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In vitro and *in vivo* antitumor activity of platinum(II) complexes with thiosemicarbazones derived from 2-formyl and 2-acetyl pyridine and containing ring incorporated at N(4)-position: Synthesis, spectroscopic study and crystal structure of platinum(II) complexes with thiosemicarbazones, potential anticancer agents

Dimitra Kovala-Demertzi [a,*], Athanassios Papageorgiou [b], Leuteris Papathanasis [a], Alexandros Alexandratos [a], Panagiotis Dalezis [b], John R. Miller [c], Mavroudis A. Demertzis [a,*]

[a] *Inorganic and Analytical Chemistry, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece*
[b] *Symeonidio Research Center, Theagenio Cancer Hospital, Thessaloniki, Greece*
[c] *Department of Biological and Chemical Sciences, University of Essex, Colchester CO4 3SQ, UK*

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Dedicated to Professor D.X. West for his contribution to Chemistry and the Chemistry of Thiosemicarbazones.

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ABSTRACT

Reactions of thiosemicarbazones of 2-formyl and 2-acetyl pyridine and containing an azepane ring (hexamethyleneiminyl ring) incorporated at N(4)-position, HL^1 (**1**) and HL^2 (**2**) with platinum(II) afforded the complexes, [Pt(L^1)Cl] (**3**) and [Pt(L^2)Cl] (**4**). Characterization of the compounds was accomplished by means of elemental analysis and spectroscopic techniques NMR, UV–vis and IR spectroscopy. The single-crystal X-ray structure of complex [Pt(L^2)Cl] (**4**) shows that the ligand monoanion coordinates in a planar conformation to the metal *via* the pyridyl N atom, the imine-N atom, and thiolato S-atom. Compounds **1**–**4** have been evaluated for antiproliferative activity *in vitro* against three human cancer cell lines: MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), A-549 (non-small cell lung carcinoma) and a mouse L-929 (a fibroblast-like cell line cloned from strain L). Ligand **2** exhibited high activity as anticancer agent against all four cancer cell lines, while ligand **1** exhibited selectivity against MCF-7, L-929 cell lines and complex **4** against A-549, T-24 cancer cell lines. Also, the acute toxicity and antitumor activity were evaluated on leukemia P388-bearing mice. Complex **3** afforded five to six cures against leukemia P388. The *in vivo* results of the antitumor activity show the two platinum complexes as very effective chemotherapeutic antileukemic agents.

1. Introduction

Thiosemicarbazones are among the most potent inhibitors of ribonucleotide reductase, RR, activity and possess a wide range of biological activity depending on the parent aldehyde or ketone. Heterocyclic thiosemicarbazones (TSCs) have aroused considerable interest in chemistry and biology due to their antibacterial, antimalarial, antineoplastic and antiviral activities and represent an important series of compounds because of potentially beneficial, biological activity [1].

The chemistry of transition metal complexes of thiosemicarbazone, TSCs, has been receiving considerable attention largely because their broad profile of pharmacological activity affords a diverse variety of compounds with different activities [2,3]. The biological properties of TSCs are often related to metal ion coordination. Firstly, lipophilicity, which controls the rate of entry into the cell, is modified by coordination and some side effects may decrease upon complexation. In addition, the complex can exhibit bioactivities which are not shown by the free ligand [4,5]. The thiosemicarbazone, HL^2, has been screened against HSV-1, HSV-2 and leukemia P388 because of its potential for medicinal use [6]. A complex of Pd(II) with HL^2 was found to exhibit significant reduction of toxicity, enhancement of cytogenic damage and a significant increase of survival time of the drug-treated leukemia bearing mice (T/C % 166) [14]. The combination of TSCs with agents like platinum(II) and palladium(II) that damage DNA may lead to improvements in the effectiveness of cancer chemotherapy regimens.

It was thought of interest to explore the platinum chemistry of TSCs, HL^1 and HL^2, as a continuation of our studies on TSCs and in order to widen the scope of investigations on the coordination behaviour of TSCs, towards platinum(II), we carried out systematic studies with the final goal to develop new biologically active pharmaceuticals.

* Corresponding authors. Fax: +30651 98786.
E-mail address: dkovala@cc.uoi.gr (D. Kovala-Demertzi).

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Here, we report the synthesis and chemical characterization of new platinum(II) complexes. The single-crystal X-ray structure of complex [Pt(L^2)Cl] (**4**) and the electronic, IR, UV–vis, and NMR spectroscopic data of the two complexes are reported. The results of the cytotoxic activity of **1**–**4** have been evaluated for anti-proliferative activity *in vitro* against cancer cell lines: MCF-7, T24, A-549 and a mouse L-929. Also, the acute toxicity and antitumor activity were evaluated on leukemia P388-bearing mice.

2. Results and discussion

2.1. Chemistry

The two thiosemicarbazones HL^1, **1** and HL^2, **2**, were prepared according to the method described in the literature [7,16]. Trans-amination of 4-methyl-4-phenyl-3-thiosemicarbazide (A) in the presence of azepane and the requisite aldehyde or ketone gives the two ligands HL^1 and HL^2, Scheme 1 [7,16]. To synthesize complexes **3** and **4**, HL^1 and HL^2 were added to an aqueous solution of $Na_2[PtCl_4]$, and the mixture was stirred for 24 h, Scheme 2. The structures were confirmed by, IR, UV–vis, ^{1}H and ^{13}C NMR spectroscopy and X-ray diffraction data for **4**.

2.2. Spectral studies

2.2.1. Infrared spectroscopy

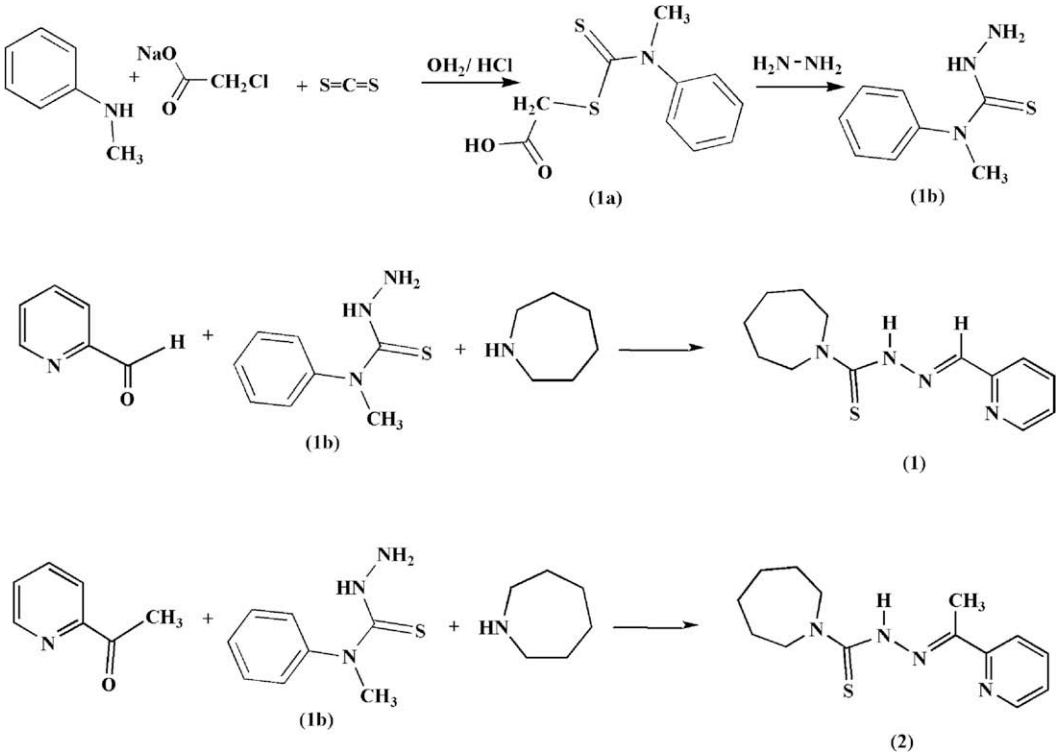
Coordination of the azomethine nitrogen atom to platinum(II) is suggested by the shift of the ν(C=N) band to lower frequencies along with the occurrence of the ν(N–N) band to higher frequency in the IR spectra of the complexes compared to that in the ligand [7–11]. The breathing motion of the pyridine ring is shifted to a higher frequency upon complexation and is consistent with pyridine ring nitrogen atom coordination. The thioamide band, which contains considerable ν(CS) character, is less intense in the complexes and is found at a lower frequency, suggesting coordination of the metal through sulfur atom after deprotonation. The platinum donor atom stretching frequencies are as follows: ν(Pt–N_{imine}), 420–430 cm^{-1}; ν(Pt–Cl), 338– 346; ν(Pt–S), 400–370 cm^{-1}; and ν(Pt–N_{py}), 320–300 cm^{-1}.

2.2.2. NMR spectroscopy

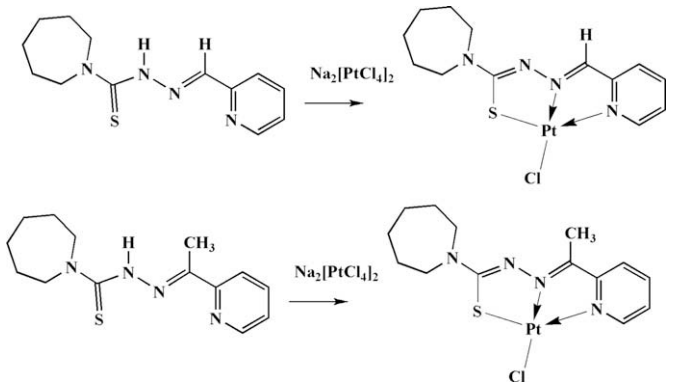
In the ^{1}H NMR spectra of the ligands **1** and **2**, H–N(3) resonated at 15.00–13.00 ppm, indicating that this H-atom is involved in H-bonding [7]. This resonance was absent in the spectra of the corresponding complexes, which indicated deprotonation of H–N(3) and, thus, anionic thiosemicarbazonato moieties. In the ^{1}H NMR spectra of the Pt(II) complexes **3** and **4**, H–C(1) was downfield shifted upon coordination, indicating decreased electron density at position 1. The pyridyl hydrogens are shifted upon coordination which indicated variations in the electron density in the pyridyl ring. The C=S resonances of the thiosemicarbazone moieties in the free ligands resonated at 183.0 ppm. In the complexes, downfield shifts were observed, indicating decreased electron density from the thiolato C-atoms [8–10].

2.3. X-ray crystallography

Crystal data and experimental details are listed in Table 1. Interatomic distances and bond angles are compiled in Table 2. The crystal structure of compound **4** is shown in Fig. 1. The anionic L^2 acts as a tridentate ligand which coordinates to platinum(II) through the pyridyl nitrogen atom, N(1), the azomethine nitrogen atom, N(2) and the thiolato sulfur atom. The tridentate ligand shows a *Z*, *E*, *Z* configuration for the donor centres nitrogen, nitrogen and sulfur atoms, respectively. The stronger coordination of the metal to the azomethine nitrogen atom compared to the pyridyl nitrogen atom is attributed to its higher basicity of N(2). The negative charge of the monoanionic ligand is delocalized over the L^2 moiety and the S–C bond distance is consistent with increased single bond character, while the imine C–N distances and both thioamide C–N distances indicate considerable double bond character. The tridentate ligand is nearly planar. The displacement from co-planarity is indicated by the dihedral angle between the pyridyl



Scheme 1. The reaction scheme for synthesis of ligands **1** and **2**.



Scheme 2. The reaction scheme for synthesis of platinum(II) complexes **3** and **4**.

ring and the plane defined by the five-membered chelate ring Pt–N(2)–C(6)-C(5)–N(1), and between the pyridyl ring and the plane defined by Pt–S–C(8)–N(3)–N(2), 1.4(1) and 2.3(7), respectively. The dihedral angle between the pyridyl ring and the azepane ring (hexamethyleneiminyl ring) is 68.2(9). There is a weak interaction between the metal atom and the C(7), the methyl carbon, and its attached hydrogen, H(7C), the metal–hydrogen distance being 3.04 Å.

The crystal packing is determined by Pt–C, Pt–π and π–π, interactions, as shown in Fig. 2.The monomers are arranged in dimers, in a head to tail fashion and are connected by strong π–π interactions. The most significant double π–π interaction is that between the pair of rings Pt–N(2)–C(6)–C(5)–N(1) and N(1)–C(1)–C(2)–C(3)–C(4)–C(5), (Cg2)–Cg(3) and Cg(3)–Cg(2), respectively; symmetry operation: $2-x, -y, 1-z$} at a centre to centre separation of 3.517(4) Å.

2.4. *Pharmacology: antiproliferative activity* in vitro

Platinum(II) complexes of *N*4-ethyl 2-formyl and 2-acetyl pyridine thiosemicarbazones showed cytotoxicity and were found to be able to overcome the cisplatin resistance of A2780/Cp8 cells [12]. Pt(II) or Pd(II) complexes with 2-acetyl pyridine N(4)-ethyl-thiosemicarbazone,

Table 1
Crystal data and structure refinement for **4**

Empirical formula	$C_{14}H_{19}ClN_4PtS$
Formula weight	505.93
Temperature	291(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	$P2_1/n$
Unit cell dimensions	$a = 7.258(4)$ Å $\alpha = 90°$ $b = 24.869(5)$ Å, $\beta = 104.27(2)°$ $c = 9.150(4)$ Å, $\gamma = 90°$
Volume	1600.7(11) Å^3
Z	4
Density (calculated)	2.099 Mg/m^3
Absorption coefficient	9.061 mm^{-1}
F(000)	968
Crystal size	0.20 × 0.25 × 0.75 mm
Theta range for data collection	1.64–26.01°
Index ranges	$0 \le h \le 8$, $0 \le k \le 30$, $-10 \le l \le 11$
Reflections collected	3376
Independent reflections	3215 [$R_{int} = 0.025$]
Completeness to theta = 26.01°	99.4%
Absorption correction	Refined empirical
Refinement method	Full matrix least squares on F^2
Data/restraints/parameters	3.125/0/192
Goodness-of-fit	1.079
Final *R* indices [$I > 1.5\sigma(I)$]	$R1 = 0.0292$, $wR2 = 0.0823$
R indices (all data)	$R1 = 0.0416$, $wR2 = 0.0879$
Extinction coefficient	0.0043(3)
Largest difference peak and hole	1.307 and −0.884 e Å^{-3}

Table 2
Selected bond lengths (Å) and angles (°) for **4**

Pt–Cl	2.310(2)	N(2)–N(3)	1.385(7)
Pt–S	2.257(2)	N(2)–C(6)	1.307(7)
Pt–N(1)	2.047(5)	N(3)–C(8)	1.322(7)
Pt–N(2)	1.942(2	N(4)–C(8)	1.346(7)
S–C(8)	1.767(6)	N(4)–C(9)	1.448(8)
N(1)–C(1)	1.342(8)	N(4)–C(14)	1.471(9)
N(1)–C(5)	1.353(7)		
Cl–Pt–S	97.20(7)	Cl–Pt–N(1)	97.15(14)
S–Pt–N(1)	165.64(14)	S–Pt–N(2)	85.40(15)
Cl–Pt–N(2)	175.96(15)	N(1)–Pt–N(2)	80.30(19)

HAc4Et were tested in a panel of human tumor cell lines of different origins (breast, colon, and ovary cancers), and cisplatin-refractory/resistant cell lines and were found to exhibit very remarkable growth inhibitory activities with mean IC_{50} values of 0.9–0.5 nM and support the hypothesis that both [Pt(Ac4Et)$_2$] and [Pd(Ac4Et)$_2$] complexes can be characterized by cellular pharmacological properties distinctly different from those of cisplatin [13].

Compounds **1**–**4** were tested for their antiproliferative activity *in vitro* against the cells of three human cancer cell lines: MCF-7 (human breast cancer cell line), T24 (bladder cancer cell line), A-549 (non-small cell lung carcinoma) and a mouse fibroblast L-929 cell line. The results of cytotoxic activity *in vitro* are expressed as IC_{50} – the concentration of compound (in μM) that inhibits a proliferation rate of the tumor cells by 50% as compared to control untreated cells, Table 3.

The ligand **2** is in the same μM range compared to cisplatin against the four cell lines, less cytotoxic against L-929 and A-549 cell lines and more cytotoxic against MCF-7 and T-24 cancer cell lines. Ligand **2** is 3.5 and 1.6 times more active than cisplatin against the T24 and MCF-7 cell lines, respectively. The platinum complex **4** is less cytotoxic against MCF-7 and L-929 cell lines and in the same μM range compared to cisplatin and the parent ligand against T-24 and A-549 cancer cell lines.

The IC_{50} values for **1** against A-549 and T-24 cell lines are 27.31 and 74.32 μM, respectively, and against MCF-7 and L-929 cell lines are 2.23 and 2.27 μM, respectively. Ligand **1** is 3.6 more active than cisplatin against MCF-7 cell line and 3.8 times less active than cisplatin against L-929 cell line.

Selectivity was exhibited from the platinum(II) complex, **4**, which was found active against A-549 and T-24 cancer cell lines. From the *in vitro* experimental results, it is indicated that the two ligands HL^1 and HL^2 are more active than their corresponding

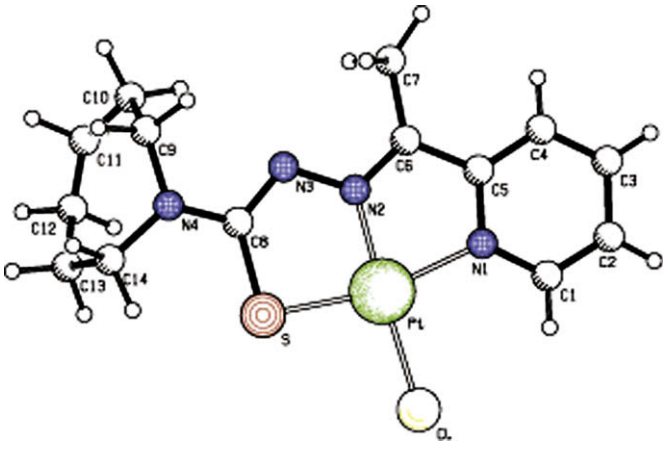


Fig. 1. Perspective view of **4**.

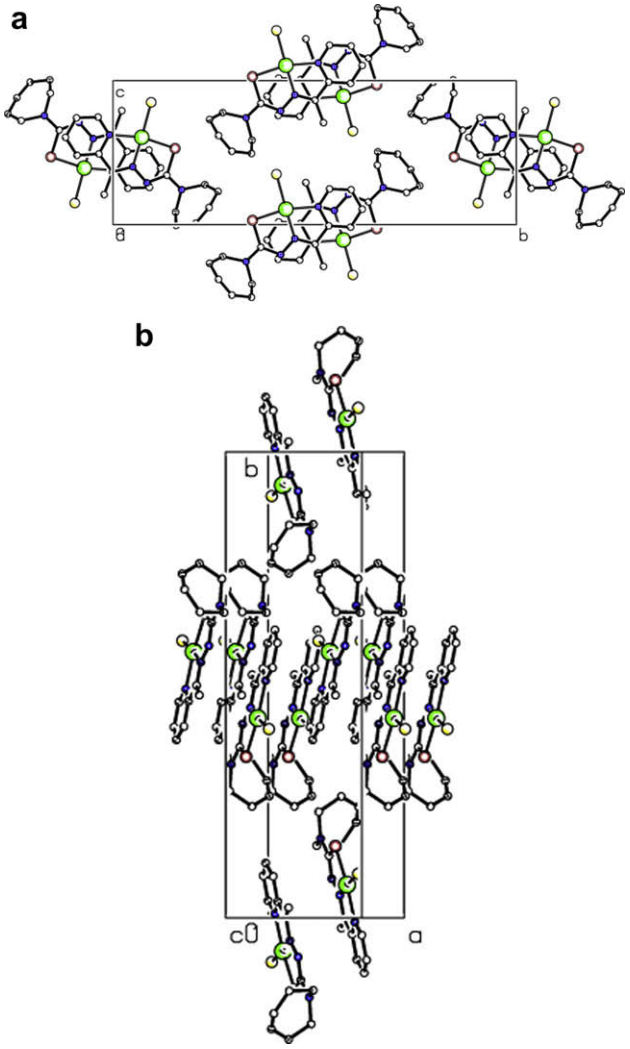


Fig. 2. (a) Packing diagram of **4** viewed along α and (b) c axis.

platinum complexes, which indicates that the mechanism of action is different.

Ligand **2** exhibited high activity as anticancer agent against all four cancer cell lines, while ligand **1** exhibited selectivity against MCF-7, L-929 cell lines and the complex **4** against A-549, T-24 cancer cell lines.

2.5. In vivo *antitumor experiments*

The acute toxicity and antitumor activity were evaluated on leukemia P388-bearing mice for compounds **1**–**4**. The acute toxicity and the antitumor activity of these compounds were correlated with the related complexes of 2-formyl pyridine and 2-acetyl pyridine thiosemicarbazone, HFoTsc and HAcTsc, respectively [5,14,15].

The ligands **1** and **2** cause acute toxicity and display some antitumor activity, while the complexes **3** and **4** show reduction of the toxicity, and very high increase of survival time of the drug-treated leukemia bearing mice, Table 4. The ligand **2** is achieving a T/C % value of 118 and the Pt(II) complex, **4**, of 417. The ligand **1** is achieving a T/C % value of 147 and the Pt(II) complex, **3**, of 298. Also, five of the six mice were cured and were considered as long-term survivors. The long-term survivors are defined as mice alive 90 days after tumor inoculation. The replacement of a methyl group at position C(7) (HL^2) by a C(7)H group (HL^1) in the 2-pyridyl position

Table 3
The *in vitro* activity of **1**–**4** (expressed as IC_{50} (μM)) against MCF-7, T-24, A-549 and L-929 cancer cell lines

Compounds	MCF-7	T-24	L-929	A-549
HL^1 (**1**)	2.23 ± 0.31	74.32 ± 6.70	2.67 ± 0.22	27.31 ± 0.10
HL^2 (**2**)	5.02 ± 0.10	11.80 ± 0.12	3.04 ± 0.34	6.55 ± 0.21
[Pt(L^1)Cl] (**3**)	20.30 ± 2.10	19.83 ± 2.11	37.70 ± 3.10	89.30 ± 6.10
[Pt(L^2)Cl] (**4**)	25.70 ± 3.12	12.83 ± 0.09	77.10 ± 5.10	6.92 ± 0.35
Cisplatin	8.00 ± 0.50	41.7 ± 2.10	0.70 ± 0.05	1.53 ± 0.15

causes marked differences on the biological results a significant increase of survival time of the drug- treated leukemia bearing mice, T/C % 147 and 118 for HL^1 and HL^2, respectively. Probably the Pt(II) complexes, [Pt(L^1)Cl], **3** and [Pt(L^2)Cl], **4**, may interact better with DNA and/or proteins than the other compounds. In conclusion, these experiments showed that the activity of the metal complexes **3** and **4** is different compared with the activity of the ligands alone and probably represent independent cytotoxic entities.

Complex **4** was proved to be the most potent antileukemic agent, comparing the T/C % value and almost non-toxic, producing low level of toxicity. It is important to remark that complex **3** gave a high T/C % value of 298 and afforded additionally five of six cures in leukemia P388. The results of their *in vivo* biological tests showed that platinum(II) complexes **3** and **4** proved less toxic than the free ligands **1** and **2**, while their antileukemic potency was significantly improved. The most potent compound **4** had significantly decreased toxicity and compound **3** exhibited impressive potency (5/6 cures against leukemia P388). The *in vivo* results of the antitumor activity show the two platinum complexes as very effective chemotherapeutics, antileukemic agents.

These compounds **1**–**4** may be considered as polyfunctional molecules, possessing an azepane ring, theiosemicarbazonato portion, N–N–CS–N and a pyridyl ring. Obviously, these groups are capable of hydrophobic, azepane ring (hexamethyleneiminyl ring), hydrogen bonding (thiosemicarbazonato portion, N–N–CS–N) and π–π interactions (pyridyl ring) with the corresponding bioreceptor. The molecules reach to the receptor microenvironment

Table 4
Acute toxicity and antitumor activity of of **1**–**4** and related TSCs and Pd(II) complexes on leukemia P388-bearing BDF1

Compound	LD_{50}[a]	LD_{10}[a]	MST ± SD[b] (days)	T/C[b] (%)	Cures
Controls	–	Saline	10.00 ± 0.41	100	0/6
HL^1 (**1**)	63	40	15.30 ± 0.47	147	0/6
HL^2 (**2**)	60	37	12.30 ± 0.44	118	0/6
[Pt(L^1)Cl] (**3**)	80	53	31.00 ± 0.51	298*	5/6
[Pt(L^2)Cl] (**4**)	107	76	43.40 ± 0.49	417	0/6
HAcTsc[c]	25	10	10 90 + 0.19	106	0/6
HFoTsc[c]		20	11.25 ± 0.21	129	0/6
[Pd(AcTsc)Cl][c]	80	50	9.40 ± 0.22	111	0/6
[Pd(AcTsc)$_2$][c]	65	40	10.80 ± 0.25	127	0/6
[Pd(FoTsc)Cl][c]		38	11.50 ± 0.19	132	0/6
[Pd(FoTsc)$_2$][c]		150	13.00 ± 0.25	149	0/6
[Pd(L_2)$_2$][d]	37	30		166	0/6
Cisplatin[e]			22.60 ± 6.5	138	

[a] LD_{50} and LD_{10} (dosage μg/g body wt)values were estimated graphically: percent deaths due to the toxicity of each dose are shown on the ordinate, while the administered doses are indicated on the abscissa on semi-logarithmic paper. LD_{50} and LD_{10} = lethal doses for 50 and 10%, respectively, of the mice used (10 animals per dose).

[b] LD_{10} was given as a single dose on the first day after tumor transplantation for antitumor evaluation. MST, mean survival time; T/C %, median survival time of the drug-treated animals *versus* corn oil-treated controls (C).

[c] Where HFoTsc and HAcTsc is the related pyridine-2-carbaldehyde and 2-acetyl pyridine Tsc, respectively [14,15].

[d] Ref. [14].

[e] Ref. [28]; dose 3 mg/kg.

and then must interact with its receptor. The receptor may have a lipophilic pocket, a hydrogen bonding surface and a π system to interact with the lipophilic, hydrogen bonding moieties and pyridine ring of compounds **1**–**4**. The goal of reducing toxicity while maintaining therapeutic efficiency can be accomplished by improving the solubility of the complexes, by slowing down degradation processes through shielding of the platinum with bulky ligands, and by increasing membrane permeability with more lipophilic ligands. In complexes **3** and **4** it is anticipated that extra lipophilicity and extra bulk to shield the platinum are both introduced by the azepane ring and the molecular shape of compounds.

3. Experimental section

3.1. Measurements

Solvents were purified and dried according to standard procedures. The two ligands were prepared according to the method described in the literature [7,16]. [Pd(FoTsc)Cl], [Pd(FoTsc)$_2$], [Pd(AcTsc)Cl] and [Pd(AcTsc)$_2$] (where HFoTsc and HAcTsc is pyridine-2-carbaldehyde and 2-acetyl pyridine thiosemicarbazone, respectively) were prepared according to Refs. [17,18]. [Pd(L^2)$_2$] was prepared according to Ref. [19]. For the platinum(II) compounds a stock 0.04 M solution of $Na_2[PtCl_4]$ was prepared by dissolving $PtCl_2$ (2.66 g, 10 mmol) in conc. HCl under reflux, filtering to remove a turbidity of undissolved material, neutralizing with Na_2CO_3 and diluting with distilled water up to 250 ml (pH = 6.0–6.5). Infrared and far-infrared spectra were recorded on a Perkin–Elmer Spectrum GX FT-IR System spectrophotometer using KBr pellets (4000–400 cm^{-1}) and nujol mulls dispersed between polyethylene disks (400–40 cm^{-1}). NMR experiments were performed using a Brüker AMX-400 MHz NMR spectrometer. Elemental analyses, C, H, N and S were performed on a Carlo Erba EA (model 1108).

3.2. Syntheses of TSCs and platinum(II) complexes

3.2.1. (E)-N′-(pyridin-2-ylmethylene)azepane-1-carbothiohydrazide, HL^1 (**1**)

4-Methyl-4-phenyl-3-thiosemicarbazide was prepared according to the method described by Scovil [16]. The crude product, 4-methyl-4-phenyl-3-thiosemicarbazide, was recrystallized from a mixture of EtOH and distilled water (3:1). To a solution of 4-methyl-4-phenyl-3-thiosemicarbazide (1.000 g, 5.52 mmol) in CH_3CN (1.50 ml), azepane (0.63 mL, 5.52 mmol) and 2-formyl pyridine (0.55 mL, 5.52 mmol) were added. The mixture was refluxed for 45 min and then left standing in a refrigerator for 12 h. The resulting light-orange precipitate was filtered off, washed with cold CH_3CN, recrystallized from CH_3CN and dried *in vacuo* over silica gel, and then at 40 °C *in vacuo* over P_4O_{10} for 3 h. [7,16]; m.p. 109 °C. Yield 53%. Elemental analysis calcd for $C_{13}H_{18}N_4S$: C, 59.5; H, 6.9; N, 21.4; S 12.2. Found: C, 60.5; H, 5.4; N, 21.7; S, 12.4%. m.p. 108 °C. Yield 53.0%. IR (KBr): 1591 ν(C=N), 1002 ν(N–N), 879 ν(C=S), 580 ν(py) cm^{-1}; ^{1}H NMR (DMSO-d^6): δ 13.38 (s, 1H, NH), 8.56 (d, J = 5.0 Hz, H1), 7.35 (t, J = 2.5 Hz, H2), 7.60 (t, J = 9.9 Hz, H3), 7.45 (d, J = 7.5 Hz, H4), 7.85 (s, H6); ^{13}C NMR (DMSO-d^6): δ 144.3(C1), 129.4(C2), 137.4(C3), 128.4(C4), 152.2(C5), 146.7(C6), 183.1(C7).

3.2.2. (E)-N′-(1-(pyridin-2-yl)ethylidene)azepane-1-carbothiohydrazide, HL^2 (**2**)

Compound **2** was prepared as described in literature [7,16]. To a solution of 4-methyl-4-phenyl-3-thiosemicarbazide (1.000 g, 5.52 mmol) in CH_3CN (5 mL), azepane (0.63 ml, 5.52 mmol) and 2-acetyl pyidine (0.61 mL, 5.52 mmol) were added. The mixture was refluxed for 15 min and then left standing in a refrigerator for 1 h. The resulting yellow precipitate was filtered off, washed with cold CH_3CN, recrystallized from CH_3CN and dried *in vacuo* over silica gel, and then at 40 °C *in vacuo* over P_4O_{10} for 3 h [7,16]; m.p. 159 °C. Yield 50%. Elemental analysis calcd for $C_{14}H_{20}N_4S$: C, 60.9; H, 7.2; N, 20.3; S, 11.6. Found: C, 61.7; H, 7.6; N, 20.7; S, 11.7; m.p. 160 °C. Yield 43%. IR (KBr): 1583 (C=N), 1014 ν(N–N), 880 ν(C=S), 594 ν(py) cm^{-1}; ^{1}H NMR (DMSO-d^6): δ 15.07 (s, 1H, NH), 8.79 (d, J = 5.0 Hz, H1), 7.58 (t, J = 9.2 Hz, H2), 7.80 (t, J = 7.7 Hz, H3), 7.99 (d, J = 7.7 Hz, H4), 2.60 (s, CH_3); ^{13}C NMR (DMSO-d^6): δ 148.6(C1), 124.1(C2), 136.5 (3), 121.8(C4), 152.8(C5),146.7(C6), 13.0 (CH_3), 182.8(C8).

3.2.3. [Pt(L^1)Cl] (**3**)

To a solution of **1** (0.55 mmol) in methanol (10 ml) was added the stock solution of $Na_2[PtCl_4]$ (15 mL, 0.6 mmol). The reaction mixture was stirred for 3 days at 24 °C and then left at 3–4 °C for 1 day. The dark-red powder was filtered off, recrystallized from hot methanol and washed with cold methanol and ether, dried *in vacuo* over silica gel, and re-dried at 70 °C *in vacuo* over P_4O_{10}. Colour dark-red and m.p. 230 °C (with decomposition). Yield 70%. Elemental analyses calcd for $C_{13}H_{17}N_4SPtCl$: C, 31.8; H, 3.5; N, 11.4; S, 6.5. Found: C, 31.8; H, 2.6; N, 10.9; S, 7.0. %. FT-IR (KBr): ν(C=N), 1595; ν(N–N), 1025; ν(C=S), 767, 757 δ(py), 618, 583; ν(Pt–N), 422; ν(Pt–Cl), 346; ν(Pt–S), 372; ν(Pt–N_{py}), 318 cm^{-1}; ^{1}H NMR (DMSO-d^6): δ 8.89 (d, J = 5.0 Hz, H1), 7.43 (t, J = 3.7 Hz, H2), 8.02 (t, J = 2.5 Hz, H3), 7.73 (d, J = 7.5 Hz, H4), 8.56 (s, H6); ^{13}C NMR (DMSO-d^6): δ 159.8(C1), 129.4(C2), 139.8(C3), 124.0(C4), 147.5(C5), 145.4(C6), 187.5(C7),

3.2.4. [Pt(L^2)Cl] (**4**)

A solution of **2** (0.55 mmol) in methanol (10 ml) was added to the stock solution of $Na_2[PtCl_4]$ [(30 mL, 0.6 mmol). The reaction mixture was stirred for 3 days at 24 °C and then left at 3–4 °C for 1 day. The orange powder was filtered off, washed with cold methanol and ether, dried *in vacuo* over silica gel, and re-dried at 70 °C *in vacuo* over P_4O_{10}. Yield 30%; m.p. 285 °C (with decomposition). Elemental analyses calcd for $C_{14}H_{19}N_4SPtCl$: C, 33.2; H, 4.0; N, 11.1; S 6.3. Found: C, 31.1; H, 4.0; N, 11.1; S, 6.3%. IR (KBr): ν(C=N), 1599; ν(N–N), 1023; ν(C=S), 770; δ (py), 619; ν(Pt–Cl), 338; ν(Pt–N), 429; ν(Pt–S), 419; ν(Pt–N_{py}), 301 cm^{-1}; ^{1}H NMR (DMSO-d^6): δ 9.05 (d, J = 5.2 Hz, H1), 7.41 (t, J = 6.7 Hz, H2), 7.93 (d, J = 7.7 Hz, H3), 8.90 (d, J = 7.0 H4), 2.32 (s, CH_3); ^{13}C NMR (DMSO-d^6): δ 147.7(C1), 124.8(C2), 139.2(C3), 122.9(C4), 152.9(C5), 149.9(C6),184.1(C8). Suitable crystals for X-ray study were obtained by crystallization from a fresh solution of DMF.

3.3. X-ray crystallography

A crystal of dimensions 0.75 × 0.25 × 0.20 mm. was glued to a glass fiber and mounted on the head of an Enraf–Nonius CAD4 diffractometer. Graphite-monochromated Mo Kα (λ 0.71073 Å) radiation was used. The unit cell was found to be monoclinic from analysis of 25 centred reflections in the range 23.9° < θ < 26°. Space group $P2_1/n$ was assigned, with μ(Mo Kα) 9.061 mm^{-1}, d(calc) 2.099 g cm^{-3}, temperature 291(1) K; 3376 reflections were collected in ω-2θ mode in the range 1.6° < θ < 26.0°. Three standard reflections were collected every 2 h. Decay of 28% *ca.* was observed and corrected during processing [17]. Lorentz and polarisation effects were corrected. An empirical absorption correction was made using ψ-scans of 9 reflections (T_{max} = 0.0181, T_{min} = 0.0075). The structure was solved by the Patterson heavy atom method and successive cycles of full matrix refinement and Fourier difference syntheses [18]. An empirical absorption correction was made using the program "DIFABS" [19]. All non-hydrogen atoms were refined anisotropically on F by full matrix least squares. Hydrogen atoms

were placed in calculated positions and used in structure factor calculations but not refined. Molecular graphics were performed from PLATON2001 [20,21].

Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-21939. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

3.4. Biological experiments

3.4.1. *Antiproliferative assay* in vitro

Compounds. Test solutions of the compounds tested (1 mg/ml) were prepared by dissolving the substance in 100 μl of DMSO completed with 900 μl of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium to reach the final concentrations of 100, 40, 10, 1, and 0.1 ng/μl. The solvent (DMSO) in the highest concentration used in test did not reveal any cytotoxic activity.

Cells. The established *in vitro* cancer cell lines are maintained in the Cell Culture Collection of the University of Ioannina. Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates at a density of 10^4 cells per well. The MCF-7 and T24 cells were cultured in the D-MEM (Modified Eagle's Medium) medium supplemented with 1% antibiotic and 10% fetal calf serum. L-929 cells were grown in Hepes-buffered RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 mg/ml). A-549 cells were grown in F-12 K Ham's medium supplemented with 1% glutamine, 1% antibiotic/antimycotic, 2% $NaHCO_3$ and 10% fetal calf serum. The cell cultures were maintained at 37 °C in a humid atmosphere saturated with 5% CO_2. Cell number was counted by the Trypan blue dye exclusion method. MCF-7, L-929 and A-549 cells were determined by the sulforhodamine B assay, while T24 cells by the MTT assay [10,25,26].

SRB assay. The details of this technique were described by Skekhan et al. [22]. The cytotoxicity assay was performed after 72-hour exposure of the cultured cells to varying concentrations (from 0.1 to 100 ng/μl) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% trichloroacetic acid (TCA) on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained for 30 min with 0.4% sulforhodamine B dissolved in 1% acetic acid. Unbound dye was removed by rinsing with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate.

MTT. This technique was applied for cytotoxicity screening against T-24 cells growing in suspension culture. An assay was performed after 72 h exposure to varying concentrations (from 0.1 to 100 ng/μl) of the tested agents. For the last 3–4 h of incubation 20 μl of MTT solution was added to each well [MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma; stock solution: 5 mg/ml]. The mitochondria of viable cells reduce a pale yellow MTT to a navy blue formazan, so if more viable cells are present in the well, more MTT will be reduced to formazan. When the incubation time was completed, 80 μl of the lysing mixture was added to each well (lysing mixture: 225 ml dimethylformamide, 67.5 g sodium dodecylsulfate (both from Sigma) and 275 ml of distilled water). After 24 h, when formazan crystals had been dissolved, the optical densities of the samples were read on an Eliza spectra max 190 photometer at 570 nm wavelength.

Each compound in given concentration was tested in triplicates in each experiment, which was repeated three times.

3.4.2. In vivo *antitumor experiments*

Mice. BALB/c, DBA/2 and BDF1 mice of both sexes, weighting 20–23 g, 6–8 weeks old were used for toxicity studies and antitumor evaluation. Mice obtained from the experimental section of the Research Center of Theagenion Anticancer Hospital, Thessaloniki, Greece, were kept under conditions of constant temperature and humidity, in sterile cages, with water and food. Leukemia P388-bearing BDF1 (DBA/2·C57BL) mice were used to evaluate the cytostatic effect. Lymphocytic P388 leukemias were maintained in ascitic form by injection of 10^6 cell at 6-day intervals, into the peritoneal cavity of DBA/2 mice.

Compounds. For intraperitoneal (ip) treatment, stock solutions of the compounds used in this study were prepared immediately before use. They were suspended in corn oil in the desired concentration following initial dissolution in 10% dimethylsulfoxide (DMSO). This concentration by itself produced no observable toxic effects

Estimation of acute toxicity. The acute toxicity of the compounds was determined following a single ip injection into BDF1 mice in groups of 10 mice per dose at three different dosages. The mice were observed for 30 days and the therapeutic dose of the compounds was determined after graphical estimation of the LD50 (30-day curves). The dose used for a single treatment was equal to the LD10.

Antileukemic evaluation. For the survival experiments, the antileukemic activity of the tested compounds against Leukemia P388 was assessed from the oncostatic parameter T/C %, that is, the increase in median life span of the drug-treated animals (T) excluding long-term survivors *versus* corn oil-treated controls (C) was expressed as a percentage. The other index of the antileukemic activity used was the number of long-term survivors defined as mice alive for 90 days after tumor inoculation. Each drug-treated group consisted of six mice while the tumor control group included eight mice; in each group, equal numbers of male and female mice were used. Experiments were initiated by implanting mice with tumor cells according to the protocol of the National Cancer Institute, USA [27]. The experiments were terminated on day 90. Statistical evaluation of the experimental data was made by the Wilcoxon test.

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