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Novel tetranuclear Pd\textsuperscript{II} and Pt\textsuperscript{II} anticancer complexes derived from pyrene thiosemicarbazones
Novel tetranuclear Pd\textsuperscript{II} and Pt\textsuperscript{II} anticancer complexes derived from pyrene thiosemicarbazones\textsuperscript{†}

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Cyclometallated palladium(\textit{i}) and platinum(\textit{i}) pyrenyl-derived thiosemicarbazone (H\textsubscript{2}PrR) complexes of the type [M\textsubscript{4}(μ-S-PrR-κ\textsubscript{3}-C,N,S)\textsubscript{4}] (M = Pd\textsuperscript{II}, Pt\textsuperscript{II}; R = ethyl, cyclohexyl) have been synthesised in good yields and fully characterised. X-ray crystallography showed that the tetranuclear complex [Pt\textsubscript{4}(μ-S-PrC\textsubscript{3}-C,N,S)\textsubscript{4}]\textsubscript{4}\textsubscript{+}(CH\textsubscript{3})\textsubscript{2}COCHCl\textsubscript{3} contains an eight-membered ring of alternating M–S atoms. The ethyl derivatives [M\textsubscript{4}(μ-S-PrEt-κ\textsubscript{3}-C,N,S)\textsubscript{4}] exhibit potent antiproliferative activity towards A2780 human ovarian cancer cells, with IC\textsubscript{50} values of 1.27 µM (for M = Pd\textsuperscript{II}), 0.37 µM (for M = Pt\textsuperscript{II}), the latter being an order of magnitude more potent than the anticancer drug cisplatin (IC\textsubscript{50} 1.20 µM). These promising complexes had low toxicity towards non-cancerous human MRCS cells, which points towards an early indication of differential toxicity between cancer and normal cells. Experiments that investigated the effects of these tetranuclear complexes on the cell cycle, integrity of the cell membrane, and induction of apoptosis, suggested that their mechanism of action of does not involve DNA targeting, unlike cisplatin, and therefore could be promising for combating cisplatin resistance.

Introduction

Thiosemicarbazones (TSCs) are well known as a promising class of compounds with a rich coordination chemistry\textsuperscript{1–3} and a range of pharmacological activities,\textsuperscript{4–6} including antiparasitic, antibacterial and antitumour activities. Some thiosemicarbazones such as the di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone Dp44mT (Chart 1) have been extensively studied because of their selective antitumour and antimitastatic properties, causing lysosomal membrane permeabilisation and cell death.\textsuperscript{7} The thiosemicarbazone derivative Triapine (Chart 1), which inhibits cancer cell growth at nanomolar concentrations, has undergone Phase 1 and Phase 2 clinical trials for the treatment of cervical cancer.\textsuperscript{8} It is well established that triapine has the ability to suppress tumour growth by inhibiting ribonucleotide reductase (RR), a key enzyme required for DNA synthesis.\textsuperscript{9} Following the successful anticancer agent cisplatin (CDDP) and its relatives carboplatin and oxaliplatin, thousands of other metal complexes have been screened in order to study the effects of the metal and their structures on the biological activity.\textsuperscript{10,11} However, the major challenge of this field, is still to eliminate strong side effects, off-target toxicity and achieve cancer-cell specificity. Accordingly, current antitumour metallo-drug research is focused on the development of compounds with new and unusual features, including metal–carbon bonds (organometallic compounds).

Organometallic complexes of palladium and platinum containing thiosemicarbazones as ligands have attracted attention. First because of their diverse range of structures, including oligomers, and second because organometallic bonding can increase the stability of the complex as a whole.\textsuperscript{12} Some cyclometallated complexes have potential antitumour

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Chart 1. Molecular structures of thiosemicarbazones Dp44mT (topoisomerase IIa inhibitor) and triapine (compound in clinical trials).
Thiosemicarbazone ligands derived from pyrene and (B) the general chemical structure of tetranuclear complexes studied in this work.

The thiosemicarbazone ligands are formed by condensation reactions of a thiosemicarbazide and an aldehyde. The ligand features depend on the aldehyde. Both thiosemicarbazide and aldehyde may provide coordination sites. In this work, we have used 1-pyrenecarboxaldehyde to incorporate a fluorescent pyrene into the thiosemicarbazones as a luminescent probe to track complexes inside cells. Furthermore, the well-known intercalating properties of pyrene might enhance inhibition of cellular DNA replication, if DNA is a target. Although there are many studies of metal thiosemicarbazone complexes, only a few reports describe the biological activity of tetracoordinated orthometallated complexes with thiosemicarbazone CNS donors. Herein, we have used potential CNS thiosemicarbazone ligands (Fig. 1A) to prepare tetracoordinated cyclometallated palladium and platinum complexes (Fig. 1B) and investigated their biological activity against A2780 human ovarian cancer cells. Since selectivity is important for reducing the severity of undesired side-effects, we have also studied the activity of the most potent complexes against a non-tumorigenic cell line.

Experimental

Materials and measurements

1-Pyrenecarboxaldehyde, 4-ethyl-thiosemicarbazide, analytical reagents grade chemicals and solvents were purchased from Sigma-Aldrich and used without further purification. The 4-cyclohexyl-3-thiosemicarbazide and trans-[PdCl2(MeCN)2]- were prepared as described previously. CHN elemental analyses were carried out on a CE-440 elemental analyzer by PerkinElmer Optima 5300 DV series ICP-OES instrument. The water (18.2 MΩ) used for analysis was doubly deionized (DDW) using a Millipore Milli-Q water purification system and a USF Elga UHQ water deionizer. ICP standards for platinum (1001 ± 2 ppb, Fluka) and palladium (1015 μg mL⁻¹, Aldrich) were diluted with 3.6% HNO₃ DDW to prepare fresh calibrants at concentrations of 50–700 ppb, which were spiked with NaCl to match the salt content of the samples being analysed. All Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) analyses were carried out on an Agilent Technologies 7500 series ICP-MS instrument. ICP standards were diluted with 3.6% HNO₃ DDW to prepare fresh calibrants at concentrations of 0.1–1000 ppb. The ICP-MS instrument was set to detect Pd and Pt with typical detection limits of ca. 2 ppt using no-gas mode, with an internal calibration standard of Er (50 pp).

Cell culture

Cell maintenance. A2780 human ovarian carcinoma cells and MRC-5 human fetal lung fibroblasts were obtained from the European Collection of Cell Cultures (ECACC). A2780 human ovarian carcinoma cells were used between passages 5 and 18. Cell lines were grown in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% of fetal calf serum, 1% of 2 mM glutamine and 1% penicillin/streptomycin. The cells were grown as adherent monolayers at 310 K in a 5% CO₂ humidified atmosphere and pasaged at ca. 70–80% confluency.

In vitro growth inhibition assay. 5000 cells were seeded per well (150 μL) in 96-well plates. The cells were pre-incubated in drug-free medium at 310 K for 48 h before adding various concentrations of the compounds to be tested. Stock solutions of
the palladium complexes were firstly prepared in DMSO (5% v/v) and a mixture 0.9% saline and RPMI-1640 medium (1:1 v/v) following serial dilutions in RPMI-1640. Drug exposure period was 24 h. After this, supernatants were removed by suction and each well was washed with PBS. A further 72 h was allowed for the cells to recover in drug-free medium at 310 K. SRB assay was used to determine cell viability. Absorbance measurements of the solubilized dye (on a BioRad iMark microplate reader using a 470 nm filter) allowed the determination of viable treated cells compared to untreated controls. IC\(_{50}\) values (concentrations which caused 50% of cell death) were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated.

**Flow cytometry.** The flow cytometry experiments were carried out using a Becton Dickinson FACScan Flow Cytometer at Warwick University (School of Life Sciences).

**Metal accumulation in cancer cells**

**Cell seeding.** Briefly, 4 × 10\(^6\) cells were seeded in a P100 Petri dish using 10 ml of culture medium. Cells were incubated at 310 K for 24 h.

**Sample preparation.** 100 \(\mu\)M solution of each compound was prepared in cell culture medium containing 5% DMSO. The exact concentration of Pt/Pd was determined by ICP-OES (using freshly prepared calibration standards, 50–700 ppb, with standard addition of sodium chloride to match the sample matrix). The stock solutions were diluted with culture medium to achieve a final working concentration equal to IC\(_{50}\).

**Drug addition.** The supernatant medium was removed, and 10 mL of each compound was added in triplicate to the Petri dishes. Cells were exposed to the drugs for 24 h.

**Drug removal.** After supernatants were removed by suction, cells were washed with PBS and detached using trypsin/EDTA and a single cell suspension was obtained using culture medium. Cells were counted using a hemocytometer, and centrifuged (1000 rpm, 5 min, 277 K) to obtain whole-cell pellets.

**Sample digestion.** Cell pellets were digested in 72% v/v nitric acid using a CEM Discovery SP microwave reactor (3 min, 393 K, 150 W, 250 psi) then diluted to a working acid concentration of 3.6% v/v. Samples were analysed using an Agilent 7500 series ICP-MS in both no-gas and He-gas mode. Calibration standards for Pt/Pd were prepared with HCl for 1.5 h at 333 K, as described previously. The purity of the free ligands was confirmed by \(^1\)H-NMR.

**Synthesis**

**Preparation of \(H_2\)PrCh and \(H_2\)PrEt.** The ligands were synthesized by refluxing a solution of 1-pyrene carboxaldehyde (1 mmol) and the desired thiosemicarbazide (1 mmol) in ethanol (15 mL) with the addition of HCl for 1.5 h at 333 K, as described previously. The purity of the free ligands was confirmed by \(^1\)H-NMR.

**Synthesis of the tetravalent palladium and platinum complexes**

**Preparation of \([M_4(\mu-S-PrR-N\_2C\_2N\_2)]_2\).** Two synthetic routes were explored to obtain the tetravalent palladium complexes. Both methods A and B worked well. However, method B gave better yields.

**Method A.** \(H_2\)PrR (0.1 mmol) was added to a solution of \(trans-[PdCl_2(\mu-MeCN)]_2\) (0.1 mmol) in 3 mL of MeCN. The mixture was stirred for 24 h at room temperature. An orange precipitate was formed during this time, which was filtered off, washed with hexane and dried in a vacuum. Afterwards, MeCN (2 mL) and Et\(_3\)N (0.5 mL) was added to the solid and the resulting mixture was kept under stirring and reflux for 5 h. The solvent was removed in vacuo and the residue was washed with methanol and then filtered. Afterwards the red solid was recrystallized from a mixture of CHCl\(_3\)/MeOH (1:1 v/v), affording a crystalline powder.

**Method B.** To a mixture of 0.1 mmol of \(trans-[PdCl_2(\mu-MeCN)]_2\) and 0.1 mmol of 1-pyrene carboxaldehyde,
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R-thiosemicarbazone in MeCN (5 mL) 0.5 mL of Et₂N was added; the mixture was then heated at 358 K for 8 h. A crystalline powder was obtained as described for method A.

**[Pd(µ-S-PrEt-k^1:C,N,S)]^1(1)**. Colour: Red. Yield: 75% mg (33 mg). M.P.: decomposes at 311 °C. IR (ν (cm⁻¹): 3398 ν(N-H), 1560, 1517 ν(C=N) + ν(C=C), 715 ν(C-S). Anal. Calcd for C₂₀H₁₅N₃PdS (435.83 g mol⁻¹): C, 55.12; H, 3.47; N, 9.64%. Found: C, 55.08; H, 3.37; N, 9.52%. UV-Vis, CH₂Cl₂ solution concentration: 5.1 × 10⁻⁵ M [ν max (ε, L mol⁻¹ cm⁻¹)]: 279.00 nm (11 710), 438.00 nm (7015), 439.00 nm (6719), 279.00 nm (11 710), 352.50 nm (7015), 438.00 nm (6719).

**[Pd(µ-S-PrCh-k^1:C,N,S)]^2** (2). Colour: Red. Yield: 81% mg (40 mg). M.P.: decomposes at 290 °C. IR (ν (cm⁻¹): 3392 ν(N-H), 1575, 1562, 1517 ν(C=N) + ν(C=C), 715 ν(C-S). Anal. Calcd for C₂₄H₂₁N₃PdS (489.92 g mol⁻¹): C, 58.84; H, 4.32; N, 8.58%. Found: C, 58.06; H, 4.26; N, 8.57%. UV-Vis, CH₂Cl₂ solution concentration: 1.12 × 10⁻⁵ M [ν max (ε, L mol⁻¹ cm⁻¹)]: 280.50 nm (26 714), 353.50 nm (17 232), 439.00 nm (13 476), 386.00 nm (12 466), 409.00 nm (12 135). MS (ESI+): m/z for C₈₀H₆₀N₁₂Pd₄S₄ [M⁺]: calcld 2096.25, 3815.21 ppm. Molar conductivity (1 × 10⁻³ mol L⁻¹ in DMSO): 0.02 μS cm⁻¹. HPLC: Rₘ = 28.18 min (at 254 nm).

**Preparation of [Pt(µ-S-PrPr-k^1:C,N,S)]^3**. The synthetic routes explored for the platinum tetratran complex are described below. Method C was the best since it provided higher purity and the formation of side products was not observed.

**Method A**. To a flask containing 0.1 mmol of [PtCl₄(COD)] dissolved in 2 mL of acetonitrile was added 0.1 mmol of the desired ligand H₂PrR. Et₂N (0.5 mL) and then the solution was refluxed for 8 h. After cooling to room temperature, a precipitate was formed which was filtered off, washed with methanol, hexane and dried under vacuum.

**Method B**. 0.1 mmol of H₂PrR dissolved in hot ethanol was added to a solution of the metal precursor K₂[PtCl₄] (0.1 mmol) in 1 mL of water. The formation of an orange coloured precipitate was observed. The reaction was kept under constant stirring at room temperature for 24 h in the absence of base. The precipitate was filtered, washed with methanol, hexane and dried under vacuum.

**Method C**. To a solution containing 0.1 mmol of K₂[PtCl₄] dissolved in 1 mL of water, a solution containing one mol equiv. H₂PrR in 3 mL of DCM/MeCN (1 : 2) mixture was slowly added. Thereafter, 0.5 mL of triethylamine was added. The reaction mixture became clear and a red solid formed. This solution was kept under constant stirring and under reflux for 24 h. Upon slow evaporation of the solvent at room temperature a microcrystalline solid was obtained. The red solid was filtered, washed with methanol and n-hexane, and dried under vacuum. The powder obtained was recrystallized from a mixture of chloroform and acetone (1 : 1 v/v).

**[Pt(µ-S-PrEt-k^1:C,N,S)]^3** (3). Colour: Red. Yield: 57% mg (30 mg). M.P.: 337–339 °C. IR (ν (cm⁻¹): 3398 ν(N-H), 1560, 1517 ν(C=N) + ν(C=C), 713 ν(C-S). Anal. Calcd for C₂₀H₁₅N₃PtS (524.50 g mol⁻¹): 45.80; H, 2.88; N, 8.01%. Found: C, 45.29; H, 2.91; N, 8.04%. UV-Vis, CH₂Cl₂ solution concentration: 5.95 × 10⁻⁵ M [ν max (ε, L mol⁻¹ cm⁻¹)]: 281.00 nm (4638), 383.00 nm (4184), 436.00 (3411), 465.00 (3058). MS (ESI⁺): m/z for C₂₀H₁₅N₃PtS [M⁺]: calcld 2096.25, found 2098.76. Molar conductivity (1 × 10⁻³ mol L⁻¹ in DMSO): 0.003 μS cm⁻¹. 195Pt NMR (CDCl₃): δ ~3801.88 and ~3815.21 ppm.

**Results and discussion**

**Synthesis and characterisation**

The reaction of 1-pyrenecarboxaldehyde with ethyl or cyclohexyl-thiosemicarbazide in the presence of a catalytic amount of HCl in methanol afforded yellow precipitates of composition H₂PrR, which were isolated in good yields, as described for a procedure reported earlier.²⁵,²⁹ The purity and identities of the free thiosemicarbazones were confirmed by IR, ¹H-NMR, ¹³C NMR, MS, HPLC and UV-Vis. The ¹³C NMR spectra for the uncoordinated thiosemicarbazones had peaks in the expected regions, with those for C≡N and C≡S at ca. 140 ppm and 176 ppm, respectively, as observed for similar structures.³⁰
Reactions of trans-[PdCl2(MeCN)]2 or K2[PtCl4] with H2PrEt in the presence of trimethylamine in MeCN under reflux afforded cyclometallated palladium and platinum complexes (Scheme 1). Two methods were used to prepare the palladium tetramers. For method A, described in the Experimental section, a dimeric product was isolated. The structure of the palladium dimers was confirmed by high resolution mass spectrometry (example in Fig. S7†). In the second method, however, the dinuclear complex was generated in situ, providing higher yields. For the preparation of the platinum tetranuclear complex, three methods were investigated, with method C being the most efficient, providing higher purity with no side products generated. The platinum dinuclear intermediate complexes could not be isolated, but they were generated in situ as described before.31 Generally, dinuclear complexes are used as intermediates in orthometallation reactions, representing the first step for the cyclometallation.32 However, since the aim was to obtain tetranuclear complexes, the isolation of such dimers was not necessary.

The IR spectra of the free ligands H2PrEt and H2PrCh show two absorptions for the ν(NH) stretching mode, while all the complexes show only one band (see Experimental section). This suggests that the ligands are deprotonated and coordinated in the thiolate form in the four complexes. Furthermore, the ν(C=N) absorption band at 1537 cm⁻¹ for H2PrEt, shifts to lower frequencies upon complexation, being found at 1560 cm⁻¹ in the spectra of both complexes 1 and 3. This suggests coordination to the metal ion through the iminic nitrogen. The same bathochromic shift was observed for the H2PrCh derived complexes after complexation, a ca. 30 cm⁻¹ shift.

The ¹H NMR spectrum of complex 3, recorded in CDCl₃ solution, shows two sets of signals, suggesting that the tetranuclear complex possesses two units in different environments. The ¹H-NMR spectrum shows two sets of peaks in the aromatic region from the four pyrene units and two sets of aliphatic hydrogen atoms related to the ethyl group from the thiosemicarbazone ligand. The spectra of the other complexes show a similar pattern. The formation of two different geometric isomers in solution probably arises from the flexibility of the eight-membered ring of alternating M–S atoms.31

The four metalloccyclic complexes were analysed by nano-electrospray-ionisation MS (nESI-MS), after dissolution of a few crystals in a mixture DCM/MeOH (25:75 v/v) + 0.3% formic acid. Without the presence of acid, ionisation was poor. Both complexes 1 and 2 showed significant peaks for protonated species at m/z 1743.0109 and 1959.1927, respectively, consistent with the corresponding proposed molecular ion peaks of the palladium tetranuclear complexes (Fig. S8 and S9†). On the other hand, the solvent + acid system did not work well for the platinum tetranuclear complexes. It was possible to observe peaks for protonated complexes 3 and 4 at m/z 2098.76 and 2313.42 respectively, in accord with the formation of the platinum tetramers; however, other species were generated by this method (see Fig. S11† as an example).

In order to verify the purity of the platinum complexes, we used ¹⁹⁵Pt NMR spectroscopy, since each fragment shows only one characteristic signal.33 The ¹⁹⁵Pt NMR spectra of the complexes 3 and 4 were recorded in CDCl₃ and CD₂Cl₂, respectively, at ambient temperature. Two broad signals at −3801.9 and −3815.2 ppm were found for 3, and at −4053.3 and −4080.7 ppm for 4 (Fig. S12†), as expected from tetrameric species. The chemical shifts for square planar Pt(n) complexes are consistent with a PtCNS₂ coordination sphere ([δ(¹⁹⁵Pt) −4000 to −3000 ppm], as reported in the literature.34

The electronic absorption spectra of the tetranuclear complexes in CH₂Cl₂ solution at room temperature display high-
energy bands at $\lambda \approx 279-287$ nm, with extinction coefficients ($\epsilon$) of $1 \times 10^4$ to $1 \times 10^5$ L mol$^{-1}$ cm$^{-1}$, and low-energy absorption bands at $\lambda = 352-495$ nm, with extinction coefficients of $1 \times 10^4$ L mol$^{-1}$ cm$^{-1}$. The high-energy absorption bands are assigned to the spin-allowed intraligand IL ($\pi \rightarrow \pi^*$) transitions of the thiosemicarbazone ligands. The low-energy absorption bands of the tetranuclear complexes may be metal-to-ligand charge-transfer (MLCT) transitions $[\sigma(\text{Pt-Pd}) \rightarrow \pi^*(\text{TSC})]$.\textsuperscript{36} as observed previously for other organometallic complexes.\textsuperscript{37}

The luminescence properties of the complexes were determined for DMSO solutions at ambient temperature, since only very weak emission bands were observed in CH$_2$Cl$_2$. The maximum emission for the complexes is blue-shifted by $\sim 270$ nm when compared to their free ligand absorption spectra. The palladium complexes show green emission around 500 nm, while the platinum complexes exhibited two emission maxima around 450 nm. Based on such large Stokes shifts, these emissions are indicative of phosphorescence.\textsuperscript{38} The large spin–orbit coupling caused by the palladium/platinum centre, indicates efficient spin–orbit coupling, often a prerequisite for phosphorescence of organometallic complexes.\textsuperscript{39} Furthermore, the presence of a fluorophore can increase the luminescence properties of the complex.\textsuperscript{40} The emission energy of the tetranuclear complexes is not significantly influenced by the R groups of the thiosemicarbazonate ligands, since both ethyl and cyclohexyl substituents are electron-donors.

Reverse phase HPLC studies confirmed the purity of the tetranuclear palladium complexes. The chromatograms obtained show only one peak for complexes 1 and 2. Complex 1 had a retention time of 28.18 min, while the cyclohexyl derivative (complex 2) had a retention time of 33.03 min. From the retention times, it can be assumed that the cyclohexyl derivative is more lipophilic than the ethyl derivative. As indicated by the mass spectra, the platinum complexes are not stable in the acid system used for HPLC studies.

Suitable crystals of $[\text{Pt}_4(\mu-\text{S-PrCh-})_3\text{C}_3\text{N}_2\text{S}_4]_2(\text{CH}_3)_2\text{COCHCl}_3$ (4) were obtained by slow evaporation of a solution of complex 4 and were studied by single crystal X-ray crystallography. A perspective view of the molecule is shown in Fig. 2. Selected bond distances are summarized in Tables S1 and S2.\textsuperscript{†} The asymmetric unit contains four tetrameric Pt complexes, and also two molecules of solvent acetone and a chloroform molecule. There was further diffuse electron density and attempts were made to model the disordered solvent, but little progress was made using several partially occupied chloroform molecules refined with many restraints. Better progress was made using the Squeeze routine in Platon. Voids located by the Squeeze routine are shown in detail in the ESL.\textsuperscript{†}

The X-ray structure shows the tetranuclear complex $[\text{Pt}_4(\mu-\text{S-PrCh-})_3\text{C}_3\text{N}_2\text{S}_4]_2(\text{CH}_3)_2\text{COCHCl}_3$ is composed of four metallated monomers held together by bridging sulfur atoms. In this way, each platinum centre possesses a coordination sphere of donor atoms from the thiosemicarbazone ligand, the imine nitrogen, a sulfur atom and an ortho carbon from the benzyl ring, while the fourth position is occupied by a sulfur atom from a neighboring monomer unit, resulting in a CNSS tetradentate coordination mode. A predominantly double bond is found for C=S in the free ligand (1.685(3) Å), while in complex 4 this bond is longer, 1.820(14) Å. The elongation of the C=S bond in the dianionic ligand is due to the deprotonation of the iminic nitrogen followed by the thieno[3,2-$d$]thiophene of the CS bond. Pt-N bond distances of $\sim 2.00$ Å and Pt-C of $\sim 2.03$ Å in the five-membered orthometallo cycle are analogous to those found in other similar complexes.\textsuperscript{41} The trans influence of the metallated carbon is reflected in the lengthening of the Pt–S distance trans to this carbon atom, Pt–S$^{\text{Schelating}}$ (ca. 2.35 Å), with respect to the Pt-S distance trans to the imine nitrogen, Pt–S$^{\text{bridging}}$ (ca. 2.03 Å). This feature has also been observed for other polynuclear complexes with similar ligands.\textsuperscript{19} Therefore, the two distinct Pt–sulfur bonds, Pt–S$^{\text{Schelating}}$ and Pt–S$^{\text{bridging}}$, show that the sulfur bridge is relatively strong. The strong M–S$^{\text{bridging}}$ bond was also apparent from treatment of the tetranuclear compounds with coordinating solvents such as DMSO, DMF and acetonitrile, which did not lead to opening of the eight-membered Pt$_4$S$_4$ ring core of the tetranuclear structure, nor to ring cleavage of the chelate. However, some papers have described the M–S$^{\text{bridging}}$ (M = Pt or Pd) bond cleavage in reactions of orthometallated TSC tetranuclear complexes with strongly binding phosphine ligands.\textsuperscript{42} The two 5-membered chelate rings are planar to each other, this planarity being extended to the pyrene fragment. Moreover, the connections between the platinum ions and the bridging sulfur atoms generate a cavity in the centre of the structure (Fig. 3). The Pt$_4$S$_4$ core consists of an eight-membered ring of alternating Pt(ii) and S atoms in a boat conformation. The size of this cavity can be assessed by the distance between two platinum centres of parallel monomers: a Pt–Pt distance of ca. 3.37 Å. More inter-metal distances are listed in Table S3.\textsuperscript{†} This Pt–Pt distance indicates little interaction between the platinum centres. This is in agreement with the reported values for tetranuclear Pt$_4$ complexes (2.54–3.51 Å).\textsuperscript{43} The geometry around the Pt ion is distorted square-planar, in accord with the bond angles C115–Pt1–S120 [166.2(4)°], C315–
The inhibition of cell proliferation in ovarian cancer cell A2780 line by the tetranuclear complexes was evaluated using the SRB method,24 Table 2. For comparison purposes, the antiproliferative activity and cellular uptake of the pyrene rings. Their orientation may be dominated by the requirements of coordination.

Metal-based compounds are sometimes unstable in solution. For this reason, it is important to ensure that the tested compound is stable in aqueous solution. Therefore, as a model system, the stability of complex 1 was investigated and monitored by UV-vis spectra in H2O with 5% of DMSO for at least 24 h period at 25 °C (see Fig. S13, ESI†). The spectra remained unchanged over the time for this assay, suggesting that compounds are stable in solution.

Antiproliferative activity and cellular uptake

The inhibition of cell proliferation in ovarian cancer cell A2780 line by the tetranuclear complexes was evaluated using the SRB method,24 Table 2. For comparison purposes, the antiproliferative activity of the antiproliferative activity of the ethyl complexes 1 and 3 was the highest, these were then screened against the non-tumourigenic cell line MRC5, human embryonal lung fibroblasts. These two complexes exhibited much higher IC50 values in MRC5 human fibroblasts (>100 µM, Table 2) compared to A2780 cells. This cell line is used routinely as an indicator to investigate differences in toxicity between cancerous and non-cancerous cells in the early-stage development of anticancer complexes and as a preliminary predictor for cancer cell selectivity. In this case, the values obtained are highly promising as there are at least two and three orders of magnitude difference (for complexes 1 and 3 respectively) between the potency in the MRC5 and the A2780 lines.

The cellular accumulation of palladium or platinum from 1 or 3, respectively, using equipotent IC50 concentrations was determined in A2780 ovarian cells in order to correlate the amount of metal accumulated with antiproliferative activity and lipophilicity. For these experiments, drug exposure time was 24 h and cells were not allowed to recover. Values are expressed as ng of Pd and Pt per million cells and were determined as independent duplicates of triplicates. The cellular accumulation of Pd for complex 1 was 6.0 ± 0.4 ng of Pd per 10^6 cells, while for complex 3 was 3.4 ± 0.2 ng of Pt per 10^6 cells, which is equivalent to a molar uptake of 3.44 and 1.62 µmol respectively, this taking into account the molecular weight of the tetramer units for complexes 1 and 3 (Table 3).

Table 2 Comparison of IC50 values (µM) for the thiosemicarbazone ligands, tetranuclear Pd and Pt complexes and cisplatin against A2780 cancer cells and MRC5 normal cells. Experiments included 24 h of drug exposure and 72 h of recovery time in drug-free medium. Cell viability results, determined by the SRB assay, are expressed as the average of duplicates of triplicates and together with standard deviations.

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<th>IC50 a (µM)</th>
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<td>H2PrEt</td>
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<td>H2PrCh</td>
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<td>CDDP</td>
<td>1.20 ± 0.03</td>
<td>10.9 ± 0.3</td>
</tr>
</tbody>
</table>

a IC50 concentration based on the molecular weights of the tetranuclear complexes.

Table 3 Cellular accumulation (ng) of Pd or Pt from complexes 1 and 3 in A2780 ovarian cancer cells treated with equipotent IC50 concentrations for 24 h with no recovery time in drug-free medium.

<table>
<thead>
<tr>
<th></th>
<th>ng Pt/Pd × 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>CDDP</td>
<td>0.51 ± 0.07</td>
</tr>
</tbody>
</table>
Due to the potent cytotoxicity of complex 3, we investigated its effects on the cell cycle (Fig. 4). Complex 3 was incubated with A2780 ovarian cancer cells for 24 h. For this study, cells were stained using propidium iodide (PI) as a fluorescent probe that interacts with DNA through intercalation. In comparison to the control population, data shown in Table S5 (ESI†) for complex 3 at two different concentrations, it is notable that there is no significant difference in the population of G1, G2/M and S phases after 24 h of exposure to the platinum complex (e.g., from 63.5 ± 1.0% for the control versus 61.8 ± 0.6% for G1 at the highest concentration tested). These results contrast dramatically with those for CDDP, which causes cell cycle arrest in the S phase given its DNA-based mechanism of action.45

One cellular response to stress stimuli is programmed cell death (apoptosis). The possibility that treatment of A2780 cancer cells with the platinum tetranuclear complex 3 leads to apoptosis was investigated. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) dual-staining allowed the detection of four different cellular populations: viable cells, non-viable cells and early-stage, and late-stage apoptosis. Early-stage apoptosis is characterised by changes in the symmetry of the phospholipid membrane, and late-stage by further disruption of the integrity of the cell membrane so it becomes permeable to PI. Staurosporine, a potent apoptosis-inducer, was used as a positive control.46 After 24 h of drug exposure, the extent of non-viable, early and late apoptotic cells is close to zero. No population of cells in either of these two stages of apoptosis was detected (Fig. 5). The compound does not affect any of the apoptotic populations, showing only viable cells (see Table S2†).

In the absence of apoptosis after 24 h of drug exposure, we investigated the cellular membrane integrity by flow cytometry. A2780 cells exposed to complex 3 at two different concentrations (1× IC₅₀ concentration and 3× IC₅₀ concentration) tested negative for increased fluorescence of the 7-ADD stain in the FL2-red channel, indicating that, under these experimental conditions, there is no cellular membrane integrity damage (Fig. 6). However, metal accumulation studies in A2780 cells indicated that complex 3 does accumulate in the cancer cells. We also attempted to investigate the interaction of complexes 1 and 3 with DNA (circular and linear dichroism) and with GSH. However, due to their poor solubility in buffer, it was not possible to perform these experiments despite many

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**Fig. 4** FL2-H histograms obtained by flow cytometry for cell cycle analysis (A) control untreated cells; (B) cells treated using 3 times IC₅₀ concentration of complex 3; (C) cells treated using 0.4 μM of complex 3. Experiments included 24 h drug exposure time.

**Fig. 5** FL2-H vs FL1-H dot plots obtained by flow cytometry for apoptosis analysis using FITC (FL1-H channel) and PI (FL2-H channel) staining. (△) cells treated using 3× IC₅₀ concentration of complex 3; (▲) Cells treated using 0.4 μM of complex 3. (■) Control untreated cells. For all experiments, drug exposure time was 24 h, cells were treated with RNase and stained using PI.

**Fig. 6** Membrane integrity assessed by flow cytometry for A2780 cancer cells (red) and after treatment with complex 3 at 1× IC₅₀ concentration (blue) and 3× IC₅₀ concentration (orange) at 310 K. Viable cell membrane (FL2−). Non-viable cell membrane (FL2+). No changes were observed after 24 h drug exposure.
Conclusions

Four tetrameric palladium(ii) and platinum(ii) complexes \([\text{M}_4(\mu-S-\text{PrR}-\kappa^2\cdot\text{C},\text{N},S)_4]\) containing tridentate thiosemicarbazones (H$_2$PrR) derived from monocyclic aromatic aldehydes and thiosemicarbazides have been synthesised by ortho-metallation reactions via C–H activation of a pyrene ring substituent. These new cyclometallated complexes were fully characterised using various analytical techniques and also for one Pt$_4$ complex by X-ray crystallography. The crystal structure confirmed that the thiosemicarbazone ligands undergo cyclometallation reactions to give tetranuclear compounds with a central core consisting of an eight-membered ring of alternating platinum and sulfur atoms. The R = ethyl PrR thiosemicarbazone complexes are potently cytotoxic to human ovarian cancer cells being as active (Pd, 1) or an order of magnitude more active (Pt, 3) than the clinical drug cisplatin. Moreover, the tetranuclear R = ethyl complexes appear more selective for cancer cells versus non-tumorigenic cells than cisplatin. In contrast to cisplatin, these complexes do not induce apoptosis, nor cause cell membrane damage. Hence, the difference in mechanism of action may provide a basis for combating cisplatin resistance which is a major current problem and causes an unmet clinical need.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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