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***cis*-Pt I₂(NH₃)₂: a Reappraisal**

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The investigation of *cis*-PtI₂(NH₃)₂, the diiodido analogue of cisplatin (*cisPtI₂* hereafter), has been unjustly overlooked so far mainly because of old claims of pharmacological inactivity. Some recent -but still fragmentary- findings prompted us to reconsider more systematically the chemical and biological profile of *cisPtI₂* in comparison to cisplatin. Its solution behaviour, interactions with DNA and cytotoxic properties versus selected cancer cell lines were thus extensively analysed through a variety of biophysical and computational methods. Notably, we found that *cisPtI₂* is highly cytotoxic *in vitro* toward a few solid tumour cell lines and that its DNA platination pattern closely reproduces that of cisplatin; *cisPtI₂* is also shown to overcome completely resistance to cisplatin in a platinum resistant cancer cell line. The differences in the biological actions of these two Pt complexes are most likely related to slight but meaningful differences in their solution behaviour and reactivity. Overall, a very encouraging and unexpected pharmacological profile emerges for *cisPtI₂* with relevant implications both in terms of mechanistic knowledge and of prospective clinical application. An *ab initio* DFT study is also included to support the interpretation of the solution behaviour of *cisPtI₂* under physiological and slightly acidic pH conditions.

Introduction

Cisplatin is a leading compound in the field of anticancer drugs. Together with its two main parent Pt drugs, *i.e.* carboplatin and oxaliplatin, it is currently used in about half of chemotherapeutic protocols for cancer treatment.¹⁻³ Since the discovery of its outstanding anticancer properties during the 60's and subsequent clinical approval by FDA, in 1978, cisplatin has been the subject of hundreds of studies to describe and understand its peculiar chemical behaviour, and elucidate the mechanisms of its biological and pharmacological effects.⁴⁻⁶

A quite wide consensus now exists concerning the likely mode of action of anticancer Pt drugs that mainly relies on direct DNA damage, impairment of fundamental DNA functions and consequent triggering of apoptosis;^{1,2} yet, several issues and details remain unexplored and/or unanswered, so that other mechanistic hypotheses -even DNA unrelated- have been advanced.^{7,8}

The great clinical success of cisplatin has inspired an intense search for platinum analogues with hopefully improved biological and pharmacological properties;⁹⁻¹³ however, the outcomes of these research activities turned out to be quite modest and disappointing, both in terms of identification of new clinically effective Pt agents and of discovery of innovative modes of action.



Fig. 1 Structure of cisplatin and its diiodido analogue

Despite the huge research efforts made on platinum analogues over the last three decades, we were surprised in learning that only rare studies were carried out on *cisPtI₂*, the strict iodo analogue of cisplatin. This is probably the consequence of an initial negative assessment by Hoeschele and Cleare,^{14a} who reported that *cisPtI₂* is nearly inactive in animal models of sarcoma 180 while warning about its extremely low aqueous solubility and the difficulty in determining the toxic dose. Accordingly, even the cytotoxicity profile of *cisPtI₂* against the 60 cancer cell lines panel of NCI has never been reported.

However, more recently, a few studies started reconsidering iodido platinum complexes as possible anticancer drug candidates. For instance, Kratochwil *et al.*, described some diamino diiodido platinum(II) complexes manifesting an appreciable cytotoxicity *in vitro*.¹⁵ In the last few years, we discovered that some mixed diamino diiodido Pt complexes

exhibit appreciable cytotoxic properties *in vitro*.¹⁶⁻¹⁸ Moreover, for some of these Pt complexes, in spite of their lower reactivity with DNA as compared to cisplatin, an unconventional reactivity toward the model protein cytochrome c was disclosed, unexpectedly characterized by the loss of the amino ligands and retention of iodido ligands.^{16,17} Furthermore, in a paper published in 2013, we structurally described the reaction of *cisPtI₂* with lysozyme, confirming the peculiar and different interaction mode toward this model protein in comparison to cisplatin.¹⁹ Yet, no study has re-evaluated so far the biological profile of *cisPtI₂*. Altogether, these arguments led us to reconsider, in a more systematic way, some key chemical and biological aspects of *cisPtI₂* with respect to cisplatin.

Specifically, we have focused our attention on the four following issues: *i*) the activation process of *cisPtI₂* in aqueous solutions both at physiological and slightly acidic pH; *ii*) a computational rationalization of the spectral results; *iii*) the investigation of the direct reaction of *cisPtI₂* with DNA; *iv*) the cellular effects of *cisPtI₂* in a small panel of cancer cell lines. Some notable results have emerged from these studies that are comprehensively analysed and discussed here in direct comparison to cisplatin, with relevant mechanistic and medical implications.

Experimental

Chemistry of *cis*-PtI₂(NH₃)₂

Synthesis of *cis*-PtI₂(NH₃)₂ and UV-Vis analysis. *cisPtI₂* was synthesized according to published procedures, using (1:4) Pt:KI in order to avoid further purifications.^{17,20} The purity of the product was checked by elemental analysis of C, N and H [Calculated C: 0%, H: 1,26%, N: 5,84%, Experimental: C: 0%, H: 1,12%, N: 5,71%], and as described in ref. 14b. The correct *cis* geometry was assessed by comparison with its *trans* analogue as describe in the literature.¹⁴ Solution behaviour of *cisPtI₂* was assessed through spectrophotometric studies performed with a Varian Cary 50 Bio UV-Vis spectrophotometer in buffered solutions without use of DMSO and in absence of NaCl. A solution of complex (10⁻⁴ M) was prepared in 50 mM phosphate buffer (with or without presence of KI) at pH=7.4 or in 20 mM ammonium acetate buffer at pH=4.5 and absorbance monitored in the range between 200 and 800 nm of wave length for 72 h at 25° C.

Density Functional Theory simulations ORCA package has been used throughout the calculations.²¹ Hybrid PBE0 functional²² and Aldrich TZVP gaussian basis sets with scalar relativistic (ZORA) corrections²³ were used for both geometry optimizations and spectra simulations. Excited states have been computed within the TDDFT approach²⁴ and only the first twenty transitions have been computed using 200 expansion vectors in the iterative solution of the CI equations. D3 Grimme's dispersion corrections²⁵ have also been included. Solvent effects have been take into account within the COSMO model^{26,27} with an ϵ value of 80.4. Highly accurate integration

grids (Grid 5 in the ORCA notation) without pruning of the inner angular part of the wave function and "TightSCF" convergence criteria has been used throughout all the calculations

LogP determination. The octanol-water partition coefficients for cisplatin and *cisPtI₂* were determined by modification of reported shake-flask method.²⁸ Water (50 mL, distilled after milli-Q purification) and n-octanol (50 mL) were shaken together for 72 h to allow saturation of both phases. Solution of the complexes were prepared in the water phase (3 x 10⁻³ M) and an equal volume of octanol was added. Biphasic solutions were mixed for ten minutes and then centrifuged for five minutes at 6000 rpm to allow separation. Concentration in both phases was determined by UV-VIS. Reported logP is defined as $\log [\text{complex}]_{\text{oct}}/[\text{complex}]_{\text{wat}}$. Final values were reported as mean of three determinations.

Uptake experiment: comparison between cisplatin and *cis*-PtI₂(NH₃)₂ HCT116 cell line. The determination of platinum concentration in the cellular pellets as well as in the corresponding supernatant samples was performed in triplicate by a Varian 720-ES Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) equipped with a CETAC U5000 AT+ ultrasonic nebulizer, in order to increase the method sensitivity. Before the analysis, samples were weighted in PE vials and digested in a thermo-reactor at 80 °C for 24 h with 2 mL of aqua regia (HCl suprapure grade and HNO₃ suprapure grade in 3:1 ratio) and 0.2 mL of H₂O₂ suprapure grade. After digestion, the samples were diluted to about 5 mL with ultrapure water (≤ 18 M Ω) and accurately weighed; 5.0 mL of each sample were spiked with 1 ppm of Ge used as an internal standard, and analysed. Calibration standards were prepared by gravimetric serial dilution from a commercial standard solution of Pt at 1000 mg L⁻¹. The wavelength used for Pt determination was 214.424 nm whereas for Ge was used the line at 209.426 nm. The operating conditions were optimized to obtain maximum signal intensity, and between each sample, a rinse solution constituted by HCl suprapure grade and HNO₃ suprapure grade in 3:1 ratio was used in order to avoid any "memory effect".

Cellular studies

Cell cultures. PANC-1, HCT116-S, HCT116-R, IGROV-1, A549 and HT29 cell lines were cultured in RPMI 1640 (Euroclone; Milan, Italy) with 10% Fetal Bovine Serum (FBS) (Euroclone Defined; Euroclone; Milan, Italy). HCT116 cells were kindly provided by Dr. R. Falcioni (Regina Elena Cancer Institute, Roma). We cultured at 37 °C in a humidified atmosphere in 5% CO₂ in air.

Pharmacology experiments. Cells were seeded in a 96-well flat-bottomed plate (Corning-Costar, Corning, NY, USA) at a cell density of 1 x 10⁴ cells per well in RPMI complete medium. Drugs were used, after solubilisation in water, without

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use of DMSO, at the final concentrations indicated in the figures and in the legends to the figures. After 24 h, viable cells (determined by Trypan blue exclusion) were counted in triplicate using a haemocytometer. Each experimental point represents the mean of four samples carried out in three separate experiments.

Trypan blue assay. Cells viability was assessed by the Trypan blue exclusion assay. In brief, 10 μL of 0.4% trypan blue solution was added to 10 μL cell suspensions in culture medium.

The suspension was gently mixed and transferred to a haemocytometer. Viable and dead cells were identified and counted under a light microscope. Blue cells failing to exclude dyes were considered nonviable, and transparent cells were considered viable. The percentage of viable cells was calculated on the basis of the total number of cells (viable plus non viable). The IC_{50} value (i.e., the dose that caused apoptosis of 50% of cells) was calculated by fitting the data points (after 24 h of incubation) with a sigmoidal curve using Calcusyn software.

Cell cycle analysis. The effect of cisplatin and *cisPtI₂* on cell cycle distribution was assessed by flow cytometry after staining cells with propidium iodide (PI). Briefly, the cells (5×10^5 cells mL^{-1}) were analyzed prior and after treatment with IC_{50} s of both drugs (20.98 μM and 7.3 μM) for 24 h. The cells were harvested, washed with PBS and resuspended in 300 μL 1X Propidium Iodide staining solution and incubate at 4 $^{\circ}\text{C}$ in the dark for 20 minutes. DNA content of the cells was measured by BD FACSCanto Flow Cytometer and the population of each phase was determined using ModFit LT 3.0 analysis software (Verity Software House, Topsham, ME USA).

DNA binding studies.

Reaction of *cisPtI₂* with DNA, its putative main target, was analysed in direct comparison with cisplatin through a robust experimental approach including quantification of Pt binding to DNA, DNA interstrand cross-linking analysis, DNA adducts characterization by ethidium bromide fluorescence, DNA unwinding and thermal stability analyses.

Quantitative evaluation of binding of *cis*-PtI₂(NH₃)₂ and cisplatin to mammalian DNA in a cell-free medium. Solutions of double-helical calf thymus (CT) DNA (42% G + C, mean molecular mass approximately 2×10^7) at a concentration of 0.032 mg mL^{-1} (1×10^{-4} M related to the phosphorus content) were incubated with *cisPtI₂* (8 μM) or cisplatin (8 μM) at a value of $r_f = 0.08$ in 0.1 mM KI or KCl,

respectively, at 37 $^{\circ}\text{C}$ (r_f is defined as the molar ratio of free platinum complex to nucleotide phosphates at the onset of incubation with DNA). Two different stock solutions of *cisPtI₂* (0.1 mM) or cisplatin (0.1 mM) were prepared. One contained the Pt^{II} complex incubated for 7 days in unbuffered KI or KCl, respectively (0.01 M, pH 6) at 37 $^{\circ}\text{C}$ in the dark, whereas the other contained Pt^{II} complexes incubated for 7 days in double distilled water at 37 $^{\circ}\text{C}$ in the dark. Fifty microliters of the Pt^{II} complex aged in KI/KCl (0.01 M) or in water were quickly mixed with 4950 μL of DNA dissolved in NaClO₄ (10 mM), and the reaction mixture was kept at 37 $^{\circ}\text{C}$. In the experiments in which the Pt^{II} complex aged in water was used, the final reaction mixture was still supplemented at the onset of the reaction with KI or KCl so that the resulting concentration of KI or KCl in the reaction mixtures was always 0.1 mM. At various time intervals, an aliquot of the reaction mixture was withdrawn and assayed by differential pulse polarography (DPP) for platinum not bound to DNA.²⁹

Platination reactions. If not stated otherwise, CT or plasmid DNAs were incubated with the platinum complex in 10 mM NaClO₄ at 37 $^{\circ}\text{C}$ in the dark. After 24 h, the samples were exhaustively dialyzed against the medium required for subsequent biochemical or biophysical analysis. An aliquot of these samples was used to determine the value of r_b (r_b is defined as the number of molecules of the platinum complex bound per nucleotide residue) by flameless atomic absorption spectrophotometry (FAAS) or by differential pulse polarography (DPP).²⁹

Unwinding of negatively supercoiled DNA. Unwinding of closed circular supercoiled pSP73KB plasmid DNA (2455 bp) was assayed by an agarose gel mobility shift assay.³⁰ The unwinding angle Φ , induced per platinum–DNA adduct was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled to the relaxed form of the plasmid was attained. Samples of pSP73KB plasmid were incubated with platinum compounds at 37 $^{\circ}\text{C}$ in the dark for 24 h. The samples were subsequently subjected to electrophoresis on 1% agarose gels running at 25 $^{\circ}\text{C}$ with tris(hydroxymethyl) aminomethane (Tris)–acetate–EDTA buffer. The gels were then stained with EtBr, followed by photography with a transilluminator.

DNA melting. The melting curves of CT DNAs were recorded by measuring the absorbance at 260 nm. The melting curves were recorded in a medium containing 10 mM or 0.1 M Na⁺ with 1 mM Tris–HCl/0.1 mM Na₂H₂EDTA, pH 7.4. The value

of the melting temperature (t_m) was determined as the temperature corresponding to a maximum on the first derivative profile of the melting curves.

Interstrand DNA cross-linking in a cell-free medium. *cisPtI₂* or cisplatin were incubated for 24 h with 0.5 μg of a linear 2686-bp fragment of pUC19 plasmid linearized by EcoRI. The linear fragment was first 3'-end-labeled by means of the Klenow fragment of DNA polymerase I in the presence of [α -³²P]dATP. The platinated samples were analyzed for DNA interstrand crosslinks by previously published procedures.^{31,32} The number of interstrand cross-links was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis had been completed, the intensities of the bands corresponding to single strands of DNA and interstrand cross-linked duplex were quantified. The frequency of interstrand cross-links was calculated as ICL/Pt (%) = $\text{XL}/5372r_b$ (the DNA fragment contained 5372 nucleotide residues), where ICL/Pt (%) is the number of interstrand cross-links per adduct multiplied by 100, and XL is the number of interstrand cross-links per molecule of the linearized DNA duplex, which was calculated assuming a Poisson distribution of the interstrand crosslinks as $\text{XL} = -\ln A$, where A is the fraction of molecules running as a band corresponding to the non-cross-linked DNA.

Characterization of DNA adducts by EtBr fluorescence. These measurements were performed on a Varian Cary fluorescence spectrophotometer using a 1 cm quartz cell.

Fluorescence measurements were performed at an excitation wavelength of 546 nm, and the emitted fluorescence was analyzed at 590 nm. The fluorescence intensity was measured at 25 °C in NaCl (0.4 M) to avoid secondary binding of EtBr to DNA.²⁶ The concentrations were 0.01 mg mL⁻¹ for DNA and 0.04 mg mL⁻¹ for EtBr, which corresponded to the saturation of all intercalation sites of EtBr in DNA.³³

Other physical methods: Absorption spectra were measured with a Beckman 7400 DU spectrophotometer equipped with a thermoelectrically controlled cell holder. The FAAS measurements were conducted with a Varian AA240Z Zeeman atomic absorption spectrometer equipped with a GTA 120 graphite tube atomizer. The gels were visualized with a FUJIFILM BAS 2500 bioimaging analyzer, and the radioactivity associated with the bands was quantified with AIDA Image Analyzer. DPP measurements were performed using an EG&G PARC Electrochemical Analyzer, model 384B.

Results

Solution Chemistry of *cis*-Pt I₂(NH₃)₂: logP determination and activation profile in aqueous buffers

We first investigated some unexplored chemical features of *cisPtI₂*; in particular we determined its logP value and analysed its

solution behaviour under various conditions and the inherent metal complex activation processes.

The logP of *cisPtI₂* was measured according to the method reported in the experimental section; a value of -0.13 was determined versus a value of -2.4 previously determined for cisplatin and here again confirmed. This result implies that *cisPtI₂*, in its intact form, is far more lipophilic than cisplatin: this might have some appreciable impact on its cellular uptake (see later).

Then, the solution behaviour of *cisPtI₂* was studied spectrophotometrically under physiological-like or slightly acidic pH conditions. Remarkably, freshly prepared aqueous solutions of *cisPtI₂*, at pH=7.4, manifest two intense bands in the UV-visible region, located at 290 and 350 nm, respectively, that allow the direct and continuous monitoring of the solution behaviour of this platinum complex (Figure 2).

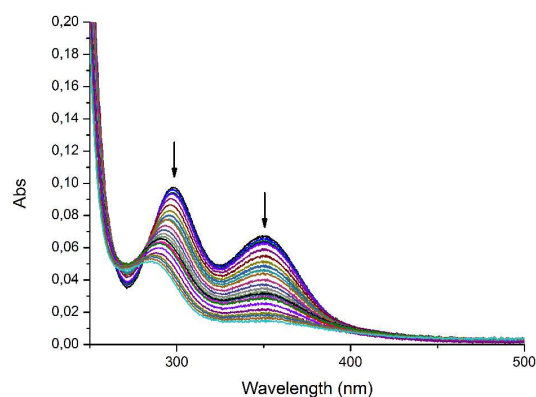


Fig. 2 time dependent UV-Vis analysis of complex *cis*-PtI₂(NH₃)₂ 10⁻⁴ M in 50 mM phosphate buffer (pH=7.4) followed for 72 h.

Some important spectral changes are slowly detected consisting in the progressive and regular decrease of the two main bands and in the appearance of a new band, of lower intensity, around 280 nm. The observed spectral changes are tentatively traceable to the progressive release of the two iodido ligands and to their replacement by water molecules according to a moderately biphasic kinetics. This would mean that the aquation process of *cisPtI₂* reproduces quite closely that of cisplatin, involving the progressive detachment of the two halide ligands while both ammonia ligands are kept as confirmed by some further experiments. In particular, addition of KI at increasing concentrations completely abolished and even reverted the above described spectral changes (see SI) strongly supporting the proposed interpretation. In any case, the rate of the aquation process for *cisPtI₂* is appreciably slower than for cisplatin (by at least a factor 2) possibly because of the greater strength of the Pt-I bond and/or greater inertness related to the increased "soft" character of iodide over chloride (more details for hydrolysis process and UV-Vis analysis are given in the SI).

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To gain further insight into the activation processes of *cisPtI₂*, we also analysed its spectral behaviour under slightly acidic conditions. Indeed, a mildly acidic environment is thought to destabilise the ammonia ligands and thus favour ammonia release over iodide release. Accordingly, *cisPtI₂* was dissolved in an acetate buffer, at pH 4.5, and its spectral changes monitored over 72 hours. The resulting spectral profile is shown in Figure 3: it is apparent that the overall spectral profile is profoundly different from that recorded at physiological pH. In particular, a new and quite intense visible band is clearly detected around 410 nm, at 72 hours, whose assignment is discussed later.

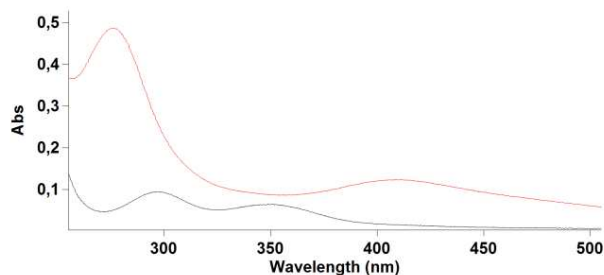


Fig. 3 UV-Vis spectral changes of complex *cis*-PtI₂(NH₃)₂ 10⁻⁴ M in 50mM Acetate buffer (pH=4.5) followed for 72h. Gray line t=0 h, red line t=72 h.

Computational studies, simulation of UV-Vis spectra

To interpret the reported spectral profiles at two distinct pH values, we performed UV-Vis spectra simulations of 5 species: the native *cisPtI₂*, two products of the Iodido aquation mechanism i.e. [cis-PtI(H₂O)(NH₃)₂]⁺ (1), [cis-Pt(H₂O)₂(NH₃)₂]²⁺ (2) and two products related to ammonia aquation mechanism i.e. [cis-PtI₂(NH₃)(H₂O)] (3), [cis-PtI₂(H₂O)₂] (4).

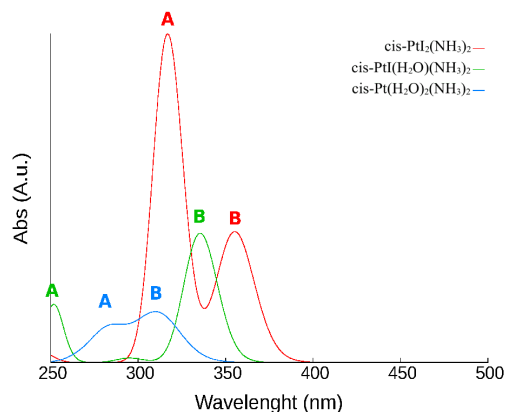
The computed spectrum of *cisPtI₂* (cfr. red line in Figure 4a) is in very good agreement with the experimental one reported in Figure 2. The calculated spectra for the mono- and di-Iodido hydrolysis species, i.e. (1) and (2), reproduce closely the experimental spectral evolution shown in Figure 2, offering strong support to the iodide release mechanism.

Simulated bands assignments were done through transition density plots reported in Figure 4b. It is possible to observe that, for *cisPtI₂*, band A is a LMCT transition, I,O(*p_{x,y}*) → Pt(*d_{x²-y²}*), while band B is a platinum *d-d* transition, Pt(*d_{xy}*) → Pt(*d_{x²-y²}*). Notably, band A changes from a LMCT to a platinum *d-d* character passing from *cisPtI₂* to species (1) and (2). For (1), both bands have a Pt(*d_{xy}*) → Pt(*d_{x²-y²}*) character. For (2) we

observed that (B) band observed for the *cisPtI₂* undergoes a blue shift until it turns into band (A).

Band B conserved the Pt(*d_{xy}*) → Pt(*d_{x²-y²}*) character observed for (2). Such behavior is in agreement with expected *d* orbitals shifts due to substitution of a strong basic □ donor as iodide with one water molecule.

a)



b)

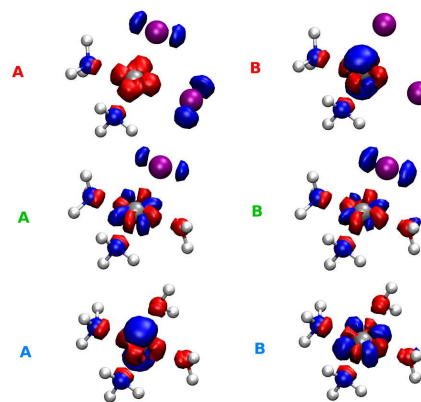
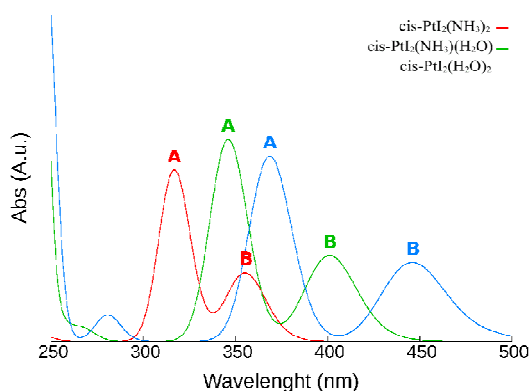


Fig. 4 (a) Evolution of UV-Vis spectral features for the Iodido hydrolysis mechanism. (b) Evolution of the electronic transition's nature of A and B bands during the iodido aquation mechanism (*cis*-PtI₂(NH₃)₂ top, [cis-PtI(H₂O)(NH₃)₂]⁺ middle and [cis-Pt(H₂O)₂(NH₃)₂]²⁺ bottom): for each transition red densities correspond to arrival orbitals and blue densities correspond to starting molecular orbitals. Platinum ions are coloured in silver, nitrogen in blue, oxygen in red, iodide in violet and hydrogen in white.

To verify the correctness of the supposed iodide hydrolysis mechanism for physiological-like pH conditions, we have calculated the UV-Vis spectra (see Figure 5a) also for the species involved in the concurrent amino hydrolysis mechanism, i.e. (3) and (4). In this case, the two *cisPtI₂* bands undergo a significant red shift keeping their shape and relative intensities practically unchanged. Interestingly, the same behavior is experimentally observed for acidic conditions (cfr. Fig. 3), where, indeed, the release of the ammonia ligands is expected. Furthermore, since dimeric species of the type [Pt(amine)I₂]₂, that are active on selected cancer cell lines, were reported to be synthesised in acidic conditions³⁴, additional ESI MS experiments were carried out at pH=4.5. Formation of dimers in solution under the latter conditions is confirmed by the presence of few weak peaks assignable to these species (see SI).

a)



b)

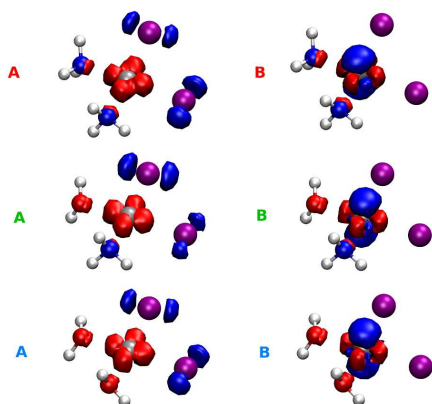


Fig. 5 (a) Evolution of UV-Vis spectral features for the ammonia hydrolysis mechanism. (b) Evolution of the electronic transition's nature of A and B bands during the ammonia aquation mechanism (*cis*-PtI₂(NH₃)₂ *top*, [*cis*-PtI₂(NH₃)(H₂O)] *middle* and [*cis*-PtI₂(H₂O)₂] *bottom*): for each transition red densities correspond to arrival orbitals and blue densities correspond to starting molecular orbitals. Colour code as in Fig. 4.

The difference transitions MOs analysis performed for this second group of molecules is shown in Figure 5b; it is observed that the nature of the main spectral transitions is retained for all species involved. In this case, only the shift of valence molecular orbitals has been observed due to the similar orbital contributions of ammonia and water molecules. Therefore bands (A) and (B) can be described as I₂O(*p_{x,y}*) → Pt(*d_{x²-y²}*), Pt(*d_{xy}*) → Pt(*d_{x²-y²}*) transitions, respectively.

DNA binding properties of *cis*-PtI₂(NH₃)₂

To determine the nature of the DNA interactions of *cisPtI₂*, the DNA binding properties of this complex were examined through a variety of methods and compared with those of cisplatin. First, we aimed at quantifying the binding of *cisPtI₂* to mammalian (CT) double-helical DNA in a cell free medium.

The amount of platinum bound to DNA increased with time and the times at which the binding reached 50% (*t*_{50%}) are summarized in Table 1.

Compound	<i>t</i> _{50%} (min) ^a
<i>cis</i> -PtI ₂ (NH ₃) ₂ aged in H ₂ O	105 ± 11
cisplatin aged in H ₂ O	64 ± 5
<i>cis</i> -PtI ₂ (NH ₃) ₂ aged in 10 mM KI	390 ± 24
cisplatin aged in 10 mM KCl	136 ± 19

^a The time at which the binding reached 50%. Values shown in the table are the means ± SEM of three separate experiments.

Table 1 Binding of *cis*-PtI₂(NH₃)₂ and cisplatin to calf thymus DNA.

Importantly, after 24 h, *cisPtI₂* and cisplatin aged in water or in 10 mM KI/KCl were quantitatively bound. As expected, the Pt^{II} complexes preincubated in water reacted with DNA significantly more rapidly than those preincubated in KI/KCl (10 mM), but the rate of the reaction of cisplatin was considerably higher than that of *cisPtI₂*.

The lower rate of the reaction of *cisPtI₂* with double-helical DNA in comparison with that of cisplatin can be interpreted to mean³⁵ that the rate of the aquation of the leaving ligands in *cisPtI₂* is significantly lower than that in cisplatin. Since interstrand and intrastrand cross-links formation are believed to be responsible for anticancer activity of bifunctional platinum compounds, we have quantitated the interstrand cross-linking efficiency of *cisPtI₂* in the linear pUC19 DNA (linearized with EcoRI restriction enzyme). Results point out that interstrand cross-linking efficiency of *cisPtI₂* (5 ± 2%) was similar to that of cisplatin (6 %) (see SI for further details).³²

Afterward, to gain more information about the adducts formed in the reaction of *cisPtI₂* with DNA, in comparison with those produced by cisplatin, experiments with the fluorescent probe ethidium bromide have been carried out. Double helical DNA was first modified by the Pt^{II} complex (*cisPtI₂* or cisplatin) for 24 h. The levels of the modification corresponded

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to the values of r_b in the range between 0 and 0.1. Modification of DNA by both platinum complexes resulted in a decrease of EtBr fluorescence (Figure 6).

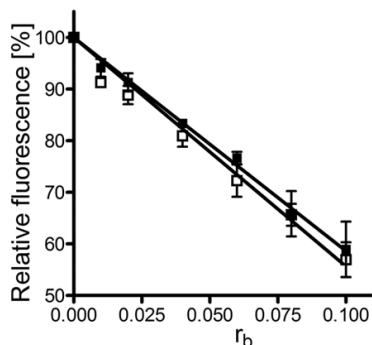


Fig. 6 Ethidium bromide fluorescence. Dependences of the EtBr fluorescence on r_b for CT DNA modified by platinum complexes in 10 mM NaClO₄ at 37 °C for 24 h. Open symbols, *cis*-PtI₂(NH₃)₂; closed symbols, cisplatin.

The formation of adducts of *cis*PtI₂ resulted in a progressive decrease of EtBr fluorescence intensity, which was similar to that of cisplatin. This result suggests that the conformational distortion induced in DNA by the adducts of *cis*PtI₂ is delocalized and extends over the same number of base pairs around the platination sites as in the case of the adducts of cisplatin.

Even DNA unwinding experiment suggests the same mode of metalation between cisplatin and its iodide analogue (see SI for further experimental details). Using this approach, we determined the DNA unwinding angles to be in the range $11 \pm 2^\circ$ for *cis*PtI₂, which is very similar to that found for cisplatin (13°).³⁰ Finally thermal stability experiment of DNA has been carried out.

CT DNA was modified by *cis*PtI₂ to the value of $r_b = 0.03$ – 0.09 . After the modification, the salt concentration was further adjusted by addition of NaCl to 0.1 M, and the samples were further supplemented by Tris-HCl (1 mM) and EDTA (0.1 mM). Thus, the melting curves for DNA modified by the platinum compounds were measured at a relatively high salt concentration (Table 2). We found that the effect of DNA platination by *cis*PtI₂ on the melting temperature of DNA (t_m) is similar to that observed for the modifications by cisplatin.

Three major factors have been invoked to account for the thermal stability of DNA modified by Pt^{II} complexes capable of DNA cross-linking and the observed change in melting temperature of DNA as a consequence of its platination reflects the relative proportion and contribution of these three factors.³⁶

These three factors are (i) a destabilizing effect of conformational distortions due to the formation of cross-links induced in DNA by platinum coordination; (ii) stabilizing effects of DNA interstrand cross-links which prevent dissociation of DNA strands; (iii) the positive charge on the Pt^{II} centers (introduction of a positive charge into the DNA molecule, e.g. by binding of positively charged ligands such as Pt^{II} moieties of platinum compounds, results in a stabilization of the DNA duplex by decreasing electrostatic repulsion of negative charges of phosphate groups located at the complementary strands).

	$r_b = 0.03$	$r_b = 0.06$	$r_b = 0.09$
<i>cis</i> -PtI ₂ (NH ₃) ₂	-1.9 ± 0.1	-5.0 ± 0.1	-7.9 ± 0.3
cisplatin	-2.1 ± 0.3	-5.2 ± 0.3	-8.3 ± 0.3

^a Δt_m is defined as the difference between the t_m values of platinated and unmodified DNAs.

^b The t_m values were measured in the medium containing NaClO₄ (0.1 M), Tris-HCl (1 mM, pH 7.4) and EDTA (0.1 mM).

^c Values shown in this table are the means \pm SEM of three separate experiments.

Table 2 Δt_m (°C) values^a of CT DNA modified by *cis*-PtI₂(NH₃)₂ or cisplatin^{b,c}.

Thus, it is reasonable to conclude that the decreases in t_m are caused by destabilizing conformational distortions and that these destabilizing factors dominate over the interstrand cross-links formed by both *cis*-platinum compounds and by positive charges on platinum moieties.

Owing to the fact that both *cis*PtI₂ and cisplatin carry the same 2+ charge on platinum moiety and form approximately identical amount of interstrand cross-links (5 or 6%, respectively, *vide supra*), the values of t_m yielded by the adducts of both complexes reflect mainly destabilizing effects of conformational distortions induced by the platinum-DNA adducts. Hence, the results of DNA melting experiments are consistent with the thesis that DNA adducts of *cis*PtI₂ distort and destabilize double helix of DNA approximately in the same extent as DNA adducts of cisplatin.

From an overall inspection of the obtained results it is evident that *cis*PtI₂ interacts with DNA through a mode of interaction that is very similar to that well documented for cisplatin. Apparently, the major difference consists of a somewhat slower kinetics of platinum association to DNA while the formed adducts are apparently the same.

Cellular actions of *cis*-Pt I₂(NH₃)₂

Afterward, the antiproliferative effects of *cisPtI₂* were evaluated in a representative panel of cancer cell lines according to the method described in the experimental section.

The cancer cell panel included the following lines: *PANC-1* (human pancreatic cancer), *IGROV-1* (human ovarian cancer), *A549* (human lung cancer), *HT29* (human colon cancer), *HCT116-S* and *HCT116-R* (human colon cancer). These cells were exposed to increasing concentrations of the drugs in the range 0-200 μM; after 24 hours viable cells (determined by Trypan blue exclusion) were counted and the IC₅₀ values determined. The measured IC₅₀ values are reported in Table 3.

cell line	cisplatin	<i>cis</i> -PtI ₂ (NH ₃) ₂
PANC-1	2.48±0.11	0.91±0.10
IGROV-1	25.30±0.40	7.36±0.31
A549	5.77±0.60	3.54±0.49
HT29	16.39±1.10	11.69±1.40
HCT116-S	7.65±0.63	13.42±0.49
HCT116-R	21.96±1.11	4.15±0.23

Table 3 IC₅₀ values (μM) of cisplatin and its iodo analogue in a panel of cancer cell lines.

From data inspection it is evident that *cisPtI₂*, in the four cisplatin sensitive cell lines, produces on the whole cytotoxic effects comparable and often greater than cisplatin. Extremely meaningful is the case of IGROV-1 where the cytotoxicity of *cisPtI₂* is far higher than cisplatin (IC₅₀ = 7.36 μM versus 25.30 μM). Moreover, very remarkably, *cisPtI₂* is found to overcome completely resistance to cisplatin in the cisplatin-resistant HCT116-R line. These results are very relevant and unexpected if one considers that *cisPtI₂* is commonly reputed as inactive.

The effects of *cisPtI₂* on the cell cycle were also analysed in HCT116-R cells in comparison to cisplatin; an arrest in G2/M phase is clearly detected in both cases as well as a decrease of the S phase (Figure 7).

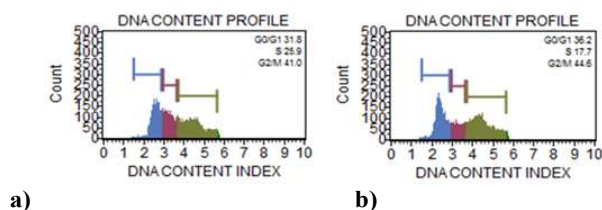


Fig. 7 Cell cycle analysis in HCT116 treated with cisplatin (a) or *cisPtI₂* (b).

Analytical determinations were then carried out to measure intracellular Pt content in HCT116-S and HCT116-R cells after

exposure to equimolar concentrations of either cisplatin or *cisPtI₂*. More precisely, cells were exposed to a 20 μM concentration of the Pt complexes for 3 h. Results expressed as Pt content per cell in the various cases and as percentage of internalised Pt are reported in Table 4.

Cell line	Drug	Platinum level	Internalised Pt %
HCT116-S	cisplatin	2.53x10 ⁻⁸ μg	0,98
HCT116-S	<i>cis</i> -PtI ₂ (NH ₃) ₂	5.37x10 ⁻⁸ μg	1,91
HCT116-R	cisplatin	1.46x10 ⁻⁸ μg	0,50
HCT116-R	<i>cis</i> -PtI ₂ (NH ₃) ₂	6.66x10 ⁻⁸ μg	2,41

Table 4 Platinum level (per cell) measured after exposure (3 hours) of cell lines to 20 μM of cisplatin and *cisPtI₂*.

From inspection of Table 4 it is evident that *cisPtI₂* manifests a greater ability to enter both types of cells than cisplatin; such difference is more evident in the Pt resistant cell line compared to the Pt sensitive one (5 folds vs 2 folds). This is possibly linked to its greater lipophilic character and larger inertness.

Furthermore, in the resistant cell line (HCT116-R), we can pinpoint the correlation occurring between the above IC₅₀ values and Pt uptake in the sense that a greater cytotoxicity is associated to a greater Pt uptake -in terms of a five fold difference- proving the capability of *cisPtI₂* to overcome chemo-resistance.

Discussion and Conclusions

The substantial lack of information concerning *cisPtI₂* despite its striking similarity to cisplatin has inspired the present study; notably, some crucial aspects of its chemical and biological profile have been eventually elucidated.

First, we have shown that *cisPtI₂* manifests a solution behaviour very similar to cisplatin when dissolved in an aqueous medium, at physiological pH; indeed, as seen for cisplatin, *cisPtI₂* undergoes activation through sequential slow release of its two iodo ligands that are replaced by water molecules. However, the process is appreciably slower than in the case of cisplatin. These statements are clearly supported both by experimental data (see SI) and computational analysis. The alternative hydrolysis path, where preferential release of NH₃ ligands is observed, could also be induced by changing the pH conditions. Even in this latter case the reaction route has been characterized by spectrophotometric analysis and spectral simulations. The large differences observed between the spectral patterns associated to the two possible mechanisms are very consistent with our interpretation.

Then, the reactions of *cisPtI₂* with DNA were investigated in detail in direct comparison to cisplatin through a robust

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experimental approach involving a variety of independent biophysical methods. Remarkably, we found that the pattern of DNA platination produced by *cisPtI₂* is highly reminiscent and nearly superimposable to that of cisplatin though the kinetics of the DNA metalation process is again appreciably slower.

Afterward, the cellular effects induced by *cisPtI₂* were explored in a small but representative panel of solid tumor cell lines; the panel included PANC-1 (human pancreatic cancer), IGROV-1 (ovarian cancer), A549 (lung cancer), HT29 (colon cancer), HCT116-S (colon cancer) and HCT116-R cells. Very remarkably, we observed that *cisPtI₂*, in contrast to expectations and to the current opinion, is on the whole more cytotoxic than cisplatin in three out of four cisplatin sensitive cancer cells; on the other hand, *cisPtI₂* was found to overcome completely resistance to cisplatin in the cisplatin resistant HCT116-R cell line. This latter result implies that *cisPtI₂* is able to circumvent the molecular mechanisms that reduce sensitivity to cisplatin in the resistant line. On the other hand, it is unlikely that dimeric Pt species (see SI) play a major role in the observed cytotoxicity as the latter species are detected only at acidic pH and in very low amounts.

Analytical determinations were carried out to measure Pt uptake in the various cases: we found that cisplatin manifested a far lower ability to enter cells than *cisPtI₂*. In addition, a pronounced reduction of Pt uptake was detected in HCT116 resistant cells in comparison to their sensitive counterpart upon cisplatin treatment implying that this is most likely the main mechanism of resistance. In contrast, *cisPtI₂* is internalised in far greater amounts by both cell types with resistant cells even showing a slightly greater Pt uptake.

However, the correlation between the cytotoxic potency and the intracellular Pt content is not straightforward for both HCT116 cellular lines. For instance, the Pt content measured upon exposure of HCT116-S cells to *cisPtI₂* is more than two times greater compared to exposure to cisplatin. Yet, cisplatin produces a greater cytotoxic effect than *cisPtI₂* (by a factor 2). Pairwise, roughly comparable amounts of intracellular Pt are found in HCT116-R and HCT116-S cells upon exposure to *cisPtI₂*; but *cisPtI₂* is far more cytotoxic in the resistant cells compared to the sensitive ones (by a factor 3). This implies that the nature of the cells and the nature of the Pt complex are still important in determining the overall antiproliferative effect.

We believe that the here reported results are very meaningful and innovative with respect to current mechanistic knowledge on cisplatin and on its analogues for the following reasons:

1. In contrast to expectations and to early claims of inactivity *cisPtI₂* is found to produce cytotoxic effects comparable and

often greater than those caused by cisplatin, at least in four representative cancer cell lines;

2. *cisPtI₂* overcomes to a large extent resistance to cisplatin in a cancer cell line showing acquired resistance to cisplatin, being taken up in a far greater amount. This implies that the recognition processes for the two Pt complexes are distinct.

3. However, the large differences measured in Pt uptake between *cisPtI₂* and cisplatin do not correlate with the large differences observed in their respective biological effects. The nature of the Pt complex and the type of cell still play major roles.

4. The aquation process of *cisPtI₂* at physiological pH reproduces closely that of cisplatin- though being slower-involving the progressive release of the two halide ligands; however, at slightly acidic pH, *cisPtI₂* is found to release preferentially its ammonia ligands at variance with cisplatin. These processes are reproduced very satisfactorily through computational analysis of the respective spectral profiles. It is very likely that these differences in reactivity between *cisPtI₂* and cisplatin may lead to different interaction modes with specific biomolecules, in particular with proteins, thus generating distinct pharmacological profiles.

5. Remarkably, *cisPtI₂* produces a kind of DNA damage very similar to cisplatin. Yet, the kinetics of Pt binding to DNA is slower and the final level of DNA platination appreciably lower. As the process of DNA platination is apparently less efficient than in the case of cisplatin it is unlikely that DNA is the only or primary target for *cisPtI₂*. when one considers that *cisPtI₂* is even more cytotoxic than cisplatin.

Altogether, the reported results delineate an extremely favourable biological profile for *cisPtI₂* and strongly warrant its further assessment in appropriate preclinical models. Moreover, at least in our opinion, these findings contain relevant implications with respect to the postulated mode of action of cisplatin and of its analogues. As DNA damage produced by *cisPtI₂* is, on the whole, less extensive and less important than that induced by cisplatin and the type of DNA lesion is basically the same, the view that the cytotoxic effects of *cisPtI₂* may arise from mechanisms other than DNA damage is supported. However, more studies are definitely needed to further substantiate this hypothesis.

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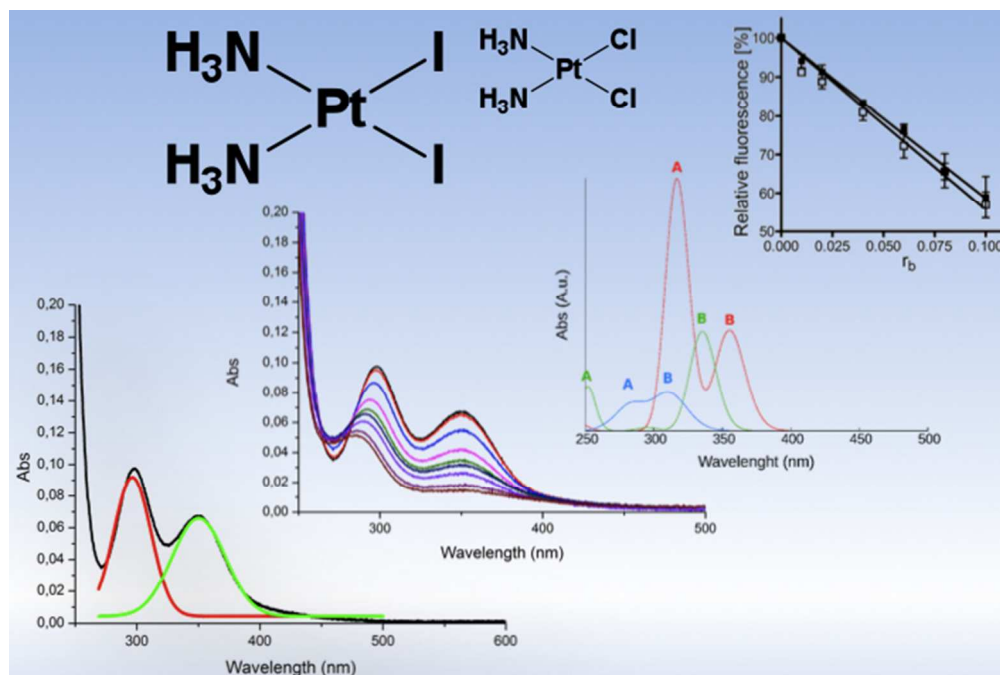
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Electronic Supplementary Information (ESI) available: UV-Vis spectrum of complex cis-Pt(NH₃)₂I₂ 10⁻⁴ M in presence of KI, kinetic studies, ESI MS experiments, DNA interstrand cross-linking in the linear pUC19 DNA, DNA unwinding See DOI: 10.1039/b000000x/

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Cis-PtI₂(NH₃)₂ is highly cytotoxic toward a few solid tumour cell lines, furthermore it is capable to overcome resistance to cisplatin.



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