and neck [1]. The mechanism of its antitumor activity has been shown to involve substitution of the mutually *cis* chloride ligands by water followed by the formation of 1,2-intrastrand cross-links with DNA [1d]. These cross links are created by the coordination of the dicationic moiety [*cis*-$Pt(NH_3)_2]^{2+}$ to adjacent guanine units. This in turns inhibits its DNA transcription and/or replication leading to cell death. Despite its remarkable success, however, cisplatin has several disadvantages including notable toxic side effects such as nephrotoxicity, neurotoxicity and emesis. In addition, some tumors exhibit inherent resistance to cisplatin while others develop resistance after initial treatment, thereby limiting its clinical usefulness [1c]. These particular disadvantages have driven the search for new compounds exhibiting high cytotoxic activity along with reduced side effects and non-cross resistance.

Since the mode of action of cisplatin involves coordination of DNA to cisplatin in a *cis* fashion, a great deal of research has focused on a variety of transition metal complexes bearing labile *cis* chloride or similar ligands. Among the candidate drugs, pseudotetrahedral metallocene complexes of the type Cp_2MCl_2 (M = Ti, V, Nb, Mo, Re;

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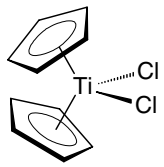
$Cp = \eta^5\text{-}C_5H_5$) represent a seemingly logical extension of cisplatin and have received much attention [2]. Of all the metallocenes investigated to date, titanocene dichloride [Cp_2TiCl_2, TDC, **2**] has been shown to exhibit the most promising chemotherapeutic activity.



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TDC has been shown to be very effective against Ehrlich ascites tumor, B16 melanoma, colon B adenocarcinoma, Lewis lung carcinoma and sarcoma 180 cells, and has undergone Phase II clinical trials [2] although interest in this direction appears to be waning [2d,2e]. TDC has significantly fewer toxic side effects than cisplatin and, despite its structural similarity to cisplatin, it is quite effective against cisplatin resistant cancer cell lines, strongly suggesting that TDC has a different mechanism of action.

The aqueous chemistry of TDC involves rapid hydrolytic substitution of the chloride ligands [3], which provides an explanation for the observation that substitution of the chloride ligands of TDC with other anionic ligands results in very little change in drug activity [2a]. There follows a series of pH dependent equilibria involving soluble ionic species such as $[(\eta^5\text{-}C_5H_5)_2Ti(Cl)(H_2O)]^+$ and, eventually, precipitation of hydrated titanium oxides and the formation of cyclopentadiene (Fig. 1) [3]. However, in the presence of nucleotides, the highly oxophilic titanium(IV) of TDC is reported to target the phosphoester groups of the nucleotides rather than the nucleotide bases [2g] targeted by platinum(II)-based drugs, at least in vitro. The corresponding Cp_2Ti–DNA adducts are believed to prevent replication and/or transcription, resulting in cell death, but the differences in mode of action likely explain, at least in part, why TDC is effective against cisplatin resistant cell lines.

Most research in the past decade has focused on modifying TDC via derivatization of the cyclopentadienyl rings, although earlier work involving the incorporation of electron donating or withdrawing groups yielded mixed results. Electron donating groups diminished the cytotoxicities of the compounds assessed [2a], a not unexpected observation if a $[Cp_2Ti]^{2+}$ cation binds to DNA since electron donating groups would diminish the Lewis acidity of a metal center. This rationale prompted earlier work in which electron withdrawing groups such as esters were incorporated into the rings in the compounds $(\eta^5\text{-}C_5H_4CO_2R)_2TiCl_2$ and $(\eta^5\text{-}C_5H_5)(\eta^5\text{-}C_5H_4CO_2R)TiCl_2$ (R = Me, *t*-Bu, Ph) [4]. However, the complexes studied exhibited little or no increase in cytotoxic potency relative to TDC.

A major disadvantage to TDC is its very low aqueous solubility, which early on resulted in the development of water soluble clinical formulations that could be administered to patients. One of the most widely employed formulations, MKT-4, is generated by reacting TDC with a polyol such as mannitol in refluxing aqueous sodium chloride solution (Fig. 2). The resulting mixture is freeze-dried to produce an air and water stable material of uncertain composition, but water soluble and hence suitable for administration [5].

More recently, research has focussed on the synthesis of novel TDC derivatives which are inherently soluble in aqueous media and which have more potent cytotoxic activities. One approach has involved substitution of the chlorides by alkoxides and thiols [6], while others involve incorporation of neutral but polar functional groups into the Cp rings [7,8]. Many of the resulting TDC derivatives exhibit promising cytotoxic activities.

Recent work in our [9] and other [10] laboratories to enhance the cytotoxic properties of TDC derivatives has involved functionalization of the cyclopentadienyl rings with pendant alkylammonium substituents. Complexes **3** and **4** and similar candidates have been shown to exhibit useful cytotoxicities (IC_{50} values in the low μM range) against several cancer cell lines [9a]. Interestingly, dicationic TDC derivatives bearing cyclic alkylammonium substituents, such as **3** and **4**, were found to exhibit *generally*

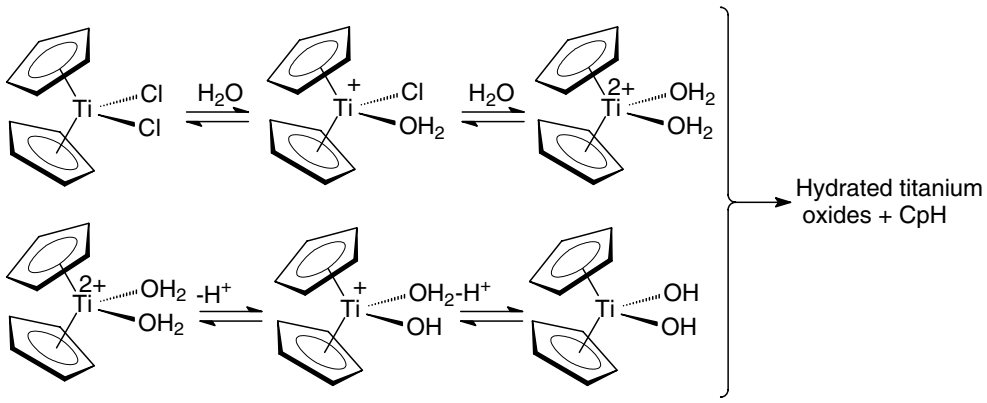


Fig. 1. Hydrolysis of TDC in water.

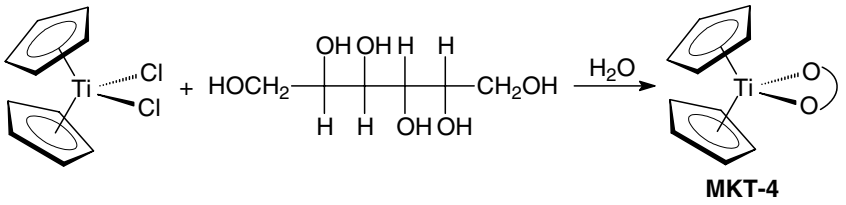
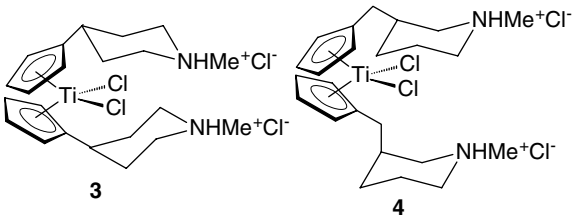


Fig. 2. Preparation of the clinical formulation MKT-4.

greater potencies than either the corresponding monocationic TDC derivatives containing the same functionalized ring plus one unsubstituted Cp ring or TDC derivatives containing acyclic pendant ammonium groups.



In an attempt to exploit these apparent correlations, especially the enhanced potencies of cyclic candidates, we are investigating a new series of cyclic TDC derivatives of the types $[Cp(\eta^5\text{-}C_5H_4R)TiCl_2]Cl$ and $[(\eta^5\text{-}C_5H_4R)_2\text{-}TiCl_2]Cl_2$ where R = cyclic pyrrolidylium, morpholinium and picolylium groups. We now report our findings, which demonstrate that while this approach can indeed yield highly potent new drugs, there remain significant, as yet unexplained variations in potency among cyclic derivatives of TDC.

2. Results and discussion

2.1. Syntheses and structures of the new TDC derivatives

The new cyclic and aryl compounds **5a**,**b**–**9a**,**b**, shown in Fig. 3, were prepared using the standard procedures developed previously [9] and outlined in Fig. 4. Thus cyclopentadienes containing the desired pendant cyclic amine functionalities were synthesized by treating solu-

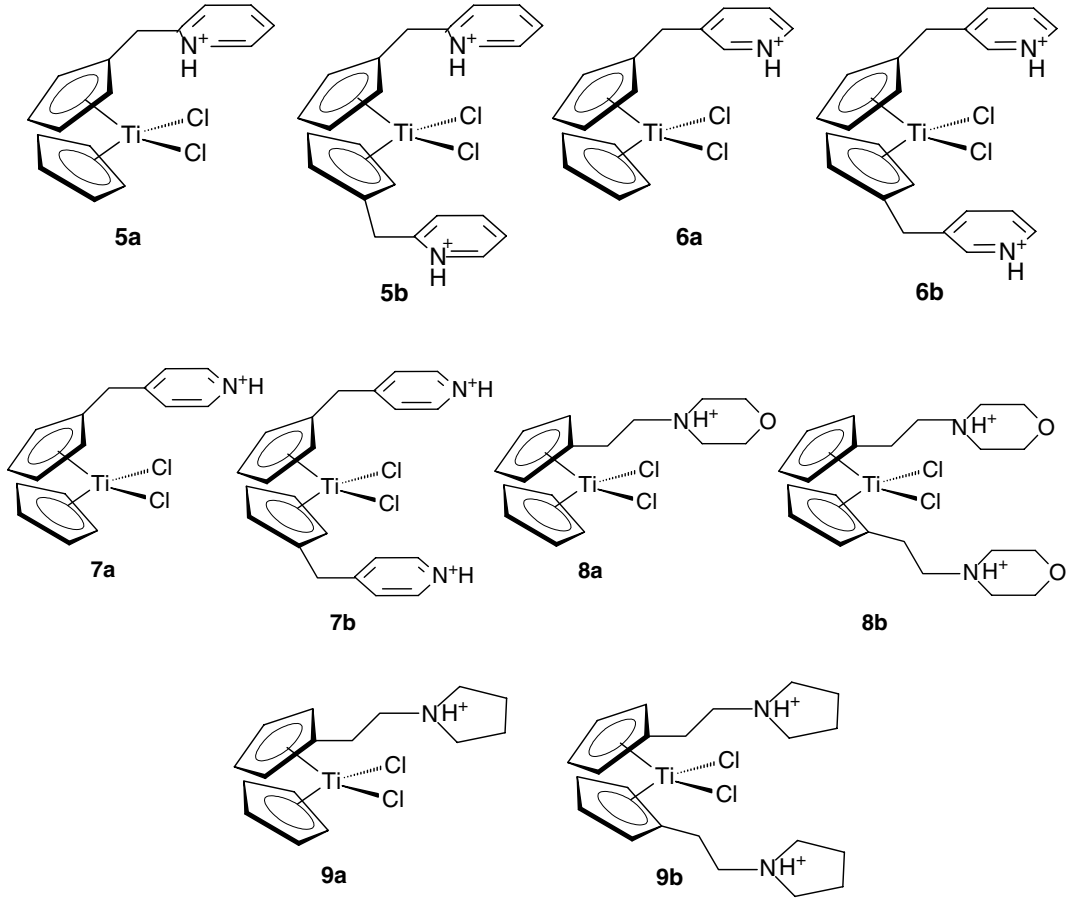


Fig. 3. Monocationic and dicationic TDC derivatives synthesized and assessed.

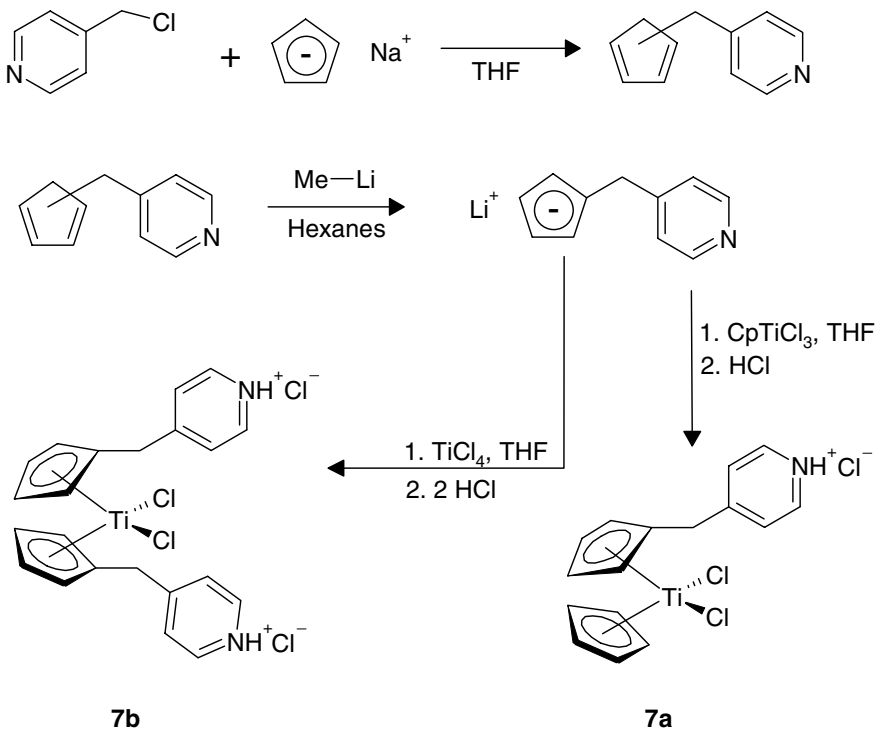


Fig. 4. Synthesis of representative complexes **7a** and **7b**.

tions of the hydrochlorides of 2-, 3- or 4-picolyl chloride, 4-(2-chloroethyl)morpholine or 1-(2-chloroethyl)pyrrolidine with a slight excess of NaCp. The resulting mixtures of isomeric cyclopentadienes were then deprotonated with methyl lithium to give the corresponding cyclopentadienyl salts, which were reacted in turn with either $CpTiCl_3$ (1:1 molar ratio) or $TiCl_4$ (2:1 molar ratio) to give respectively mono- and disubstituted TDC derivatives. In situ treatment of the THF solutions of these with 2 M HCl in ether then resulted in orange–red precipitates of the products, which were recrystallized as appropriate. All were obtained analytically pure as chloride salts and were characterized by ^{1}H and ^{13}C NMR spectroscopy and electrospray mass spectrometry (see Section 4). All exhibit at least millimolar solubilities in water.

Interestingly, recrystallizations of several of the new complexes, **5b**, **7a** and **9b**, were found to yield crystals suitable for X-ray crystallography, and the molecular structures of these three complexes were determined to add to the previously reported data base [9a] of this class of TDC derivatives. The structures are shown in Fig. 5, crystallographic data are shown in Table 1 and important bond lengths and angles are summarized in Table 2 where they are compared with analogous data for TDC and **3** [9a]. Complete structural information is available in Supplementary material.

As can be seen in Fig. 5 and Table 2, the structures of the three derivatives consist of the anticipated pseudotetrahedral arrangements of ligands about the titanium atoms, with the important bond lengths and angles being very similar to those of TDC and **3**. These results are consistent with previous findings [9a] that modification of the alkylammonium substituents on the rings has little effect on the ground state structures of the compounds and thus that differences in potencies (see below) are not to be related to fundamental structural differences.

2.2. Cytotoxicity studies

Using procedures described previously [9a], hydrochloride salts of the ten complexes **5a**,**b**–**9a**,**b** were tested initially for their cytotoxic effects on the benchmark human non-small cell lung cancer cell line A549. The cell line A549 is used as a preliminary screening test because it is particularly robust. This series embraces a range of structures which complement and add to the previously reported series of cyclic and acyclic alkylammonium complexes typified best by **3** and **4**, and includes aromatic (**5a**,**b**–**7a**,**b**) and saturated cyclic (**8a**,**b**, **9a**,**b**) candidate drugs. In addition, to eliminate the possibility that the ligand rather than the complex was the cytotoxic species, the hydrochloride salt of (3-picolyl)cyclopentadiene (**6c**) was prepared and its cytotoxicity was also assessed against A549 cells.

The IC_{50} data obtained are shown in Table 3 and, as is readily evident, there are clearly wide variations in potency for compounds with what appear to be very subtle structural differences. Thus the compounds **5a**,**b** and **7a**, which contain aromatic rings similar to **6a**,**b** and **7b**, nonetheless

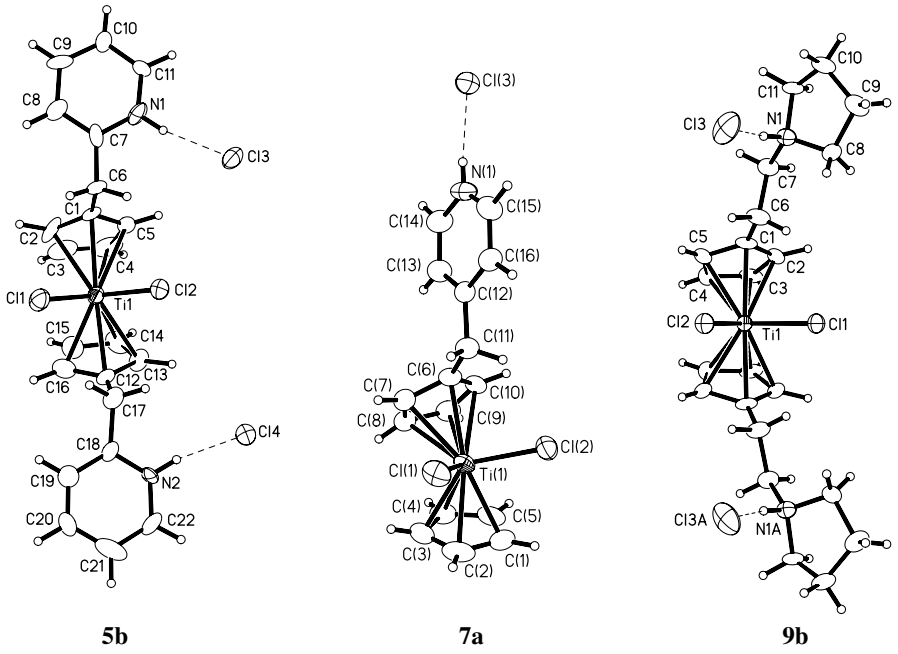


Fig. 5. Molecular structures of **5b**, **7a** and **9b**.

all exhibit very low potencies against A549 cells with IC_{50} values above the upper limit of the concentration range studied (200 μM). In contrast, the 3-picolyl complexes **6a**,**b** and the 4-picolyl complex **7b** exhibit much higher potencies than do MKT-4 or any of the other new complexes studied. Indeed, the IC_{50} values of the *dicationic* **6b** and **7b** were found respectively to be only factors of ~5 and ~3 times that of cisplatin (congruent with previous findings that dicationic analogues are generally more potent than their monocationic analogues [9a]). Furthermore the potency of **7b**, which contains pyridinium groups, is comparable with those of any other TDC derivative [7,9,10]. In contrast, **8a**,**b** and **9a**,**b**, which also contain cyclic alkylammonium groups, are much less potent than are **3** and **4**. As expected, the hydrochloride salt (3-picolyl)-cyclopentadiene **6c** is not effective, its IC_{50} value being >200 μM.

Table 1
Crystallographic data for compounds **5b**, **7a**, and **9b**

Compound	**5b**	**7a**	**9b**
Empirical formula	$C_{24}H_{28}Cl_4N_2OTi$	$C_{16}H_{16}Cl_3NTi$	$C_{22}H_{35}Cl_4N_2O_{0.5}Ti$
Formula weight	550.18	376.55	525.22
Crystal system	Monoclinic	Triclinic	Orthorhombic
Lattice parameters			
a (Å)	10.692(3)	7.6841(9)	32.160(11)
b (Å)	16.829(5)	9.6246(12)	11.639(4)
c (Å)	28.000(9)	11.9385(15)	6.547(2)
α (°)	90	108.374(2)	90
β (°)	93.751(6)	100.760(2)	90
γ (°)	90	93.465(2)	90
V (Å^3)	5027(3)	816.42(17)	2450.4(14)
Space group	$P2_1/n$	$P\bar{1}$	$Ama2$
Z	8	2	4
ρ_{calc} (Mg m^{-3})	1.454	1.532	1.424
μ (Mo-Kα) (Å)	0.71073	0.71073	0.71073
Temperature (K)	180(2)	180(2)	180(2)
$2\theta_{max}$ (°)	52	50	52
Reflections collected	31,347	4843	7682
Data/restraints/parameters	9881/1/487	2851/0/254	2130/1/135
R Indices ($I > 2\sigma I$)			
R	0.0975	0.0445	0.0487
wR_2	0.1309	0.0916	0.0711
R Indices (all data)			
R	0.3240	0.0677	0.1093
wR_2	0.1679	0.0976	0.0821
Largest peak final difference in map (e Å^{-3})	0.510	0.472	0.536

In view of their potency against A549 cells, complexes **6a**, **6b** and **7b** were tested further on additional human cell lines derived from solid tumors that are known to frequently display inherent resistance to many cytotoxic agents. The cell lines tested included the small cell lung cancer cell line H69, the widely used cervical carcinoma cell line HeLa, the ovarian carcinoma cell line A2780 and its cisplatin resistant derivative A2780/CP. As summarized in Table 3, all three compounds exhibited potencies in all cell lines comparable to or better than those observed with the A549 cells. In addition and surprisingly, complex **7b** is actually more potent than cisplatin for HeLa cells. We note also that the mechanism responsible for cisplatin resistance in the A2780/CP cells does not confer resistance to any of the TDC derivatives, suggesting the potential usefulness of these compounds in drug resistant tumors. These observations indicate that further studies of these agents for their activity in solid tumor models is warranted.

Table 2
Selected bond distances and angles of **5b**, **7a**, **9b**, **3** and TDC

Compound	Ti–Cl (Å)	Ti–Cp′(cent)[a] (Å)	Cl–Ti–Cl (°)	Cent-Ti-cent (°)[b]
5b	2.342(3), 2.360(3), 2.337(3), 2.355(3)	2.046, 2.063, 2.077, 2.057	94.17 (12) 92.43 (12)	133.4, 132.6
7a	2.3546(11), 2.3546(12)	2.061, 2.052	93.66 (4)	131.4
9b	2.355(4), 2.367(3)	2.048, 2.048	92.04(9)	132.5
3	2.3638(7), 2.3638(7)	2.079	93.87(4)	130.4
TDC	2.367(2), 2.361(1), 2.363(1), 2.365(2)	2.058	94.43(6), 94.62(6)	131.6

[a] Cp′ = Cp or substituted cyclopentadienyl; cent = ring centroid.
[b] Angle calc. using PLATON [11].

Table 3
Effect of TDC analogues on human tumor cell viability

Compound	Cell line (IC_{50}, μM)[a]				
	A549	H69	HeLa	A2780	A2780/CP
5a	>200				
5b	>200				
6a	135.9 ± 23.9	29.2 ± 11.1	114.2 ± 57.0	22.9, 22.8	32.9, 61.2
6b	41.2 ± 16.9	28.1 ± 17.9	55.9 ± 16.2	21.1, 12.4	26.1, 12.8
6c	>200				
7a	>200				
7b	24.7 ± 6.9	13.3 ± 3.2	10.8 ± 0.6	12.1 ± 6.1	25.4 ± 7.7
8a	>200				
8b	>200				
9a	>200				
9b	>200				
MKT-4	>200	>200	>200	>200	>200
Cisplatin	8.6 ± 2.5	1.9 ± 1.3	26.7, 18.9	0.9, 1.6	7.4, 4.0

[a] Data are means (±SD) of 3–5 independent experiments. Where <3 experiments were done, individual results are shown.

3. Summary

Ten new water soluble TDC derivatives, all similar in functionality to previous compounds which exhibit promising cytotoxic activities, have been synthesized and characterized. The cytotoxicities of the ten compounds against the human lung cancer cell line A549 have been assessed, and the dicationic compounds **6b** and **7b** exhibit IC_{50} values as low as any for this class of compounds. The IC_{50} values of the analogous monocationic compound **6a** and **7a** are consistent with the literature, while the two pyrrolidylium compounds **9a, 9b** and two morpholynium compounds **8a**,**8b** exhibit very low potencies ($IC_{50} > 200$ μM). Thus, in agreement with previous findings on similar compounds but for reasons which at present are unknown, there are remarkable variations in the potencies of drug candidates exhibiting similar structural features. Nonetheless the potency of **6b** demonstrates that this approach to the development of new TDC-based anticancer drugs remains very deserving of attention.

4. Experimental

All synthetic procedures were carried out under an atmosphere of argon purified by passage through a column of BASF catalyst heated to 140 °C and a column of 5 Å molecular sieves. Manipulation of air-sensitive materials employed standard Schlenk line techniques and an Mbraun Labmaster glovebox. Solvents were taken directly from anhydrous and deoxygenated grade solvents from Aldrich after passing through activated alumina columns. All chemicals were purchased from Aldrich and were purified as appropriate before use.

The ^{1}H, ^{13}C NMR and 2-D spectra were run on a Bruker Avance 600 MHz NMR spectrometer with the residual proton resonances of the deuterated solvents serving as internal references. In the case of ^{13}C spectra, the carbon resonances of the solvents or a small addition of methanol were used as internal references. Mass spectra were obtained on a Quatro Fisons Pro Quadrupole mass spectrometer in ES+ mode with a solution of nitromethane and 5% methanol used as a solvent. Canadian Microanalytical Services of Delta, B.C., performed elemental analyses. The X-ray crystallographic structure determination was carried out using a Bruker SMART CCD 1000 X-ray diffractometer with graphite-monochromated Mo-Kα radiation ($\lambda = 0.71073$ Å) controlled with Crysostream Controller 700. Typically a crystal was mounted on a glass fiber with epoxy glue. No significant decay was observed during data collection. Data were processed on a Pentium PC using the Bruker AXS Windows NT SHELXTL software package (version 5.10) (see Supplementary material). The raw intensity data were converted (including corrections for scan speed, background, and Lorentz and polarization effects) to structure amplitudes and their esds using the program SAINT, which corrects for *Lp* and decay. Absorption

corrections were applied using the program SADABS. All non-hydrogen atoms were refined anisotropically. The positions for all hydrogen atoms were calculated, and their contributions were included in the structure factor calculations.

4.1. Synthesis of mixture of isomers of (2-picolyl)-cyclopentadiene

2-Picolyl chloride hydrochloride (25.52 g, 0.156 mol) was added to an aqueous solution of NaOH (8.6 g, 0.215 mol). The solution was extracted three times with toluene (50 mL), and the organic layers were combined and dried with $MgSO_4$ and then slowly added dropwise to a solution of NaCp in THF (2 M, 85 mL, 0.17 mol) at 0 °C. An off-white precipitate formed during the addition and the reaction mixture turned brown. After allowing the mixture to stir overnight an excess of H_2O was added. The layers were separated and the organic layer was dried with $MgSO_4$. Removal of the solvent gave a dark, golden oil which was purified by vacuum distillation (80–84 °C, 0.54 mm Hg) to give a clear, colorless oil. Yield: 13.2 g, 54%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.53 (m, 2H), 7.57 (m, 2H), 7.14 (m, 2H), 7.09 (m, 2H), 6.5–6.0 (m, 6H), 3.93, 3.90 (2 × s, 4H), 2.98, 2.91 (2 × s, 4H).

4.2. Synthesis of (2-picolyl)cylopentadienyllithium

Freshly distilled (2-picolyl)cyclopentadiene (2.32 g, 14.8 mmol) was dissolved in hexanes (100 mL) and a solution of methyllithium (1.6 M, 15 mmol) in ethyl ether was added dropwise to give immediately an orange precipitate. The reaction mixture was stirred for 30 min and the product was collected with a Schlenk filter. After washing the precipitate with cold hexanes (2 × 50 mL), the product was dried overnight under vacuum. Yield: 2.0 g, 84%. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.30 (d, 1H, $^3J = 4.8$ Hz), 7.51 (m, 1H), 7.28 (d, 1H, $^3J = 7.8$ Hz), 6.99 (m, 1H), 5.20 (m, 4H), 3.83 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 166.4, 147.5, 135.0, 123.1, 119.4, 114.6, 103.6, 102.8, 40.9.

4.3. Synthesis of **5a**

(2-Picolyl)cyclopentadienyllithium (0.932 g, 5.72 mmol) was dissolved in THF (50 mL) and the solution was added slowly to a solution of $CpTiCl_3$ (1.30 g, 5.93 mmol) in THF (100 mL) at 0 °C. The reaction mixture turned dark red and formed a light brown precipitate during the addition. The mixture was stirred for 2 h and then filtered, and the resulting dark red filtrate was treated with an excess of HCl (2 M) to give an orange precipitate. The red supernatant was discarded and the product was dissolved in ethanol (10 mL) and added dropwise to diethyl ether (250 mL), resulting in precipitation of a red powder. The product was filtered and dried overnight in vacuo. The compound was dissolved in ethanol and recrystallized by slow evaporation, giving dark red crystals suitable for elemental analysis. Yield: 1.6 g, 74%. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.77 (d, 1H, $^3J = 4.2$ Hz), 8.40 (m, 1H), 7.87 (d, 1H, $^3J = 7.2$ Hz), 7.82 (m, 1H), 6.79 (m, 2H), 6.75 (s, 5H), 6.65 (m, 2H), 4.38 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 155.0, 145.1, 142.5, 130.7, 126.8, 124.6, 123.9, 120.7, 116.5, 34.5. Mass spectra (ES, m/z (%)): 340 (1) [M+H], 304 (100) [M-Cl], 285 (30), 220 (10), 158 (50). Analytical calc for **5a**: C, 51.0; H, 4.28; N, 3.72. Found: C, 50.6; H, 4.42; N, 3.61%.

4.4. Synthesis of **5b**

(2-Picolyl)cyclopentadienyllithium (0.837 g, 5.13 mmol) was dissolved in THF (100 mL), and $TiCl_4$ (1 M, 2.6 mmol) solution in toluene was slowly added dropwise at 0 °C. During the addition the reaction mixture turned dark red with the formation of a brown precipitate. After complete addition, the reaction mixture was stirred for 2 h and then filtered. The resulting dark red filtrate was treated with an excess of HCl (2 M in ether) resulting in the immediate formation of an orange precipitate. The light red supernatant was discarded and the solid was dissolved in ethanol (15 mL). Red crystals immediately began to form that were suitable for elemental and x-ray crystallographic analyses. The supernatant was added dropwise to diethyl ether (400 mL) and a dark orange powder precipitated. The orange powder was isolated by filtration and dried overnight under vacuum. Yield: 1.0 g, 78%. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.79 (d, 2H, $^3J = 4.8$ Hz), 8.45 (m, 2H), 7.93 (d, 2H, $^3J = 7.8$ Hz), 7.87 (m, 2H), 6.84 (m, 4H), 6.73 (m, 4H), 4.42 (s, 4H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 154.7, 145.5, 142.3, 130.9, 127.0, 125.0, 123.6, 117.2, 34.4. Mass Spectra (ES, m/z (%)): 429 (5) [M−Cl+MeOH], 395 (100) [M−Cl], 239 (12), 216.5 (100) $[M+H]^{+2}$, 198 (80), 157 (50). Anal. Calc. for **5b**: C, 52.42; H, 4.40; N, 5.56. Found: C, 52.19; H, 4.46; N, 5.34%.

4.5. Synthesis of mixture of isomers of (3-picolyl)-cyclopentadiene

This compound was prepared as above as a yellow oil in 46.8% yield using 3-picolyl chloride hydrochloride. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.48 (2 × s, 2H), 8.44 (2 × m, 2H), 7.48 (2 × m, 2H), 7.18 (2 × m, 2H), 6.5–5.9 (m, 6H), 3.71, 3.67 (2 × s, 4H), 2.96, 2.83 (2 × s, 4H).

4.6. Synthesis of (3-picolyl)cyclopentadiene hyrdrochloride (**6c**)

(3-Picolyl)cyclopendiene (2.35 g, 0.0150 mol) was dissolved in diethyl ether (100 mL) and treated with an excess of HCl in diethyl ether (2 M). During the addition a fine, white precipitate formed which was then filtered and washed with ether. Yield: 2.4 g, 83%. ^{1}H NMR (500 MHz, D_2O): δ 8.49–8.54 (m, 4H), 7.53 (m, 2H), 7.24 (m, 2H), 6.01–6.47 (m, 6H), 3.77, 3.73 (2 × s, 4H), 3.02, 2.89 (2 × s, 4H).

4.7. Synthesis of (3-picolyl)cyclopentadienyllithium

This compound was prepared as above as an off-white precipitate in 76% yield. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.37 (s, 1H), 8.22 (d, 1H, $^3J = 4.2$ Hz), 7.54 (d, 1H, $^3J = 7.2$ Hz), 7.15 (d of d, 1H), 5.19 (m, 2H), 5.16 (m, 2H), 3.71 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 149.7, 145.2, 141.9, 135.7, 122.6, 115.7, 103.1, 102.8, 35.2.

4.8. Synthesis of **6a**

This compound was prepared as was **5a** from the reaction of (3-picolyl)cyclopentadienyllithium and $CpTiCl_3$. Dark red crystals were obtained in 85% yield. ^{1}H NMR (600 MHz, D_2O): δ 8.68 (d, 1H, $^3J = 6.0$ Hz), 8.62 (s, 1H), 8.45 (d, 1H, $^3J = 7.8$ Hz), 8.02 (d of d, 1H), 6.66 (s and m, 7H), 6.54 (s, 2H), 4.09 (s, 2H). ^{13}C NMR (151 MHz, D_2O): δ 148.2, 141.4, 140.5, 140.1, 136.9, 127.9, 119.7, 119.2, 117.6, 32.6. Mass spectra (ES, *m/z* (%)) = 340 (90) [M+H], 304 (20) [M−Cl],158 (15), 99 (15). Anal. Calc. for **6a**: C, 51.0; H, 4.28; N, 3.72. Found: C, 50.9; H, 4.29; N, 3.71%.

4.9. Synthesis of **6b**

This compound was prepared as was **5b** from the reaction of (3-picolyl)cyclopentadienyllithium and $TiCl_4$ in a 2:1 ratio. The dark red compound was obtained in 83% yield. ^{1}H NMR (600 MHz, D_2O): δ 8.69 (d, 2H, $^3J = 6.0$ Hz), 8.65 (s, 2H), 8.47 (d, 2H, $^3J = 8.4$ Hz), 8.03 (d of d, 2H), 6.64 (m, 4H), 6.56 (m, 4H), 4.12 (s, 4H). ^{13}C NMR (151 MHz, D_2O): δ 148.0, 141.4, 140.5, 140.0, 135.8, 127.9, 118.7, 117.7, 32.6. Mass spectra (ES, *m/z* (%)) 431 (50) [M+H], 427 (20) [M−Cl+MeOH], 395 (25) [M−Cl], 313 (30), 239 (10), 158 (100). Anal. Calc. for **6b**: C, 52.4; H, 4.40; N, 5.56. Found: C, 51.7; H, 4.34; N, 5.51%.

4.10. Synthesis of mixture of isomers of (4-picolyl)-cyclopentadiene

This compound was prepared as above as a yellow oil in 61% yield from 4-picolyl bromide hydrobromide. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.49 (m, 4H), 7.11 (m, 4H), 6.5–6.0 (m, 6H), 3.71, 3.68 (2 × s, 4H), 2.98, 2.83 (2 × s, 4H).

4.11. Synthesis of (4-picolyl)cyclopentadienyllithium

This compound was prepared as above as an light orange precipitate in 95% yield. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.29 (d, 2H, $^3J = 6.0$ Hz), 7.17 (d, 2H, $^3J = 6.0$ Hz), 5.20 (m, 2H), 5.17 (m, 2H), 3.67 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 155.5, 148.5, 124.1, 114.4, 103.2, 102.9, 37.3.

4.12. Synthesis of **7a**

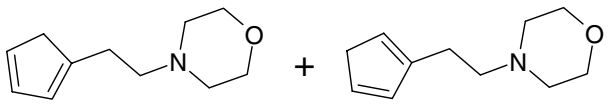
This compound was prepared as was **5a** from the reaction of (4-picolyl)cyclopentadienyllithium and $CpTiCl_3$. The supernatant from this reaction was layered with ether producing crystals that were suitable for elemental and x-ray crystallographic analyses. An orange powder was obtained in 75% yield. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.82 (d, 2H, $^3J = 6.6$ Hz), 7.92 (d, 2H, $^3J = 6.6$ Hz), 6.80 (m, 2H), 6.72 (s, 5H), 6.51 (m, 2H), 4.26 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 159.7, 141.7, 132.1, 127.1, 123.8, 120.5, 116.5, 36.2. Mass spectra (ES, *m/z* (%)): 340 (30) [M+H], 304 (100) [M−Cl], 171 (10) $[M+H]^{2+}$, 157 (35). Anal. Calc. for **7a**: C, 51.0; H, 4.28; N, 3.72. Found: C, 50.9; H, 4.43; N, 3.80%.

4.13. Synthesis of **7b**

This compound was prepared as was **5b** from the reaction of (4-picolyl)cyclopentadienyllithium and $TiCl_4$ in 2:1 ratio. The dark orange compound was obtained in 88% yield. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.86 (d, 4H, $^3J = 6.0$ Hz), 7.97 (d, 4H, $^3J = 6.0$ Hz), 6.83 (m, 4H), 6.58 (m, 4H), 4.28 (m, 4H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 160.0, 141.3, 132.1, 127.3, 123.5, 116.9, 36.2. Mass spectra (ES, *m/z* (%)): 431 (20) [M+H], 429 (40) [M−Cl+MeOH], 395 (40) [M−Cl], 313 (25), 270 (8), 239 (10). Anal. Calc. for **7b**: C, 52.4; H, 4.40; N, 5.56. Found: C, 51.7; H, 4.30; N, 5.39%.

4.14. Synthesis of mixture of isomers of [2-(1-morpholinyl)-ethyl]cyclopentadiene

This compound was prepared as above as a colorless oil in 74% yield using 4-(2-chloroethyl)morpholine hydrochloride. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.4–5.9 (m, 6H), 3.66 (m, 8H), 2.89, 2.85 (2 × s, 4H), 2.56 (m, 4H), 2.49 (m, 4H), 2.38 (m, 8H).



4.15. Synthesis of [2-(1-morpholinyl)ethyl]cyclopentadienyl lithium

This compound was prepared as above as an off-white precipitate in 72% yield. ^{1}H NMR (600 MHz, C_5D_5N): δ 6.34 (m, 2H), 6.26 (m, 2H), 3.69 (m, 4H), 3.02 (t, $^{3}J = 7.5$ Hz, 2H), 2.80 (t, $^{3}J = 7.5$ Hz), 2.46 (m, 4H). ^{13}C NMR (151 MHz, C_5D_5N): δ 118.1, 104.2, 104.1, 67.6, 63.6, 54.8, 28.9.

*4.16. Synthesis of **8a***

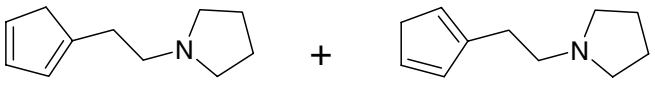
This compound was prepared as was **5a** from the reaction of [2-(1-morpholinyl)ethyl]cyclopentadienyllithium and $CpTiCl_3$. An orange powder was obtained in 63% yield. ^{1}H NMR (600 MHz, D_2O): δ 6.66 (2 × s, 7H), 6.50 (m, 2H), 4.12, 3.81 (2 × m, 4H), 3.54, 3.21 (2 × m, 4H), 3.41 (t, $^{3}J = 7.2$ Hz), 2.96 (t, $^{3}J = 7.2$ Hz). ^{13}C NMR (151 MHz, D_2O): δ 135.9, 119.5, 118.3, 117.5, 64.4, 56.6, 52.5, 24.3. Mass Spectra (ES, *m/z* (%)) = 362 (25) [M+H], 344 (90) [M−H_2O], 326 (100) [M−Cl], 308 (15), 264 (12). Anal. Calc. for **8a** · H_2O: C, 46.1; H, 5.81; N, 3.36. Found: C, 45.8; H, 5.52; N, 3.71%.

*4.17. Synthesis of **8b***

This compound was prepared as was **5b** from the reaction of [2-(4-morpholinyl)ethyl]cyclopentadienyllithium $TiCl_4$ in a 2:1 ratio. The orange–red compound was obtained in 96% yield. ^{1}H NMR (600 MHz, D_2O): δ 6.407 (s, 4H), 6.319 (s, 4H), 3.893, 3.593 (2 × m, 8H), 3.326, 2.997 (2 × m, 8H), 3.188 (t, $^{3}J = 7.8$ Hz), 2.734 (t, $^{3}J = 7.8$ Hz). ^{13}C NMR (151 MHz, D_2O): δ 134.0, 117.4, 116.3, 63.4, 56.0, 51.5, 23.4. Mass spectra (ES, *m/z* (%)) = 475 (100) [M+H], 471 (40) [M−Cl+MeOH], 457 (70) [M−H_2O], 324 (12), 292 (25), 288 (40), 238 (15) $[M]^{+2}$. Anal. Calc. for **8b** · $2H_2O$: C, 45.2; H, 6.56; N, 4.79. Found: C, 44.4; H, 6.31; N, 4.78%.

4.18. Synthesis of [2-(1-pyrrolidyl)ethyl]cyclopentadiene

This compound was prepared as above as a colorless oil in 66% yield using 1-(2-chloroethyl)pyrrolidine hydrochloride. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.5–6.0 (m, 6H), 2.91, 2.87 (2 × s, 4H), 2.60 (s, 4H), 2.51 (s, 4H), 2.49 (m, 8H,5), 1.76 (m, 8H, 6).



4.19. Synthesis of [2-(1-pyrrolidyl)ethyl]cyclopentadienyl-lithium

This compound was prepared as above as an off-white precipitate in 67.3% yield. ^{1}H NMR (600 MHz, C_5D_5N): δ 6.32 (m, 2H), 6.24 (m, 2H), 3.02 (t, 2H, $^{3}J = 7.2$ Hz), 2.79 (t, 2H, $^{3}J = 7.2$ Hz), 2.45 (m, 4H), 1.45 (m, 4H). ^{13}C NMR (151 MHz, C_5D_5N): δ 117.9, 104.7, 103.6, 61.6, 54.7, 30.9, 24.1.

*4.20. Synthesis of **9a***

This compound was prepared as was **5a** from the reaction of [2-(1-pyrrolidyl)ethyl]cyclopentadienyllithium (0.498 g, 2.95 mmol) and $CpTiCl_3$. Dark red crystals were obtained in 71.8% yield. ^{1}H NMR (600 MHz, D_2O): δ 6.63 (2 × s, 7H), 6.50 (s, 2H), 3.61, 3.06 (2 × m, 4H), 3.40 (t, 2H, $^{3}J = 7.2$ Hz), 2.90 (t, 2H, $^{3}J = 7.2$ Hz), 2.11, 1.96 (2 × m, 4H). ^{13}C (151 MHz, D_2O): δ 135.1, 118.7, 117.8, 116.7, 54.3, 54.0, 25.8, 22.6. Mass spectra (ES, *m/z* (%)) = 346 (80) [M+H], 344 (10) [M−Cl+MeOH], 311 (10) [M−Cl], 84 (100). Anal. Calc. for **9a**: C, 50.2; H, 6.06; N, 3.66. Found: C, 50.6; H, 5.57; N, 4.40%.

*4.21. Synthesis of **9b***

This compound was prepared as was **5b** from the reaction of [2-(1-pyrrolidyl)ethyl]cyclopentadienyllithium and $TiCl_4$ in a 2:1 ratio. The dark red compound was obtained in 49% yield. Recrystallization from layering a dichloromethane solution with hexanes produced crystals suitable for x-ray crystallographic analysis. ^{1}H NMR (600 MHz, D_2O): δ 6.65 (s, 4H), 6.56 (s, 4H), 3.66, 3.11 (2 × m, 8H), 3.45 (t, 4H, $^{3}J = 7.5$ Hz), 2.94 (t, 4H, $^{3}J = 7.5$ Hz), 2.16, 2.01 (2 × m, 8H). ^{13}C (151 MHz, D_2O): δ 134.2, 117.8, 117.0, 54.4 (2 × s), 25.9, 22.7. Mass spectra (ES, *m/z* (%)) = 443 (40) [M+H], 407 (90) [M−Cl], 280 (8), 262 (8), 180 (40), 163 (80). Analytical calc for **9b** · H_2O: C, 49.5; H, 6.79; N, 5.24. Found: C, 48.9; H, 6.76; N, 5.36%.

4.22. Cell culture and chemosensitivity testing

The human tumor cell lines used to measure the cytotoxicity of the titanocene derivatives have been described previously [12]. The H209, H209/CP and A549 lung tumor cell lines were cultured in RPMI 1640 medium containing 5% calf serum while the A2780 and A2780/CP ovarian tumor cell lines were cultured in DMEM medium with 7.5% fetal bovine serum. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

The cytotoxic potencies of the various compounds were determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as developed by Mosmann [13a] and adapted for chemosensitivity testing of human tumor cells [13b,13c]. Cells (>90% viable as determined by trypan blue exclusion) were suspended in culture medium and dispensed into 96-well microtitre plates

in a volume of 100 μL. H209 cells were plated at 2.5×10^4 cells per well, A549 cells at 1.0×10^4 cells per well and A2780 cells at 0.5×10^4 cells per well, based on preliminary experiments indicating these to be optimal cell densities for the MTT assay conditions chosen. After incubation of the cells for 24 h, compounds were added in a volume of 100 μL to bring the total volume of culture medium in the wells to 200 μL.

All solutions and dilutions of the titanocene derivatives were prepared immediately before addition to the cultured cells to limit the possibility of precipitation upon standing. The compounds were first dissolved in approximately 5 mL of medium and the volume was adjusted such that the final drug concentration was 2 mM. The stock solutions were then diluted as required and added to the wells. Final drug concentrations ranged from 0.001 to 100 μM in initial screening assays and from 0.1 to 200 mM in subsequent assays. Each drug concentration was added to four replicate wells. Cisplatin (Sigma) was tested in each set of assays as a positive control.

After addition of the drugs, the microtitre plates were returned to the incubator for 4 days. Three hours before completion of the incubation time, 100 μL of medium were removed from each well, and 25 μL of MTT (Sigma M2128) solution (2 mg/mL in phosphate buffered saline) were added. The plates were then returned to the 37 °C incubator for 3 h to allow reduction of the tetrazolium salt by viable cells. Subsequently, 100 μL of 1 M HCl–isopropanol (1:24) were added to each well followed by vigorous mixing with a multichannel pipette to dissolve any dark blue formazan crystals formed by MTT reduction. Absorbance values at 570 nm were then measured using a EL_X800 UV spectrophotometer. Controls consisted of wells with untreated cells and provided the baseline absorbance. Mean values (±SD) of the quadruplicate determinations were calculated and results expressed as a percentage of the baseline absorbance at 570 nm. Using GraphPAD Prism v3.02 software, IC_{50} values (defined as the drug concentration that reduced the absorbance to 50% of control values) were obtained from the best fit of the data to a sigmoidal curve.

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Appendix A. Supplementary material

CCDC 641096, 641097, and 641098 contain the supplementary crystallographic data for **5b**, **7a** and **9b**. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +(44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2007.04.024.

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