New bioactive 2,6-diacetylpyridine bis(p-chlorophenylthiosemicarbazone) ligand and its Pd(II) and Pt(II) complexes: synthesis, characterization, cytotoxic activity and DNA binding ability.

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Abstract

Preparation and characterization of 2,6-diacetylpypyridine bis(N-p-chlorophenylthiosemicarbazone) ligand, H₂L, and its palladium(II) and platinum(II) complexes [PdL] and [PtL], is described. The molecular structure of the two new complexes has been determined by single crystal X-ray diffraction. The ligand act as dianionic tetradentate donor coordinating to the metal center in a square planar geometry through the pyridine nitrogen atom and the azomethine nitrogen and thione sulfur atoms from one thiosemicarbazone arm, the fourth coordination position is occupied by the hydrazine nitrogen atom of the other arm. New free ligand and its metal complexes have been evaluated for antiproliferative activity in vitro against NCI-H460, T-47D, A2780 and A2780cisR human cancer cell lines. The cytotoxicity data suggest that these compounds may be endowed with important antitumor properties, especially H₂L and [PtL] since they are capable of not only circumvent cisplatin resistance in A2780cisR cells but also exhibit high antiproliferative activity in breast cancer T-47D cells. The interaction of H₂L with calf thymus DNA was also investigated and its binding constant (Kb) determined.
1. Introduction

Platinum metallo-drugs are among the most effective agents for the treatment of cancer however its clinical utility is restricted due to the frequent development of drug resistance, the limited spectrum of tumors against which these drugs are active and also the severe normal tissue toxicity [1-5]. These disadvantages have driven the development of improved platinum-based anticancer drugs whose structure and mode of action differ from that of cisplatin, especially those that interact with specific molecular targets as for example are the processes associated to DNA: transcription, replication and repair [6-9].

In this regard, of particular interest are compounds targeting ribonucleotide reductase (RR), enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides and provides the building blocks for the de novo DNA synthesis in all living cells. Cancer cells require increased RR activity to meet the demand for deoxyribonucleotides that are needed to support their rapid proliferation. Thus inhibition of RR activity leads to inhibition of DNA synthesis and repair, and also induces cell cycle arrest and apoptosis [10-12].

α-(N)-heterocyclic thiosemicarbazones, (N)-TSCs, have been reported to be among the most effective RR inhibitors yet identified and many efforts have been devoted to the study of the structure–activity relationship of thiosemicarbazone derivatives. The anticancer activity of (N)-TSCs is closely related to the nature of the heterocyclic ring of the parent aldehyde or ketone, metal chelation ability and terminal amino substitution [13-20]. In this sense pyridine ring itself is a part of many natural and synthetically prepared pharmaceuticals and moreover it plays a significant role in many biological processes like nicotinamide adenine dinucleotide phosphate NADP or the important vitamins niacin and pyridoxine (vitamins B3 and B6) [21, 22].
Keeping in view the above observations and as part of our systematic investigation on the coordination chemistry of thiosemicarbazone derivatives we recently reported palladium(II) and platinum(II) complexes derived of 2,6-diacetylpyridine bis(4-N-o-tolylthiosemicarbazone) and 2,6-diacetylpyridine bis(4-N-p-tolylthiosemicarbazone) ligands. The \textit{in vitro} antitumor studies have shown that these complexes exhibit important antiproliferative activity in A2780 and A2780cisR human cancer cell lines and these results encouraged us to further investigate their cytotoxic properties as well as those of novel derivatives [23].

This work is aimed to determine if the presence of an aryl ring with an electron withdrawing substituent (such as \textit{p}-chlorophenyl group) results beneficial for the antitumor activity of bis(4-N-substituted thiosemicarbazones) ligands derived from 2,6-diacetylpyridine. Therefore here we describe the synthesis and chemical characterization of the new 2,6-diacetylpyridine bis(4-N-\textit{p}-chlorophenylthiosemicarbazone) ligand, \( \text{H}_2\text{L} \), (Scheme 1) and its palladium(II) and platinum(II) complexes, \([\text{PdL}]\) and \([\text{PtL}]\).

Insert Scheme 1

The cytotoxic activity of the new compounds synthesized and cisplatin (assumed as the reference antitumor drug) against four human cancer cell lines: NCI-H460 (non-small cell lung cancer), T-47D (breast cancer), A2780 and A2780cisR (epithelial ovarian cancer) has been studied. The interaction of \( \text{H}_2\text{L} \) with calf thymus DNA (CT-DNA) was also investigated and its binding constant (\( \text{K}_b \)) determined.
2. Experimental

2.1. Measurements

Elemental analyses were performed on a LECO CHNS-932 microanalyzer. Fast atom bombardment (FAB) mass spectra (MS) were performed on a VG AutoSpec spectrometer. $^1$H NMR spectra were recorded on Bruker AMX-300 spectrometer. All cited physical measurements were obtained out by the Servicio Interdepartamental de Investigación (SIdI) of the Universidad Autónoma de Madrid.

Melting points were determined with a Stuart Scientific SMP3 apparatus. Infrared spectra were recorded on a Bomen–Michelson spectrophotometer. $^{13}$C NMR spectra were recorded on a 400 Advance Bruker Fourier Transform spectrometer. Electronic spectra were recorded on a Thermo Scientific Evolution 260 Bio UV-visible (UV-VIS) spectrophotometer.

2.2. Materials

Solvents were purified and dried according to standard procedures. Hydrazine hydrate, 2,6-diacetylpyridine, $p$-chlorophenyl isothiocyanate, PdCl$_2$(PPh$_3$)$_2$ and PtCl$_2$(PPh$_3$)$_2$ were commercially available.

2.3. Synthesis of compounds

2,6-Diacetylpyridine bis(4-$p$-chlorophenylthiosemicarbazone), H$_2$L. An ethanolic solution of hydrazine hydrate (0.250 g, 5 mmol) was added dropwise with constant stirring to an ethanolic solution of $p$-chlorophenyl isothiocyanate (0.848 g, 5 mmol). The reaction mixture was stirred for one more hour and then the white product $p$-chlorophenylthiosemicarbazide formed was filtered, washed with cold ethanol and diethyl ether, dried *in vacuo* and recrystallized from ethanol. An ethanolic solution of the $p$-chlorophenylthiosemicarbazide (0.402 g, 2 mmol) was then stirred with 2,6-diacetylpyridine (0.163 g, 1 mmol) for 5 h. The resulting solution was reduced to half
volume and the pale yellow solid formed was filtered, washed with ethanol, diethyl ether and finally dried *in vacuo*.

Yield (80%), mp 220 °C (decomposes). Elemental analysis found, C, 52.20; H, 4.25; N, 18.00; S, 12.20. \(\text{C}_23\text{H}_21\text{N}_7\text{S}_2\text{Cl}_2\) requires C, 52.07; H, 3.99, N, 18.48; S, 12.09 %. MS (FAB\(^+\) with \(\text{mNBA}\): nitrobenzyl alcohol matrix) \(m/z\) 530.0 for \([\text{H}_2\text{L}+\text{H}]^+\). IR (KBr pellet): \(\nu/\text{cm}^{-1}\) 3335, 3306, 3210 (w, NH); 1588 (s, CN); 827, 809 (w, CS-thioamide IV); 585 (pyridine ring). \(^1\text{H} \text{NMR}\) (\(\text{d}^6\)-DMSO, ppm), \(\delta=10.80 \ [\text{s}, 2\text{NH}, 2\text{H}]; 10.20 \ [\text{s}, 4\text{NH}, 2\text{H}]; 8.55 \ [\text{d}, J=7.9 \text{ Hz}, \text{CH-pyridine}, 2\text{H}]; 7.85 \ [\text{t}, J=7.9 \text{ Hz}, \text{CH-pyridine}, 1\text{H}]; 7.60 \ (\text{d}, J=8.7 \text{ Hz}, \text{aromatic-thiosemicarbazide}, 4\text{H}); 7.45 \ (\text{d}, J=8.7 \text{ Hz}, \text{aromatic-thiosemicarbazide}, 4\text{H}); 2.50 \ (\text{s}, \text{CH}_3\text{-diacetylpyridine}, 6\text{H}). \(^{13}\text{C} \text{NMR}\) (\(\text{d}^6\)-DMSO, ppm), \(\delta=178.3 \ (\text{C}=\text{S}); 153.78 \ (\text{C}2,\text{C}6\text{-pyridine}); 149.85 \ (\text{C}=^{1}\text{N}); 138.54 \ (\text{C}4\text{-pyridine}); 137.17 \ (\text{C}3,\text{C}5\text{-pyridine}); 130.08 \ (\text{aromatic-thiosemicarbazide}); 128.49 \ (\text{aromatic-thiosemicarbazide}); 128.34 \ (\text{aromatic-thiosemicarbazide}); 127.37 \ (\text{aromatic-thiosemicarbazide}); 121.96 \ (\text{aromatic-thiosemicarbazide}); 12.98 \ (\text{CH}_3\text{-diacetylpyridine}). \n \nUV/VIS (DMSO): \(\lambda/\text{nm}\) 250, 337.

2,6-Diacetylpyridine \ bis(4\text{N-p-chlorophenylthiosemicarbazonato}) palladium(II), \([\text{PdL}]\). The reaction of \(\text{H}_2\text{L}\) ligand with \(\text{PdCl}_2(\text{PPh}_3)_2\), in toluene, in presence of \(\text{Et}_3\text{N}\), in 1:1 molar ratios over 20 h at room temperature led to the formation of an orange solution which was filtered and left to stand at ambient temperature for two days. The brown solid formed was filtered, washed several times with hot water, diethyl ether and finally dried *in vacuo*.

Yield (55%), mp >250 °C. Elemental analysis found, C, 43.35; H, 3.35, N, 15.10; S, 10.05; \(\text{C}_{23}\text{H}_{19}\text{N}_7\text{S}_2\text{Cl}_2\text{Pd}\) requires C, 43.51; H, 3.02, N, 15.44; S, 10.10 %. MS (FAB\(^+\) with \(\text{mNBA}\) matrix) \(m/z\) 636 for \([\text{PdL}+\text{H}]^+\). IR (KBr pellet): \(\nu/\text{cm}^{-1}\) 3243 (s, NH); 1595 (s, CN); 830, 804 (vw) (CS-thioamide IV); 603 (pyridine ring). \(^1\text{H} \text{NMR}\) (300 MHz, \(\text{d}^6\)-
DMSO, ppm), δ=10.75, 10.18 [s, 4NH, 2H]; 8.43-8.10 [m, CH-pyridine, 3H]; 7.71-7.34 (m, aromatic-thiosemicarbazide, 8H); 2.72, 2.62 (s, CH$_3$-diacetylpyridine, 6H). UV/VIS (DMSO): λ/nm 267, 340, 410, 470.

Recrystallization from DMSO led to the isolation of orange crystals of [PdL]-DMSO that were suitable for X-ray-diffraction.

2,6-Diacetylpyridine bis(4N-p-chlorophenylthiosemicarbazonato)platinum(II), [PtL]. It was prepared by the same procedure as described for [PdL] by reaction of H$_2$L with PtCl$_2$(PPh$_3$)$_2$ and afforded a brown solid.

Yield (35%), mp 192 °C (decomposes). Elemental analysis found, C, 37.90; H, 2.55, N, 13.60; S, 8.60; C$_{23}$H$_{19}$N$_7$S$_2$Cl$_2$Pt requires C, 38.18; H, 2.65, N, 13.55; S, 8.86 %. MS (FAB$^+$ with mNBA matrix) $m/z$ 724 for [PtL+H]$^+$. IR (KBr pellet): $\nu$/cm$^{-1}$ 3288, 3208 (s, NH); 1590 (s, CN); 826, 809 (vw) (CS-thioamide IV); 591 (pyridine ring). $^1$H NMR (300 MHz, d$_6$-DMSO, ppm), δ=11.00, 10.30 [s, 4NH, 2H]; δ=8.56-8.10 [m, CH-pyridine, 3H]; δ=7.76-7.70 (m, aromatic-thiosemicarbazide, 8H); δ=2.78, 2.71 (s, CH$_3$-diacetylpyridine, 6H). UV/VIS (DMSO): λ/nm 249, 267, 366.

Recrystallization from DMSO led to the isolation of orange crystals of [PtL]-DMSO that were suitable for X-ray-diffraction.

2.4. Crystallography

Data were collected on a Bruker X8 APEX II CCD. Crystallographic data and selected interatomic distances and angles are listed in Table 1. For all compounds, the software package SHELXTL was used for space group determination, structure solution, and refinement [24]. The structures were solved by direct methods, completed with difference Fourier syntheses, and refined with anisotropic displacement parameters.
CCDC 981268 and 981269 contain the supplementary crystallographic data for compounds [PdL] and [PtL] respectively. These data can be obtained free of charge at 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; e-mail: deposit@ccdc.cam.ac.uk].

2.5. *In vitro* antiproliferative activity

The human cancer cells: A2780 and A2780cisR (epithelial ovarian cancer), T-47D (breast cancer) and NCI-H460 (non-small cell lung cancer); were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine in an atmosphere of 5% CO₂ at 37 °C.

Cell proliferation was evaluated by the sulforhodamine B assay. Cells were plated in 96-well sterile plates at a density of 1.5x10⁴ (for NCI-H460), 4x10³ (for A2780 and A2780cisR) or 5x10³ (for T-47D) cells per well with 100μL of medium and were then incubated for 24 h (A2780, A2780cisR and NCI-H460) or 48 h (T-47D). After attachment to the culture surface the cells were incubated with various concentrations of the compounds tested freshly dissolved in DMSO (1 mg/mL) and diluted in the culture medium (DMSO final concentration 1%) for 48 h (for NCI-H460) or 96 h (for A2780, A2780cisR and T-47D). The cells were fixed by adding 50 μL of 30% trichloroacetic acid (TCA) per well.

The plates were incubated at 4 °C for 1 h and then washed five times with distilled water. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B dissolved in 1% acetic acid for 10 min. Unbound dye was removed by rinsing with 0.1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 515 nm) in a Tecan Ultra Evolution spectrophotometer.
The effects of compounds were expressed as corrected percentage inhibition values according to the following equation:

\[
\text{% inhibition} = [1 - (T/C)] \times 100
\]

where T is the mean absorbance of the treated cells and C the mean absorbance in the controls.

The inhibitory potential of compounds was measured by calculating concentration–percentage inhibition curves, these curves were adjusted to the following equation:

\[
E = \frac{E_{\text{max}}}{[1 + (IC_{50}/C)^n]}
\]

where E is the percentage inhibition observed, \(E_{\text{max}}\) is the maximal effects, \(IC_{50}\) is the concentration that inhibits 50% of maximal growth, C is the concentration of compounds tested and n is the slope of the semi-logarithmic dose–response sigmoid curves. This non-linear fitting was performed using GraphPad Prism software [25].

For comparison purposes, the antiproliferative activity of cisplatin was evaluated under the same experimental conditions. All compounds were tested in two independent studies with triplicate points. These experiments were carried out at the Unidad de Evaluación de Actividades Farmacológicas de Compuestos Químicos (USEF), Universidad de Santiago de Compostela.

2.6. DNA-Binding Experiments

Calf thymus DNA (CT-DNA) stock solution was prepared by dissolving the lyophilized sodium salt in Tris-buffer (NaCl 50 mM, Tris-HCl 5 mM, pH was adjusted to 7.2 with NaOH 0.5 M) by stirring for 5 hours. The CT-DNA solution was standardized spectrophotometrically [26] by using its known molar absorption coefficient at 260 nm (6600 M\(^{-1}\cdot\text{cm}^{-1}\)). The ratio of UV absorbance at 260 and 280 nm,
A<sub>260</sub>/A<sub>280</sub>, of ca. 1.9, indicating that the DNA was sufficiently free of protein. Stock solution was kept frozen until the day of the experiment.

Concentrated stock solutions (5x10<sup>-3</sup> M and 5x10<sup>-5</sup> M) of H<sub>2</sub>L were prepared dissolving the compound in DMSO. From these stock solutions, for all experiments the desired concentration of compound was achieved by dilution with Tris-buffer (NaCl 50 mM, Tris-HCl 5 mM, pH was adjusted to 7.2 with NaOH 0.5 M) to give homogeneous solutions with DMSO content of less than 2.5%.

To investigate the binding mode, spectrophotometric titrations were performed at a fixed DNA concentration equal to 1.7x10<sup>-4</sup> M with increasing concentration of H<sub>2</sub>L (0-125 μM) and monitoring the absorbance change at the wavelength maximum 260 nm after incubation (10 min. at 37ºC).

To calculate the binding parameters, the spectrophotometric titrations were performed with increasing concentration of DNA (0-40 μM) at a fixed compound concentration equal to 50 μM and monitoring the absorbance change in one characteristic charge transference band of the compound after incubation (10 min. at 37ºC).

3. Results and discussion

3.1. Synthesis and spectroscopic characterization

A new 2,6-diacetylpypyridine bis(4<sup>N</sup>-monosubstituted thiosemicarbazone) ligand has been synthesized with high purities and acceptable yields. The yellow compound obtained is stable to air and moisture and was characterized by elemental analysis, FAB<sup>+</sup> spectrometry and IR and NMR (1<sup>H</sup> and 13<sup>C</sup>) spectroscopy.

Reaction of 2,6-diacetylpypyridine bis(4<sup>N</sup>-p-chlorophenylthiosemicarbazone) ligand with equimolar amount of MCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>, where M = Pd(II) or Pt(II), led to the
isolation of neutral mononuclear complexes [PdL] and [PtL] in which the bis(thiosemicarbazone) behaves as dianionic ligand with deprotonation of hydrazine (2NH) protons and [NNNS] donor set.

Both complexes were characterized by routine analytical and spectroscopic techniques. Analytical data are consistent with the formulation given, thus the FAB+ mass spectra exhibited a very weak ion at m/z = 636 for [PdL] and m/z = 724 for [PtL] which corresponded to the predicted molecular weight of the [M+H]+ ions and moreover the isotopic patterns of this signal fit well with the theoretical isotopic distributions.

The significant IR vibrational bands and the 1H chemical shift values of the free ligand and its complexes are listed in the Experimental section and Scheme 1 shows the numbered structure of the free ligand. As the X-ray study has shown, during metal complexation, the ligand behaves as a tetradeate dianionic forming two five-membered and one six-membered chelate rings around the metal center. The high delocalization and the asymmetric coordination hinder the IR analysis. The stretching vibration υ(C=N) and the in-plane pyridine deformation bands are slightly shifted to higher wavenumbers which are consistent with the implication of imine and pyridine nitrogen atoms in the coordination however this induces only minor changes in υ(C=S) thioamide IV band which decreases slightly in intensity.

In the 1H NMR spectrum of the double-armed H2L ligand, two independent singlets at δ = 10.80 and 10.20 ppm are observed for >C=N-2NH- and -C(S)-4NH-protons respectively. A doublet at δ = 8.55 ppm and a triplet at δ = 7.85 ppm are assigned to pyridyl ring protons. Aromatic p-chlorophenyl protons appear as a multiplet at δ = 7.60-7.45 ppm and a sharp singlet at δ = 2.50 is assigned to the methyl protons. In [PdL] and [PtL] complexes, the anionic coordination of the ligand is evidenced by
the disappearance of the signal corresponding to the \( >\text{C}=\text{N}^-\text{NH}^- \) protons. On the other hand, the \(-\text{C(S)}^-\text{NH}^-\) protons appear two independent signals (10.75 and 10.18 ppm for [PdL] and 11.00 and 10.30 ppm for [PtL]) due to the asymmetric coordination. The pyridyl ring protons appear as a multiplet at \( \delta = 8.43\text{-}8.10 \) ppm for [PdL] and \( \delta = 8.56\text{-}8.10 \) ppm for [PtL] and the rest of the proton signals appear at nearly identical positions if they are compared with the corresponding ligand signals.

\(^{13}\text{C}\) NMR spectrum of the free ligand shows carbon signals supporting the \(^1\text{H}\) NMR assignments however due to the low solubility of the complexes it was not possible to get \(^{13}\text{C}\) NMR spectra of reasonably quality.

The electronic absorption spectra of both \([\text{H}_2\text{L}]\) ligand and [PdL] and [PtL] complexes exhibit two intense band in the region 250-400 nm, which can ascribed to ligand-centered n→π* and π→π* transitions. In addition the spectra of metal complexes exhibit other less energetic bands assigned to a ligand to metal (LMCT) and metal to ligand charge transfer (MLCT) transitions [27].

### 3.2. Description of the crystal structures

[PdL]-DMSO and [PtL]-DMSO were isolated as neutral compounds. The most significant crystallographic data for these complexes are shown in Table 1, whereas selected bond lengths and bond angles are presented in Table 2.

Insert Table 1

The structures together with the atom labeling schemes are shown in Figures 1 and 2. Both compounds are isostructural hence displaying nearly identical cell parameters, crystallize in the triclinic \( P\bar{1} \) space group with \( Z=2 \) and the asymmetric units
contain one molecule of the neutral complex and one dimethyl sulfoxide solvent molecule.

The metal ion presents a square planar geometry where the bis(thiosemicarbazonate) ligand is coordinated to the metal ion through the pyridine nitrogen atom and the azomethine nitrogen and thione sulfur atoms from one thiosemicarbazone arm and being the fourth coordination position occupied by the hydrazine nitrogen atom of the other thiosemicarbazone arm generating two typical five membered (PdSCNN and PdNCCN or PtSCNN and PtNCCN) and one six membered (PdNNCCN or PtNNCCN) chelate rings. Coordination by hydrazine nitrogen atom instead of the azomethine nitrogen atom, although uncommon, has been found in the bibliography for some d^8 bis(thiosemicarbazone) complexes [23, 28-31].

The M–N and M–S bond distances are similar to those found in other palladium(II) and platinum(II) complexes. It is important to note that the two thiosemicarbazone moieties, which are symmetrically deprotonated, coordinate in a different fashion. Upon coordination the bidentate-N,S arm undergoes significant evolution from the thione to the thiol form which is reflected in C–S distance of 1.779(4) for [PdL]·DMSO and 1.804(10) for [PtL]·DMSO while the monodentate-thiosemicarbazone [N(3), hydrazine nitrogen atom] arm maintains its thione form as reflects in their C–S bond length of 1.673(5) for [PdL]·DMSO and 1.672(3) Å for [PtL]·DMSO, typical of double C=S bond.
Comparison of C–N and N–N bond distances with typical lengths of single and double bonds [C-N 1.47, C≡N 1.28, N-N 1.45, N≡N 1.25Å] suggests extensive charge delocalization over the thiosemicarbazone moieties [32, 33] and also agree with the thiolate tautomeric form of the bidentate thiosemicarbazonate arm and the thione tautomeric form of the monodentate one (Scheme 2).

Inspection of the angles formed between the metal ion (M=Pd^{2+}, Pt^{2+}) and the coordinated atoms shows that the metal is contained within a slightly distorted square-planar environment being the bond angles between adjacent coordinating atoms in the 80.5-104.2º range.

The crystal structures are stabilized by intermolecular hydrogen interactions involving the N(7) atom of the bidentate thiosemicarbazonate arms and the oxygen atom of DMSO solvent molecule being the N(7)-H(7)···O(1) contact distance 2.83 Å for both complexes and <(NHO) angle 170.4º for [PdL] and 169.2º for [PtL]. Further stabilization of the crystal packing is provided by intermolecular π-π stacking interactions involving the whole planar bis(thiosemicarbazone)-metal skeleton (Figure 3), with an interplane separation about ≈ 3.5 Å.

3.3. In vitro antiproliferative activity

To assess the antitumor potential of the synthesized compounds, its antiproliferative activity (in powder solid form) was tested in vitro against a panel of human cancer cells lines containing examples of lung (NCI-H460), breast (T-47D) and
ovarian (A2780 and A2780cisR) cancers. For comparison purposes, the cytotoxicity of cisplatin was always evaluated under the same experimental conditions.

Table 3 shows that both the $p$-chlorophenyl substituted free ligand $H_2L$ and its platinum(II) complex $[PtL]$ present important antiproliferative activity in the low-micromolar range, against ovarian (A2780, cisplatin sensitive, and A2780cisR, cisplatin resistant) and breast (T-47D) cancer cells. It is remarkable to note that both compounds exhibit better cytotoxic effects against T-47D cells than cisplatin by comparing their IC$_{50}$ values.

The A2780cisR cell line encompasses all of the known major mechanisms of resistance to cisplatin: reduced drug transport, enhanced DNA repair/tolerance, and elevated GSH levels. The ability of $H_2L$ and $[PtL]$ compounds to circumvent cisplatin-acquired resistance was confirmed from the resistance factor values, RF (defined as IC$_{50}$ in A2780cisR/IC$_{50}$ in A2780) since both have a much better RF value than cisplatin. An RF value of $<2$ was considered to denote non-cross-resistance and therefore these compounds are able to circumvent cisplatin resistance [34,35].

From a chemical point of view, analysis of these data together with those of our previous study [23] in which the related ligand 2,6-diacetylpyridine bis($^4N$-$p$-tolylthiosemicarbazone), $H_2L^2$, resulted inactive in both A2780 and A2780cisR cell lines and its $[PtL^2]$ complex showed a slightly lower antiproliferative activity than $[PtL]$ complex (see table 3) suggests that the presence of one electron withdrawing group attached to the $^4N$ atom of the thiosemicarbazone moiety results beneficial for the antiproliferative activity of the bis(thiosemicarbazone) ligands of the 2,6-diacetylpyridine series.

Insert Table 3
3.4. DNA Interaction Studies

In order to initially address if any direct interaction with DNA is part of the mechanism of action of the compounds, UV-visible absorption spectra in absence and presence of CT-DNA were carried out for H₂L ligand, which have a significant effect against the tested cell lines.

**Absorption spectral studies**

The binding affinity between DNA and H₂L ligand can be detected by UV-Vis absorption spectroscopy by measuring the changes in the absorption properties of: a) DNA (for a variable H₂L concentration) or b) H₂L ligand molecule (for a variable DNA concentration).

The UV-Vis absorption spectrum of the typical β-form DNA exhibits a characteristic $\pi \rightarrow \pi^*$ band at 260 nm as consequence of the chromophoric groups in purine and pyrimidine moieties. Compounds binding with DNA through intercalation are consistent with hypochromism (decrease in DNA band absorption), resulted of a stacking interaction between the aromatic ligand chromophore and the base pair of DNA. In case of compounds binding with DNA through external contact (including groove binding and electrostatic attraction) usually hyperchromism (increase in DNA band absorption) is observed which is attributable of a contraction and overall damage of the secondary structure of DNA [36].

Thus, the absorption spectrum of CT-DNA in presence of H₂L was recorded, by keeping constant CT-DNA concentration (1.7x10⁻⁴ M) in diverse [CT-DNA]/[H₂L] mixing ratios (R = 0.5-1.5) and monitoring the change in the absorption intensity of the typical CT-DNA spectral band at 260 nm. As Figure 4 shows, when the concentration of H₂L is gradually increased a significant increase in absorption of the DNA band
occurs being the percentage of hyperchromism observed [% hyperchromism = \( \frac{A_{\text{DNA bound}} - A_{\text{DNA free}}}{A_{\text{DNA bound}}} \)] about 40%.

Insert Figure 4

These characteristics suggest non-covalent surface (major or minor groove) binding along outside of DNA helix. The above observations are comparable to those reported earlier for various neutral bis(thiosemicarbazone) palladium and platinum complexes [37, 38].

The \( \text{H}_2\text{L} \) ligand exhibit, in DMSO:Tris buffer (2.5:100) mixture, one broad intense band of intraligand \( \pi-\pi^* \) transition at 250 nm and other less intense of intraligand \( n-\pi^* \) transition at 337 nm and any interaction with DNA could perturb it.

Thus, in order to determine the intrinsic binding constant \( (K_b) \), absorption titration experiments were performed by maintaining a constant \( \text{H}_2\text{L} \) concentration (50 \( \mu \text{M} \)) while gradually increasing the concentration of DNA (0 - 40 \( \mu \text{M} \)) and monitoring the change in the absorption intensity of the intraligand charge transfer band. While measuring the absorption spectra, an equal amount of DNA was added to both the test solution and the reference solution to eliminate the absorbance of DNA itself. The data were then fitted to the following equation, that is only valid for low compound:DNA ratios (i.e., far from the DNA saturation) and assumes no binding cooperativity [39]:

\[
\frac{[\text{DNA}]}{(\varepsilon_a - \varepsilon_f)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b (\varepsilon_b - \varepsilon_f)}
\]

where [DNA] is the concentration of the nucleic acid in base pairs, \( \varepsilon_a \) is the apparent absorption coefficient obtained by calculating \( A_{\text{obs}}/\text{[compound]} \), and \( \varepsilon_f \) and \( \varepsilon_b \) are the absorption coefficients of the free and the fully bound compound, respectively.
A plot (Figure 5) of \([\text{DNA}] / (\varepsilon_b - \varepsilon_i) \) versus [DNA], gives a slope of \(1 / (\varepsilon_b - \varepsilon_i)\) and a Y-intercept equal to \(1 / (K_b(\varepsilon_b - \varepsilon_i))\). The intrinsic binding constant \(K_b\) is calculated as the ratio of the slope to the Y-intercept.

Insert Figure 5

On titration of CT-DNA a slight increase in the absorptivity of this band is observed which is indicative of interaction between the electronic states of the ligand chromophore with that of DNA bases. The magnitude of intrinsic binding constant was calculated to be \(7.03 \times 10^4 \text{ M}^{-1}\) (correlation coefficient \(R^2 = 0.99\)) which is modest, however it should be kept in mind that the biological activity of \(\alpha\)-(N)-heterocyclic thiosemicarbazones is not only due to their non-covalent DNA binding but they are also potent inhibitors of DNA synthesis and repair through \(\text{RR}\) inactivation. This fact could explain the good cytotoxic activity that both free ligand and platinum complex have demonstrated. Further studies and more practical experiments are required to elucidate the biochemical mechanisms involved in their activity.

4. Conclusions

A new family of Pt(II) and Pd(II) bis(thiosemicarbazone) compounds of the 2,6-diacetylpyridine series containing an aryl ring with an electron withdrawing substituent (\(p\)-chlorophenyl group) has been successfully prepared and characterized.

This study has identified both the free ligand \(\text{H}_2\text{L}\) and the Pt(II) complex \([\text{PtL}]\) as having high antiproliferative activity since they are capable of not only circumvent cisplatin resistance in A2780cisR cells but they also exhibit high antiproliferative activity against breast (T-47D) cancer cells.
Acknowledgments

We are grateful to Ministerio de Economía y Competitividad, Instituto de Salud Carlos III of Spain (PI1100659) for financial support.
References


CAPTIONS

Scheme 1. Structure of 2,6-diacetylpyridine bis(4-N-p-chlorophenylthiosemicarbazone), H₂L ligand.

Scheme 2. Delocalization System in the Thiosemicarbazone moiety

Figure. 1. Molecular structure of [PdL]. The displacement ellipsoids are drawn at the 50% probability.

Figure. 2. Molecular structure of [PtL]. The displacement ellipsoids are drawn at the 50% probability.

Figure. 3. Crystal packing view of [PdL]-DMSO along a axis, dashed lines denote π···π interactions.

Figure. 4. UV absorption spectrum of CT-DNA in the absence (black curve) and presence of increasing amounts of compound H₂L. The data were collected for [CT-DNA] = 1.7x10⁻⁴ M and [H₂L] = 0, 2.5x10⁻⁶, 6.0x10⁻⁵, 8.0x10⁻⁵, 1.25x10⁻⁴ M (the arrow shows the changes upon increasing amounts of complex).

Figure. 5. UV absorption spectrum of H₂L in the absence (black curve) and presence of increasing amounts of compound CT-DNA. The data were collected for [H₂L] = 5x10⁻⁵ M and [CT-DNA] = 0, 20x10⁻⁶, 60x10⁻⁶, 70x10⁻⁶, 75x10⁻⁶ M. The insert shows a fitting of the absorbance data used to obtain the binding constants.
New bioactive 2,6-diacetylpipridine bis(\textit{para}-chlorophenylthiosemicarbazone) ligand and its Pd(II) and Pt(II) complexes: synthesis, characterization, cytotoxic activity and DNA binding ability.

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\textbf{Keywords}: Antitumor activity, Asymmetric \textit{N}_3\textit{S} coordination, 2,6-Diacetylpipridine, Palladium and Platinum complexes, Thiosemicarbazone.

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Abstract

Preparation and characterization of 2,6-diacetylpyridine bis(4-N-para-chlorophenylthiosemicarbazone) ligand, H₂L, and its palladium(II) and platinum(II) complexes [PdL] and [PtL], is described. The crystal and molecular structure of the two new complexes has been determined by single crystal X-ray diffraction. The ligand acts as dianionic tetradeptate donor coordinating to the metal center in a square planar geometry through the pyridine nitrogen Npyridinic atom and the azomethine nitrogen Niminic and thione sulfur S atoms from one thiosemicarbazone arm, the fourth coordination position is occupied by the hydrazine nitrogen atom Nhydrazinic of the other arm. New free ligand and its metal complexes have been evaluated for antiproliferative activity in vitro against NCI-H460, T-47D, A2780 and A2780cisR human cancer cell lines. The cytotoxicity data suggest that these compounds may be endowed with important antitumor properties, especially H₂L and [PtL] since they are capable of not only circumvent cisplatin resistance in A2780cisR cells but also exhibit high antiproliferative activity in breast cancer T-47D cells. The interaction of H₂L with calf thymus DNA was also investigated and its binding constant (Kb) determined.
1. Introduction

Platinum metallo-drugs are among the most effective agents for the treatment of cancer however its clinical utility is restricted due to the frequent development of drug resistance, the limited spectrum of tumors against which these drugs are active and also the severe normal tissue toxicity [1-5]. These disadvantages have driven the development of improved platinum-based anticancer drugs whose structure and mode of action differ from that of cisplatin, especially those that interact with specific molecular targets as for example are the processes associated to DNA: transcription, replication and repair [6-9].

In this regard, of particular interest are compounds targeting ribonucleotide reductase (RR), enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides and provides the building blocks for the de novo DNA synthesis in all living cells. Cancer cells require increased RR activity to meet the demand for deoxyribonucleotides that are needed to support their rapid proliferation. Thus inhibition of RR activity leads to inhibition of DNA synthesis and repair, and also induces cell cycle arrest and apoptosis [10-12].

α-(N)-heterocyclic thiosemicarbazones, (N)-TSCs, have been reported to be among the most effective RR inhibitors yet identified and many efforts have been devoted to the study of the structure–activity relationship of thiosemicarbazone derivatives. The anticancer activity of (N)-TSCs is closely related to the nature of the heterocyclic ring of the parent aldehyde or ketone, metal chelation ability and terminal amino substitution [13-20]. In this sense pyridine ring itself is a part of many natural and synthetically prepared pharmaceuticals and moreover it plays a significant role in many biological processes like nicotinamide adenine dinucleotide phosphate NADP or the important vitamins niacin and pyridoxine (vitamins B3 and B6) [21, 22].
Keeping in view the above observations and as part of our systematic investigation on the coordination chemistry of thiosemicarbazone derivatives we recently reported palladium(II) and platinum(II) complexes derived of 2,6-diacyetylpyridine bis(4N-ortho-tolylthiosemicarbazone) and 2,6-diacyetylpyridine bis(4N-para-tolylthiosemicarbazone) ligands. The in vitro antitumor studies have shown that these complexes exhibit important antiproliferative activity in A2780 and A2780cisR human cancer cell lines and these results encouraged us to further investigate their cytotoxic properties as well as those of novel derivatives [23].

This work is aimed to determine if the presence of an aryl ring with an electron withdrawing substituent (such as para-chlorophenyl group) results beneficial for the antitumor activity of 2,6-diacyetylpyridine derived bis(4N-substituted thiosemicarbazones) ligands derived from 2,6-diacyetylpyridine. Therefore here we describe the synthesis and chemical characterization of the new 2,6-diacyetylpyridine bis(4N-para-chlorophenylthiosemicarbazone) ligand, H2L, (Scheme 1) and its palladium(II) and platinum(II) complexes, [PdL] and [PtL].

![Scheme 1. Structure of 2,6-diacyetylpyridine bis(4N-para-chlorophenylthiosemicarbazone), H2L ligand](image)

The cytotoxic activity of the new compounds synthesized and cisplatin (assumed as the reference antitumor drug) against four human cancer cell lines: NCI-H460 (non-small cell lung cancer), T-47D (breast cancer), A2780 and A2780cisR (epithelial
epithelial ovarian cancer) has been studied. The interaction of H\textsubscript{2}L with calf thymus DNA (CT-DNA) was also investigated and its binding constant (K\textsubscript{b}) determined.

2. Experimental

2.1. Measurements

Elemental analyses were performed on a LECO CHNS-932 microanalyzer. Fast atom bombardment (FAB) mass spectra (MS) were performed on a VG AutoSpec spectrometer (mNBA—nitrobenzyl alcohol matrix). \textsuperscript{1}H NMR spectra (DMSO-d\textsubscript{6}) were recorded on Bruker AMX-300 spectrometer. All cited physical measurements were obtained out by the Servicio Interdepartamental de Investigación (SIdI) of the Universidad Autónoma de Madrid.

Melting points were determined with a Stuart Scientific SMP3 apparatus. Infrared spectra (KBr pellets) were recorded on a Bomen–Michelson spectrophotometer (4000–400 cm\textsuperscript{-1}). \textsuperscript{13}C NMR spectra were recorded on a 400 Advance Bruker Fourier Transform spectrometer. Electronic spectra were recorded on a Thermo Scientific Evolution 260 Bio UV-visible (UV-VIS) spectrophotometer.

2.2. Materials

Solvents were purified and dried according to standard procedures. Hydrazine hydrate, 2,6-diacetylpyridine, \textit{para}-chlorophenyl isothiocyanate, PdCl\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2} and PtCl\textsubscript{2}(PPh\textsubscript{3})\textsubscript{2} were commercially available.

2.3. Synthesis of compounds

2,6-Diacetylpyridine bis(\textit{4}-\textit{para}-chlorophenylthiosemicarbazone), H\textsubscript{2}L. An ethanolic solution of hydrazine hydrate (0.250 g, 5 mmol) was added dropwise with constant stirring to an ethanolic solution of \textit{para}-chlorophenyl isothiocyanate (0.848 g, 5 mmol). The reaction mixture was stirred for one more hour and then the white product
para-chlorophenylthiosemicarbazide formed was filtered, washed with cold ethanol and diethyl ether, dried in vacuo and recrystallized from ethanol. An ethanolic solution of the para-chlorophenylthiosemicarbazide (0.402 g, 2 mmol) was then stirred with 2,6-diacetylpyridine (0.163 g, 1 mmol) for 5 h. The resulting solution was reduced to half volume and the pale yellow solid formed was filtered, washed with ethanol, diethyl ether and finally dried in vacuo.

Yield (80%), mp 220 °C (decomposes). Elemental analysis found, C, 52.20; H, 4.25; N, 18.00; S, 12.20; C_{23}H_{21}N_{7}S_{2}Cl_{2} requires C, 52.407; H, 4.093.99, N, 18.5048; S, 12.409 %.

MS (FAB+ with mNBA: nitrobenzyl alcohol matrix) m/z 530.0 for [H_{2}L+H]^+. IR (KBr pellet): υ/cm⁻¹ 3335, 3306, 3210 (w, NH); 1588 (s, CN); 827, 809 (w, CS-thioamide IV); 585 (pyridine ring).

$^1$H NMR (300 MHz, d$_{6}$-DMSO, ppm), δ=10.80 [s, 2NH, 2H]; 10.20 [s, 4NH, 2H]; 8.55 [d, J=7.9 Hz, CH-pyridine, 2H]; 7.85 [t, J=7.9 Hz, CH-pyridine, 1H]; 7.60 (d, J=8.7 Hz, aromatic-thiosemicarbazide, 4H); 7.45 (d, J=8.7 Hz, aromatic-thiosemicarbazide, 4H); 2.50 (s, CH$_3$-diacetylpyridine, 6H).

$^{13}$C NMR (d$_{6}$-DMSO, ppm), δ=178.3 (C=S); 153.78 (C$_2$,C$_6$-pyridine); 149.85 (C=¹N); 138.54 (C$_4$-pyridine); 137.17 (C$_3$,C$_5$-pyridine); 130.08 (aromatic-thiosemicarbazide); 128.49 (aromatic-thiosemicarbazide); 128.34 (aromatic-thiosemicarbazide); 127.37 (aromatic-thiosemicarbazide); 121.96 (aromatic-thiosemicarbazide); 12.98 (CH$_3$-diacetylpyridine).

UV/VIS (DMSO): λ/nm 250, 337.

2,6-Diacetylpyridine bis(4-N-para-chlorophenylthiosemicarbazonato)palladium(II), [PdL]. The reaction of H$_2$L ligand with PdCl$_2$(PPh$_3$)$_2$, in toluene, in presence of Et$_3$N, in 1:1 molar ratios over 20 h at room temperature led to the formation of an orange solution which was filtered and left to stand at ambient temperature for two days. The brown solid formed was filtered, washed several times with hot water, diethyl ether and finally dried in vacuo.
Yield (55%), mp >250 °C. Elemental analysis found, C, 43.35; H, 3.35, N, 15.10; S, 10.05; C_{23}H_{19}N_{7}S_{2}Cl_{2}Pd requires C, 43.50; H, 3.00; N, 15.40; S, 10.10 %. MS (FAB$^+$ with mNBA matrix) m/z 636 for [PdL$^+$+H]$^+$. IR (KBr pellet): $\nu$/cm$^{-1}$ 3243 (s, NH); 1595 (s, CN); 830, 804 (vw) (CS-thioamide IV); 603 (pyridine ring). $^1$H NMR (300 MHz, d$_6$-DMSO, ppm), $\delta$=10.75, 10.18 [s, $^4$NH, 2H]; 8.43-8.10 [m, CH-pyridine, 3H]; 7.71-7.34 (m, aromatic-thiosemicarbazide, 8H); 2.72, 2.62 (s, CH$_3$-diacetylpyridine, 6H). UV/VIS (DMSO): $\lambda$/nm 267, 340, 410, 470.

Recrystallization from DMSO led to the isolation of orange crystals of [PdL]-DMSO that were suitable for X-ray-diffraction.

**2,6-Diacetylpyridine bis($^4$N-para-chlorophenylthiosemicarbazonato)platinum(II), [PtL].** It was prepared by the same procedure as described for [PdL] by reaction of H$_2$L with PtCl$_2$(PPh$_3$)$_2$ and afforded a brown solid.

Yield (35%), mp 192 °C (decomposes). Elemental analysis found, C, 37.90; H, 2.55, N, 13.60; S, 8.60; C$_{23}$H$_{19}$N$_7$S$_2$Cl$_2$Pt requires C, 38.20; H, 2.60; N, 13.55; S, 8.85 %. MS (FAB$^+$ with mNBA matrix) m/z 724 for [PtL+H]$^+$. IR (KBr pellet): $\nu$/cm$^{-1}$ 3288, 3208 (s, NH); 1590 (s, CN); 826, 809 (vw) (CS-thioamide IV); 591 (pyridine ring). $^1$H NMR (300 MHz, d$_6$-DMSO, ppm), $\delta$=11.00, 10.30 [s, $^4$NH, 2H]; $\delta$=8.56-8.10 [m, CH-pyridine, 3H]; $\delta$=7.76-7.70 (m, aromatic-thiosemicarbazide, 8H); $\delta$=2.78, 2.71 (s, CH$_3$-diacetylpyridine, 6H). UV/VIS (DMSO): $\lambda$/nm 249, 267, 366.

Recrystallization from DMSO led to the isolation of orange crystals of [PtL]-DMSO that were suitable for X-ray-diffraction.

**2.4. Crystallography**

Data were collected on a Bruker X8 APEX II CCD. Crystallographic data and selected interatomic distances and angles are listed in Table 1. For all compounds, the software package SHELXTL was used for space group determination, structure
solution, and refinement [24]. The structures were solved by direct methods, completed with difference Fourier syntheses, and refined with anisotropic displacement parameters.

CCDC 981268 and 981269 contain the supplementary crystallographic data for compounds [PdL] and [PtL] respectively. These data can be obtained free of charge at 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; e-mail: deposit@ccdc.cam.ac.uk].

2.5. **In vitro antiproliferative activity**

The human cancer cells: A2780 and A2780cisR (epithelial ovarian cancer), T-47D (breast cancer) and NCI-H460 (non-small cell lung cancer); were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine in an atmosphere of 5% CO\textsubscript{2} at 37 °C.

Cell proliferation was evaluated by the sulforhodamine B assay. Cells were plated in 96-well sterile plates at a density of 1.5·10\textsuperscript{4} (for NCI-H460), 4·10\textsuperscript{3} (for A2780 and A2780cisR) or 5·10\textsuperscript{3} (for T-47D) cells per well with 100\,μL of medium and were then incubated for 24 h (A2780, A2780cisR and NCI-H460) or 48 h (T-47D). After attachment to the culture surface the cells were incubated with various concentrations of the compounds tested freshly dissolved in DMSO (1 mg/mL) and diluted in the culture medium (DMSO final concentration 1%) for 48 h (for NCI-H460) or 96 h (for A2780, A2780cisR and T-47D). The cells were fixed by adding 50 \,μL of 30% trichloroacetic acid (TCA) per well.

The plates were incubated at 4 °C for 1 h and then washed five times with distilled water. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B dissolved in 1% acetic acid for 10 min. Unbound dye was removed
by rinsing with 0.1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 515 nm) in a Tecan Ultra Evolution spectrophotometer.

The effects of compounds were expressed as corrected percentage inhibition values according to the following equation:

\[
\% \text{ inhibition} = [1 - (T/C)] \times 100
\]

where T is the mean absorbance of the treated cells and C the mean absorbance in the controls.

The inhibitory potential of compounds was measured by calculating concentration–percentage inhibition curves, these curves were adjusted to the following equation:

\[
E = E_{\text{max}} /[1 + (\text{IC}_{50}/C)^n]
\]

where E is the percentage inhibition observed, \(E_{\text{max}}\) is the maximal effects, \(\text{IC}_{50}\) is the concentration that inhibits 50% of maximal growth, C is the concentration of compounds tested and n is the slope of the semi-logarithmic dose–response sigmoid curves. This non-linear fitting was performed using GraphPad Prism software [25].

For comparison purposes, the antiproliferative activity of cisplatin was evaluated under the same experimental conditions. All compounds were tested in two independent studies with triplicate points. These experiments were carried out at the Unidad de Evaluación de Actividades Farmacológicas de Compuestos Químicos (USEF), Universidad de Santiago de Compostela.

2.6. DNA-Binding Experiments

CT-DNA stock solution was prepared by dissolving the lyophilized sodium salt in Tris-buffer (NaCl 50 mM, Tris-HCl 5 mM, pH was adjusted to 7.2 with NaOH 0.5
M) by stirring for 5 hours. The CT-DNA solution was standardized spectrophotometrically [26] by using its known molar absorption coefficient at 260 nm (6600 M\(^{-1}\).cm\(^{-1}\)). The ratio of UV absorbance at 260 and 280 nm, \(A_{260}/A_{280}\), of ca. 1.9, indicating that the DNA was sufficiently free of protein. Stock solution was kept frozen until the day of the experiment.

Concentrated stock solutions \((5 \times 10^{-3} \text{ M} \text{ and } 5 \times 10^{-5})\) of \(\text{H}_2\text{L}\) were prepared dissolving the compound in DMSO. From these stock solutions, for all experiments the desired concentration of compound was achieved by dilution with Tris-buffer (NaCl 50 mM, Tris-HCl 5 mM, pH was adjusted to 7.2 with NaOH 0.5 M) to give homogeneous solutions with DMSO content of less than 2.5%.

To investigate the binding mode, spectrophotometric titrations were performed at a fixed DNA concentration equal to \(1.7 \times 10^{-4} \text{ M}\) with increasing concentration of \(\text{H}_2\text{L}\) (0-125 µM) compounds \((R = [\text{CT-DNA}] / [\text{compound}])\) and monitoring the absorbance change at the wavelength maximum 260 nm after incubation (10 min. at 37ºC).

To calculate the binding parameters, the spectrophotometric titrations were performed with increasing concentration of DNA (0-40 µM) at a fixed compound concentration equal to 50 µM \((r = [\text{compound}] / [\text{DNA}])\) and monitoring the absorbance change in one characteristic charge transference band of the compound after incubation (10 min. at 37ºC).

3. **Results and discussion**

3.1. **Synthesis and spectroscopic characterization**

A new 2,6-diacetylpyridine bis\((^4N\)-monosubstituted thiosemicarbazone) ligand has been synthesized with high purities and acceptable yields. The yellow compound
obtained is stable to air and moisture and was characterized by elemental analysis, FAB$^+$ spectrometry and IR and $^1$H NMR ($^1$H and $^{13}$C) spectroscopy.

Reaction of 2,6-diacetylpyridine bis($^4$N-para-chlorophenylthiosemicarbazone) ligand with equimolar amount of MCl$_2$(PPh$_3$)$_2$, where M = Pd(II) or Pt(II), led to the isolation of neutral mononuclear complexes [PdL] and [PtL] in which the bis(thiosemicarbazone) behaves as dianionic ligand with deprotonation of hydrazinic hydrazine ($^2$NH) protons and [NNNS] donor set.

Both complexes were characterized by routine analytical and spectroscopic techniques. Analytical data are consistent with the formulation given, thus in the FAB$^+$ mass spectra exhibited a very weak ion the molecular ion is seen as a small peak at m/z = 636 for [PdL] and m/z = 724 for [PtL] which corresponded to the predicted molecular weight of the [M+H]$^+$ ions and moreover the isotopic patterns of this signal fit well with the theoretical isotopic distributions.

The significant IR vibrational bands and the $^1$H chemical shift values of the free ligand and its complexes are listed in the Experimental section and Scheme 1 shows the numbered structure of the free ligand. As the X-ray study has shown, during metal complexation, the ligand behaves as a tetradentate dianionic forming two five-membered and one six-membered chelate rings around the metal center. The high delocalization and the asymmetric coordination hinder the IR analysis. The stretching vibration $\nu$(C=N) and the in-plane pyridine deformation bands are slightly shifted to higher wavenumbers which are consistent with the implication of iminic imine and pyridinic pyridine nitrogen atoms in the coordination however this induces only minor changes in $\nu$(C=S) thioamide IV band which decreases slightly in intensity.

In the $^1$H NMR spectrum of the double-armed H$_2$L ligand, two independent singlets at $\delta = 10.80$ and 10.20 ppm are observed for $^2$N hydrazinic and $^4$N amidic
hydrogens >C=N-^2NH- and –C(S)-^4NH- protons respectively. A doublet at \( \delta = 8.55 \) ppm and a triplet at \( \delta = 7.85 \) ppm are assigned to pyridyl ring protons. Aromatic para-chlorophenyl protons appear as a multiplet at \( \delta = 7.60-7.45 \) ppm and a sharp singlet at \( \delta = 2.50 \) is assigned to the methyl protons. In [PdL] and [PtL] complexes, the anionic coordination of the ligand is evidenced by the disappearance of the signal corresponding to 2N-hydradinic hydrogens the >C=N-^2NH- protons. On the other hand, 4N-amidic hydrogens the –C(S)-^4NH- protons appear two independent signals (10.75 and 10.218 ppm for [PdL] and 11.00 and 10.30 ppm for [PtL]) due to the asymmetric coordination. The pyridyl ring protons appear as a multiplet at \( \delta = 8.43-8.10 \) ppm for [PdL] and \( \delta = 8.56-8.10 \) ppm for [PtL] and the rest of the proton signals appear at nearly identical positions if they are compared with the corresponding proton signals.

\(^{13}\)C NMR spectrum of the free ligand shows carbon signals supporting the \(^1\)H NMR assignments however due to the low solubility of the complexes it was not possible to get \(^{13}\)C NMR spectra of reasonably quality.

The electronic absorption spectra of both [H\(_2\)L] ligand and [PdL] and [PtL] complexes exhibit two intense band in the region 250-400 nm, which can ascribed to ligand-centered \( \pi \rightarrow \pi^* \) and \( \pi \rightarrow \pi^* \) transitions. In addition the spectra of metal complexes exhibit other less energetic bands assigned to a ligand to metal (LMCT) and metal to ligand charge transfer (MLCT) transitions [27].

3.2. Description of the crystal structures

[PdL]-DMSO and [PtL]-DMSO were isolated as neutral compounds. The most significant parameters crystallographic data for these complexes are shown in Tables 1, whereas selected bond lengths and bond angles are presented in Table and 2.
Table 1 Crystal data and structure refinement for [PdL]-DMSO and [PtL]-DMSO compounds.

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<th>[PtL]-DMSO</th>
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<td>1.606 and -2.739</td>
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</table>

The structures together with the atom labeling schemes are shown in Fig. Figures 1 and 2. Both compounds are isostructural hence displaying nearly identical cell parameters, crystallize in the triclinic Pī space group with Z=2 and the asymmetric units contain one molecule of the neutral complex and one dimethyl sulfoxide solvent molecule.
Fig. 1. Molecular structure of [PdL] complex. The displacement ellipsoids are drawn at the 50% probability.

Fig. 2. Molecular structure of [PtL] complex. The displacement ellipsoids are drawn at the 50% probability.

The metal ion presents a square planar geometry being where the bis(thiosemicarbazone) ligand attached is coordinated to the metal ion through the
pyridine nitrogen atom and the azomethine nitrogen and the thione sulfur atoms from one thiosemicarbazone arm and being the fourth coordination position occupied by the hydrazine nitrogen atom of the other thiosemicarbazone arm generating two typical five membered (PdSCNN and PdNCCN or PtSCNN and PtNCCN) and one six membered (PdNNCCN or PtNNCCN) chelate rings. Coordination by hydrazine nitrogen atom instead of iminic azomethine nitrogen atom, although uncommon, has been found in the bibliography for some d⁸ bis(thiosemicarbazone) complexes [23, 28-31].

The M–N and M–S bond distances are similar to those found in other palladium(II) and platinum(II) complexes. It is important to note that the two thiosemicarbazone moieties, which are symmetrically deprotonated, coordinate in a different fashion. Upon coordination the bidentate-NS,S arm undergoes significant evolution from the thione to the thiol form which is reflected in C–S distance of 1.779(4) for [PdL]·DMSO and 1.804(10) for [PtL]·DMSO while the monodentate-thiosemicarbazone (N, hydrazine nitrogen atom) arm maintains its thione form as reflects in their C–S bond length of 1.673(5) for [PdL]·DMSO and 1.672(3) Å for [PtL]·DMSO, typical of double C=S bond.

Table 2. Selected bond distances (Å) and angles (º) for [PdL]·DMSO and [PtL]·DMSO.

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<th>Bond lengths (Å)</th>
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<th>[PtL]·DMSO</th>
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Table 2. Selected bond distances (Å) and angles (º) for [PdL]·DMSO and [PtL]·DMSO.
Comparison of C–N and N–N bond distances with typical lengths of single and double bonds [C-N 1.47, C=N 1.28 Å, N-N 1.45, N=N 1.25 Å] suggests extensive charge delocalization over the thiosemicarbazone moieties [32, 33] and also agree with the thiolate tautomeric form of the bidentate thiosemicarbazonate arm and the thione tautomeric form of the monodentate one (Scheme 2).

Scheme 2. Delocalization System in the Thiosemicarbazonate moiety
Inspection of the angles formed between the metal ion (M=Pd$^{2+}$, Pt$^{2+}$) and the coordinated atoms shows that the metal is contained within a slightly distorted square-planar environment being the bond angles between adjacent coordinating atoms in the range 80.5-104.2º range.

The crystal structures are stabilized by intermolecular hydrogen interactions involving the N(7) atom of the bidentate thiosemicarbazone arms and the oxygen atom of DMSO solvent molecule being the N(7)-H(7)···O(1) contact distance 2.83 Å for both complexes and <(NHO) angle 170.4º for [PdL] and 169.2º for [PtL]. Further stabilization of the crystal packing is provided by intermolecular $\pi$-$\pi$ stacking interactions involving the whole planar bis(thiosemicarbazone)-metal skeleton (Figure 3), with an interplane separation of about $\approx$ 3.5 Å.

![Fig 3](image)

**Fig 3.** Crystal packing view of [PdL]-DMSO along a axis, dashed lines denote $\pi$-$\cdot$-$\pi$ interactions.

### 3.3. In vitro antiproliferative activity

To assess the antitumor potential of the synthesized compounds, its antiproliferative activity (in powder solid form) was tested *in vitro* against a panel of
human cancer cells lines containing examples of lung (NCI-H460), breast (T-47D), and ovarian (A2780 and A2780cisR) cancers. For comparison purposes, the cytotoxicity of cisplatin was always evaluated under the same experimental conditions.

Table 3 shows that both the $p$-chlorophenyl substituted free ligand $\text{H}_2\text{L}$ and its platinum(II) complex [PtL] present important antiproliferative activity in the low-micromolar range, against ovarian (A2780, cisplatin sensitive, and A2780cisR, cisplatin resistant) and breast (T-47D) cancer cells. It is remarkable to note that both compounds exhibit better cytotoxic effects against T-47D cells than cisplatin by comparing their IC$_{50}$ values.

The A2780cisR cell line encompasses all of the known major mechanisms of resistance to cisplatin: reduced drug transport, enhanced DNA repair/tolerance, and elevated GSH levels. The ability of $\text{H}_2\text{L}$ and [PtL] compounds to circumvent cisplatin-acquired resistance was confirmed from the resistance factor values, RF (defined as IC$_{50}$ in A2780cisR/IC$_{50}$ in A2780) since both have a much better RF than cisplatin. An RF value of $< 2$ was considered to denote non-cross-resistance and therefore these compounds are able to circumvent cisplatin resistance [34,35].

From a chemical point of view, analysis of these data together with those of our previous study [23] in which the related ligand 2,6-diacetylpyridine bis($^4$N-para-tolylthiosemicarbazone), $\text{H}_2\text{L}^2$, resulted inactive in both A2780 and A2780cisR cell lines and its [PtL$^2$] complex showed a slightly lower antiproliferative activity than [PtL] complex (see table 3) [23] suggests that the presence of one electron withdrawing group attached to the $^4$N atom of the thiosemicarbazone moiety results beneficial for the antiproliferative activity of the bis(thiosemicarbazone) ligands of the 2,6-diacetylpyridine series.
Table 3. *In vitro* antiproliferative activity of bis(thiosemicarbazone) compounds $H_2L$, $[PdL]$ and $[PtL]$ complexes and $H_2L_2$, $[PtL_2]$ and cisplatin, evaluated in human T-47D (breast cancer), A2780 and A2780cisR (epithelial ovarian cancer) cell lines.

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<td>IC$_{50}$±SD (µM)</td>
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<td>$H_2L$</td>
<td>7.16±0.14</td>
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<tr>
<td>$[PdL]$</td>
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<td>$[PtL]$</td>
<td>7.12±0.21</td>
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<tr>
<td>$H_2L_2$</td>
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</tr>
<tr>
<td>$[PtL_2]$</td>
<td>20±2&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Cisplatin</td>
<td>0.88±0.01</td>
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The IC$_{50}$ values are averages of two independent determinations.

ND = not determined

<sup>a</sup> Values taken from Ref. [23]

3.4. DNA Interaction Studies

In order to initially address if any direct interaction with DNA is part of the mechanism of action of the compounds, UV-visible absorption spectra in absence and presence of calf thymus DNA (CT-DNA) were carried out for $H_2L$ ligand, which have a significant effect against the tested cell lines.

Absorption spectral studies

The binding affinity between DNA and $H_2L$ ligand can be detected by UV-Vis absorption spectroscopy by measuring the changes in the absorption properties of: a)
DNA (for a variable $H_2L$ concentration) or b) $H_2L$ ligand molecule (for a variable DNA concentration).

The UV-Vis absorption spectrum of the typical $B\beta$-form DNA exhibits a characteristic $\pi \rightarrow \pi^*$ band at 260 nm as consequence of the chromophoric groups in purine and pyrimidine moieties. Compounds binding with DNA through intercalation are consistent with hypochromism (decrease in DNA band absorption), resulted of a stacking interaction between the aromatic ligand chromophore and the base pair of DNA. In case of compounds binding with DNA through external contact (including groove binding and electrostatic attraction) usually hyperchromism (increase in DNA band absorption) is observed which is attributable of a contraction and overall damage of the secondary structure of DNA [36].

Thus, the absorption spectrum of CT-DNA in presence of $H_2L$ was recorded, by keeping constant CT-DNA concentration ($1.7 \times 10^{-4}$ M) in diverse [CT-DNA]/[H$_2$L] mixing ratios ($R = 0.5-1.5$) and monitoring the change in the absorption intensity of the typical CT-DNA spectral band at 260 nm. As Figure 4 shows, when the concentration of $H_2L$ is gradually increased a significant increase in absorption of the DNA band occurs being the percentage of hyperchromism observed [$\%$ hyperchromism = ($A_{DNA \ bound} - A_{DNA \ free}$) / $A_{DNA \ bound}$] about 40 %.
Fig. 4. UV absorption spectrum of CT-DNA in the absence (black curve) and presence of increasing amounts of compound H$_2$L. The data were collected for [CT-DNA] = 1.7 · 10$^{-4}$ M and [H$_2$L] = 0, 2.5 · 10$^{-6}$, 6.0 · 10$^{-5}$, 8.0 · 10$^{-5}$, 1.25 · 10$^{-4}$ M [CT-DNA]/[H$_2$L] mixing ratios R = 0.5–1.5 (the arrow shows the changes upon increasing amounts of complex).

These characteristics suggest non-covalent surface (major or minor groove) binding along outside of DNA helix. The above observations are comparable to those reported earlier for various neutral bis(thiosemicarbazone) palladium and platinum complexes [37, 38].

The H$_2$L ligand exhibit, in DMSO:Tris buffer (2.5:100) mixture, one broad intense band of intraligand π-π* transition at 250 nm and other less intense of intraligand n-π* transition at 337 nm and any interaction with DNA could perturb it.

So Thus, in order to determine the intrinsic binding constant (K$_b$), absorption titration experiments were performed by maintaining a constant H$_2$L concentration (50 μM) while gradually increasing the concentration of DNA (0 - 40 μM) and monitoring the change in the absorption intensity of the intraligand charge transfer band. While measuring the absorption spectra, an equal amount of DNA was added to both the test
solution and the reference solution to eliminate the absorbance of DNA itself. The data were then fitted to the following equation, that is only valid for low compound:DNA ratios (i.e., far from the DNA saturation) and assumes no binding cooperativity [39]:

\[
\frac{[\text{DNA}]}{(\varepsilon_a - \varepsilon_t)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_t)} + 1 \left/ \left( K_b \left( \varepsilon_b - \varepsilon_f \right) \right) \right.
\]

where \([\text{DNA}]\) is the concentration of the nucleic acid in base pairs, \(\varepsilon_a\) is the apparent absorption coefficient obtained by calculating \(A_{obs}/[\text{compound}]\), and \(\varepsilon_f\) and \(\varepsilon_b\) are the absorption coefficients of the free and the fully bound compound, respectively.

A plot (Figure 5) of \([\text{DNA}] / (\varepsilon_b - \varepsilon_t)\) versus \([\text{DNA}]\), gives a slope of \(1 / (\varepsilon_b - \varepsilon_t)\) and a Y-intercept equal to \(1 / (K_b(\varepsilon_b - \varepsilon_t))\). The intrinsic binding constant \(K_b\) is calculated as the ratio of the slope to the Y-intercept.

Fig. 5. UV absorption spectrum of H₂L in the absence (black curve) and presence of increasing amounts of compound CT-DNA. The data were collected for \([H_2L] = 5 \cdot 10^{-5}\) M and
On titration of CT-DNA a slight increase in the absorptivity of this band is observed which is indicative of interaction between the electronic states of the ligand chromophore with that of DNA bases. The magnitude of intrinsic binding constant was calculated to be $7.03 \times 10^2$ M$^{-1}$ (correlation coefficient $R^2 = 0.99$) which is modest, however it should be kept in mind that the biological activity of $\alpha$-(N)-heterocyclic thiosemicarbazones is not only due to their non-covalent DNA binding but they are also potent inhibitors of DNA synthesis and repair through RR inactivation. This fact could explain the good cytotoxic activity that both free ligand and platinum complex have demonstrated. Further studies and more practical experiments are required to elucidate the biochemical mechanisms involved in their activity.

4. Conclusions

A new family of Pt(II) and Pd(II) bis(thiosemicarbazone) compounds of the 2,6-diacetylpyridine series containing an aryl ring with an electron withdrawing substituent (para-chlorophenyl group) has been successfully prepared and characterized.

This study has identified both the free ligand H$_2$L and the Pt(II) complex [PtL] as having high antiproliferative activity since they are capable of not only circumvent cisplatin resistance in A2780cisR cells but they also exhibit high antiproliferative activity against breast (T-47D) cancer cells.

Acknowledgments

We are grateful to Ministerio de Economía y Competitividad, Instituto de Salud Carlos III of Spain (PI1100659) for financial support.
References


Table 1 Crystal data and structure refinement for [PdL]·DMSO and [PtL]·DMSO.

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Table 2. Selected bond distances (Å) and angles (°) for [PdL]-DMSO and [PtL]-DMSO.

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<td><strong>Bond lengths (Å)</strong></td>
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The IC$_{50}$ values are averages of two independent determinations.
ND = not determined
$^a$ Values taken from Ref. [23]
Scheme 1
Scheme 2
Figure 2

Click here to download Figure(s): Figure 2.docx
**Fig. S1.** MS spectrum for H$_2$L
Fig. S2. $^1$NMR spectrum for $H_2L$
Figure S3. $^{13}$C NMR spectrum for H$_2$L
Fig. S4. MS spectrum for [PdL]
Fig. S5. $^1$NMR spectrum for [PdL]
Fig. S6. MS spectrum for [PtL]
Fig. S7. $^1$NMR spectrum for [PtL]