Cisplatin and its dibromido analogue: a comparison of chemical and biological profiles.

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Abstract. The dibromido analogue of cisplatin, *cis*-$PtBr_2(NH_3)_2$ (*cis*$PtBr_2$ hereafter), has been prepared and characterised. Its solution behaviour in standard phosphate buffer, at pH 7.4, was investigated spectrophotometrically and found to reproduce quite closely that of cisplatin; indeed, progressive sequential release of the two halide ligands typically occurs as in the case of cisplatin, with a roughly similar kinetics. Afterward, patterns of reactivity toward model proteins and standard ctDNA were explored and the nature of the resulting interactions elucidated. The antiproliferative properties were then evaluated in four representative cancer cell lines, namely A549 (human lung cancer), HCT116 (human colon cancer), IGROV-1 (human ovarian cancer) and FLG 29.1 (human acute myeloid leukaemia). Cytotoxic properties in line with those of cisplatin were highlighted. From these studies an overall chemical and biological profile emerges for *cis*$PtBr_2$ closely matching that of cisplatin; the few slight, but meaningful differences that were underscored might be advantageously exploited for clinical application.

Keywords: Cancer • Platinum • Cisplatin Analogues • Mass Spectrometry • Circular dichroism

Introduction

Cisplatin is a leading and established anticancer compound in widespread clinical use. It is very effective against a few cancer types such as testicular and ovarian cancer but scarcely active against other important and more frequent solid tumors such as colorectal, ovarian and lung cancer (De simone et al. 1986; Wang and Wu 2006; Rosell 2002) . While thousands of analogues of cisplatin have been prepared and tested so far, quite surprisingly the immediate parent Pt compounds that are obtained through simple replacement of the two chlorides with different halides as metal ligands (in particular the diiodido and dibromido derivatives) were poorly investigated.

Most likely, this situation arises from the early misconception and/or generalisation that chloride replacement with other halides may result into substantial loss of the anticancer activity (Wilson and Lippard 2014; Johnstone et al. 2014; Cleare and Hoeschele 1973). These arguments led us to explore this kind of modification in a more systematic way and analyse its chemical and biological consequences.

Recently, we demonstrated that the diiodido analogue of cisplatin manifests truly interesting biological properties and warrants a more extensive pharmacological evaluation (Marzo et al. 2015). This encouraging result prompted us to prepare and evaluate even the dibromido analogue of cisplatin (Fig.1). Accordingly, we report here the synthesis and chemical characterisation of *cis*$PtBr_2$ as well as an initial and comparative assessment of its biological and pharmacological profile.

Figure 1. Structure of cisplatin and its dibromido analogue.

Materials and methods

Chemistry of *cis*-$PtBr_2(NH_3)_2$, synthesis and characterisation

The synthesis of this Pt complex was performed through a slight modification of Dhara's synthesis for cisplatin (Dhara 1970). A solution of 400 mg (2.4 mmol) of KI in 3 mL of water was added to an aqueous solution (5 mL) of 250 mg of $K_2[PtCl_4]$ (0.6 mmol) which was quantitatively converted into a dark solution containing $K_2[PtI_4]$ after five minutes of stirring at room temperature. Then the addition of two equivalents of ammonium hydroxide as a 40% solution results in the separation of *cis*-$PtI_2(NH_3)_2$ as a bright yellow compound, leaving behind a colorless solution. The solid was filtered off and thoroughly washed with water (yield 90%).

A suspension of 200 mg of *cis*-$PtI_2(NH_3)_2$ (0.41 mmol) in water (5mL) was mixed with a solution of $AgNO_3$ (0.82 mmol) in water (1,5 mL) and stirred in the dark (40ºC) until a pale yellow solution was formed over the suspension. The AgI formed was then filtered off using neutral celite over a solution of two equivalents of KBr in 7 mL of water. The colorless solution was stored until orange crystalline precipitation was observed. The bright orange crystals of *cis*-$PtBr_2(NH_3)_2$ were filtered off, washed with water and dried in air. Yield was 25%. Purity of the product was assessed through elemental analysis of C, N and H [calculated C: 0%, H: 1.45%, N: 7.20%, experimental: C: 0,54%, H: 1.45%, N: 7.18%], ^1H, ^{195}Pt NMR and IR analysis (see SI). Cis geometry was checked as described in (Nakamoto 1997). Solution behaviour of *cis*-$PtBr_2(NH_3)_2$ was assessed through spectrophotometric experiments performed with a Varian Cary 50 Bio UV-Vis spectrophotometer (1 cm pathlength quartz cell) in buffered solutions without the use of DMSO and NaCl. A solution of the complex (10^{-4} M) was prepared in 50 mM phosphate buffer at pH = 7.4. The absorbance was monitored in the wavelength range between 200 and 800 nm for 72 h at 25° C.

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Log P determination

The octanol–water partition coefficients for *cis*$PtBr_2$ was determined by modification of the reported shake flask method (Marzo et al. 2015). 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 complex was prepared in the aqueous phase (3×10^{-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 $[complex]_{oct}$ / $[complex]_{wat}$. Final value was reported as the mean of three determinations.

Electrospray mass spectrometry, interaction with lysozyme

A solution of *cis*-$PtBr_2(NH_3)_2$ (10^{-4} M) was incubated with lysozyme (3:1 metal/protein ratio) for 72 h at 37° C in 20 mmol L^{-1} ammonium acetate at pH=6.8. ESI MS spectra were recorded after 24 and 72 h. After a 20-fold dilution with water, ESI MS spectra have been recorded by direct introduction at 5 µl min^{-1} flow rate in an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source. The working conditions were the following: spray voltage 3.1 kV, capillary voltage 45 V, capillary temperature 220°C, tube lens voltage 230 V. The sheath and the auxiliary gases were set, respectively, at 17 (arbitrary units) and 1 (arbitrary units). For acquisition, Xcalibur 2.0. software (Thermo) was used and monoisotopic and average deconvoluted masses were obtained by using the integrated -Xtract tool. For spectrum acquisition, a nominal resolution (at m/z 400) of 100,000 was used.

Circular Dichroism experiments, interaction with ctDNA

CD spectra were measured with a Jasco J-715 spectropolarimeter with the following conditions: scan speed 50 nm/min; response 1 sec; data pitch 0.1 nm; bandwidth 2.0 nm; 8 accumulations. Calf thymus DNA (ctDNA) 52.0 µM in phosphate buffer 50 mM, pH=7.4 was additioned with *cis*-$PtBr_2(NH_3)_2$ 2.3 mM in H_2O or cisplatin 1.0 mM in H_2O, to 0.5:1 and 1:1 molar ratio directly in a 1-cm quartz cylindrical cell, volume 2.5 ml. Concentrations and molar ratios are indicated per base. All samples (except one, see results) were incubated at room temperature (22°C) for 72h before measurement.

The ctDNA had a base-pair length of ca. 800, obtained with a standardised procedure by sonication (Biver et al. 2003). The DNA concentration was checked by absorbance measurement at 260 nm (0.34 u.A., 1 cm cell). Phosphate buffer was used in all case to measure the blank spectrum.

Cellular studies

Cell cultures. A549 cells were cultured in DMEM High glucose (Euroclone; Milan, Italy) with 10% Fetal Bovine Serum (FBS) (Euroclone Defined; Euroclone; Milan, Italy). HCT-116, FLG 29.1 and IGROV-1 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). All the cell lines were cultured at 37 °C in humidified atmosphere containing 5% CO_2 in air.

Pharmacology experiments. Cells were seeded in 96-well flat-bottomed plates (Corning-Costar, Corning, NY, USA) at a cell density of 1×10^4 cells per well in either RPMI or DMEM complete medium. Drugs were used, after solubilization in water, without using DMSO, at concentrations ranging from 0 to 200µM. After 24 h, viable cells (determined by Trypan blue exclusion test) were counted in triplicate using a haemocytometer. Each experimental point represents the mean of a single experiment carried out in triplicate.

Trypan blue exclusion test. Cells viability was assessed by the Trypan blue exclusion assay. In brief, 10 µL of a 0.4% trypan blue solution were added to 10 µL of 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 the dye were considered non viable, 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 (Biosoft, Cambridge, UK).

Cell cycle analysis. The effect of cisplatin and cis$Pt(NH_3)_2Br_2$ on cell cycle distribution was assessed by flow cytometry after staining the cells with propidium iodide (PI). Briefly, the cells (5×10^5 cells per mL) were analysed prior to and after treatment with IC_{50}s of both drugs for 24 h. The cells were harvested, washed with PBS and resuspended in 300 µL of propidium iodide staining solution and incubated at 4 °C in the dark for 20 minutes. DNA content of the cells was measured by BD FACSCanto (Becton Dickinson, Franklin Lakes, NJ, USA) Flow Cytometer and the cell population residing in each phase cell cycle phase was determined using ModFit LT 3.0 analysis software (Verity Software House, Topsham, ME USA).

Annexin/PI assay. The effects of the two compounds on FLG 29.1 cells was investigated through the Annexin V/propidium iodide test (Annexin-VFLUOS staining kit; Roche Diagnostics, Mennheim, Germany). Cells were seeded at $2x10^5$/well in 6-well plates and incubated with either drug for 24 hours. Cells were then harvested, washed with PBS, re-suspended in 100 µL of binding buffer and incubated with FITC-conjugated Annexin V and propidium iodide for 15 min. Flow cytometry analysis was performed with BD FACSCanto (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed with BD FACSDiva Software 6.1.3. Each experiment was performed in triplicate.

Results and discussion

Solution behaviour of *cis*-$PtBr_2(NH_3)_2$, activation profile and logP determination

cis$PtBr_2$ shows an acceptable solubility in the reference phosphate buffer, at physiological pH; this allows to carry out experiments in the absence of DMSO or other organic solvents, thus avoiding potential interferences. Freshly prepared solutions of *cis*-$PtBr_2(NH_3)_2$ manifest typical absorption bands in the UV-visible, with a main band at 330 nm and a pronounced shoulder at 300 nm (Fig. 2a).

These bands are tentatively assigned to a d-d and a LMCT transition, respectively, in analogy with other cisplatin analogs (Marzo et al. 2015). After dissolution of the study compound in the reference buffer, progressive spectral changes are observed that are ascribed to the progressive release of the two bromide ligands. The nature of the process and its kinetics are similar to what observed in

the case of cisplatin despite release of halides in the case of *cis*$PtBr_2$ appears to be slight slower than in case of cisplatin (see SI for details and numerical data on the hydrolysis processes). By comparative inspection of the spectral profiles of *cis*$PtBr_2$ and cisplatin, monitored over 24 h, their progressive convergence was noticed, indicating that the final species are the same for both drugs (see SI for further details).

Furthermore, log P determination was carried out on *cis*$PtBr_2$ and a value of -1.04 was found. Remarkably this value is intermediate between those previously measured for cisplatin (-2.4) and *cis*-$PtI_2(NH_3)_2$ (-0.13) (Marzo et al. 2015), thus, being the dibromido analogue of cisplatin far more lipophilic than cisplatin itself, it is well conceivable that this difference may have some appreciable effect in terms of pharmacological activity.

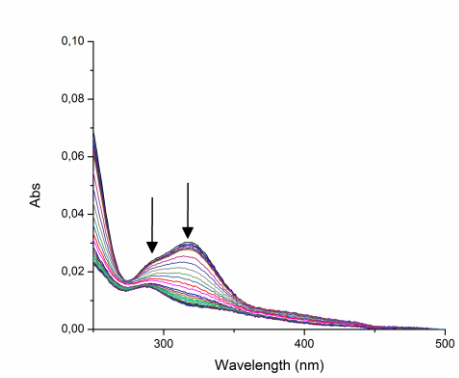

Figure 2. Time course spectra of *cis*$PtBr_2$ 10^{-4} M in 50mM phosphate buffer over 24 h, RT.

Mechanistic studies: biomolecular interactions

To investigate the possible interactions occurring between *cis*$PtBr_2$ and its potential biomolecular targets we first performed a few ESI MS experiments for samples where the study complex was incubated with the model protein lysozyme for increasing time intervals (Fig. 3).

First attempts to characterise the protein metalation process through ESI MS detection were performed by analysing samples after 24 h of incubation, but no clear evidence of adduct formation was gained. However, upon increasing the incubation time up to 72 h, under identical solution conditions, a number of peaks of higher molecular weight than the native protein, were instead observed that were straightforwardly assigned to metallodrug-lysozyme adducts.

From careful inspection of the spectrum, it is evident that Pt coordination occurs through a mechanism that involves preferential detachment of halide ligands and full retention of ammonia ligands; assignments were validated by theoretical calculations (see SI for comparison between experimental and theoretical peaks). This kind of protein metalation strictly resembles that produced by cisplatin resulting in an adduct characterised by selective Pt coordination to His15 following the release of the two chloride ligands (Casini et al. 2007).

Remarkably, this behaviour being in accord with cisplatin is in contrast with that of *cis*PtI_2, where metalation of lysozyme occurs through protein binding of metal fragments resulting from preferential release of the ammonia ligands (Messori et al. 2012).

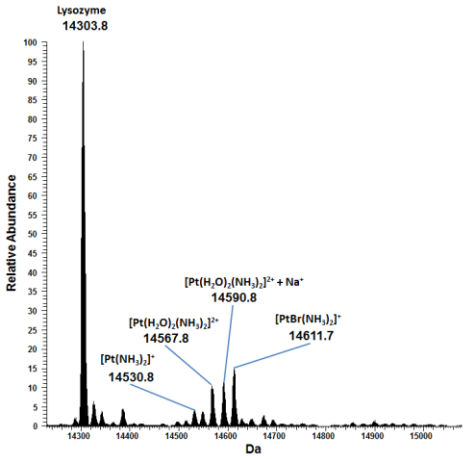

Figure 3. Deconvoluted ESI MS spectra of lysozyme treated with 3 x 10^{-4} mol L^{-1} of *cis*$PtBr_2$ recorded after 72 h of incubation at 37 °C, metal : protein ratio = 3:1; ammonium acetate buffer 20 mmol L^{-1} at pH=6.8.

CD experiments, interaction with ctDNA

Since nuclear DNA is believed to be the primary target for the pharmacological effect of cisplatin, (Siddik 2003) we next characterized the interaction of *cis*$PtBr_2$ with calf thymus DNA through CD experiments, and compared the results with those of cisplatin.

The CD spectrum of ctDNA, before addition of the drugs is in keeping with literature (Tamburro et al. 1977) (Fig. 4). Addition of 0.5 equiv of *cis*-$PtBr_2(NH_3)_2$ did not lead to any immediate change in the CD spectrum (data not shown). However, after incubation at 22°C for 72 h, the spectrum changed appreciably showing clear differences on the whole wavelength range (Fig. 5). In particular, both CD bands at 280 and 250 nm increased in intensity, the first by about 15%.

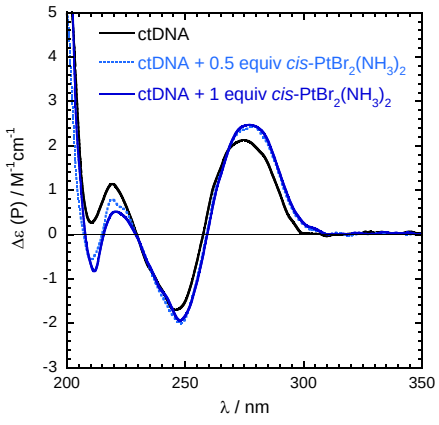

Figure 4. CD spectra of ctDNA without and with *cis*-$PtBr_2(NH_3)_2$, 0.5 and 1 equiv, after 72 h incubation.

Furthermore, no significant spectral changes were found after increasing the amount of complexes up to 1 equiv, being the two spectral profiles at 0.5 or 1 equiv superimposable (Fig. 4). Evidence that *cis*$PtBr_2$ interacts with ctDNA similarly to cisplatin derives from

the comparative analysis of the CD spectra of ctDNA after incubation with 1 equivalent of *cis*$PtBr_2$ or cisplatin (Fig. 5).

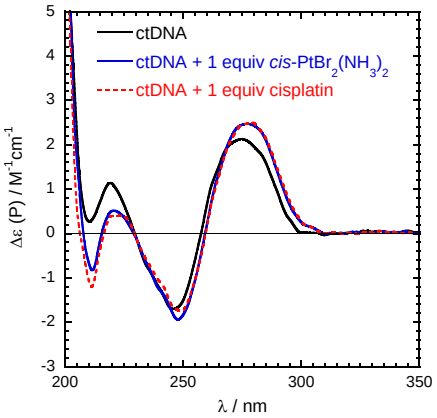

Figure 5. CD spectra of ctDNA with *cis*-$PtBr_2(NH_3)_2$ and cisplatin, 1 equivalent, after 72h incubation.

Modification in the CD spectral profiles are well detectable, consistent and superimposable for the two complexes. A direct comparison with previous CD investigations on the ctDNA/cisplatin system (Tamburro et al. 1977; Sristava 1978) is complicated by relevant differences in the applied solution conditions and in the nature of ctDNA samples (non-sonicated vs. sonicated). Qualitatively speaking, the differences observed upon binding occur in the same spectral regions and to a similar extent. However, our fragmented ctDNA (800 bp) shows a full enhancement of the main CD bands, already with 0.5 equivalent of ligand per base.

Cellular effects

We then investigated the effects of *cis*$PtBr_2$ on cell vitality of a small panel of human cancer cell lines, applying the Trypan blue exclusion test according to the method described in the experimental section.

The cancer cell panel included the following lines: A549 (human lung cancer), HCT116 (human colon cancer), IGROV-1 (human ovarian cancer) and FLG 29.1 (human acute myeloid leukaemia). These cells were exposed to increasing concentrations of the drug, in the 0–200 μM range, and treated for 24 hours; IC_{50} values were determined (Table 1). It is evident that *cis*-$PtBr_2(NH_3)_2$ produces cytotoxic effects almost superimposable to those of cisplatin in all the cancer cell lines. A slightly greater effect of *cis*$PtBr_2$ compared to cisplatin was observed in FLG 29.1 leukaemia cells. These encouraging results led us to deepen our investigation.

The effects of *cis*$PtBr_2$ on cell cycle and apoptosis were further evaluated in FLG 29.1 cells. Upon treatment with *cis*-$PtBr_2(NH_3)_2$ a reduction in the percentage of cells in the G0/G1 phase accompanied by an increase in G2/M cells was observed. Such effect was more evident than that produced by cisplatin (see Figure 6). We next evaluated the effect of *cis*-$PtBr_2(NH_3)_2$ on the induction of apoptosis by Annexin/PI test. As reported in Figure 7 *cis*-$PtBr_2(NH_3)_2$ determined a more evident pro-apoptotic effect compared to cisplatin, in FLG 29.1 cells (percentage of Annexin+/PI- cells: f 51.85±4.5 vs 41.15±2.75, respectively). Finally, we determined the effects of either *cis*$PtBr_2$ or cisplatin (both at 19 μM concentration) on proliferation of FLG 29.1 cells. Live cell number was monitored for 72 hours. As shown in Figure 8 the dibromido analogue impaired leukaemia cell proliferation even better than cisplatin, especially at longer incubation times.

	IC_{50} Cisplatin (μM) mean±SD	**IC_{50} *cis*-$PtBr_2(NH_3)_2$ (μM)** mean±SD
A549	8.61±1.26	10.08±1.26
FLG 29.1	24.33±1.30	14.66±1.10
HCT-116	20.04±1.65	28.28±5.39
IGROV-1	24.12±0.85	25.63±0.43

Table 1. Evaluation of IC_{50} on a panel of tumor cell lines. A549,FLG,HCT-116 and IGROV-1 cell lines were exposed to IC_{50} concentrations of cisplatin and *cis*$PtBr_2$ for 24 hours and cell viability was evaluated via Trypan Blue exclusion assay. Means and SDs are relative to one experiment mounted in triplicate.

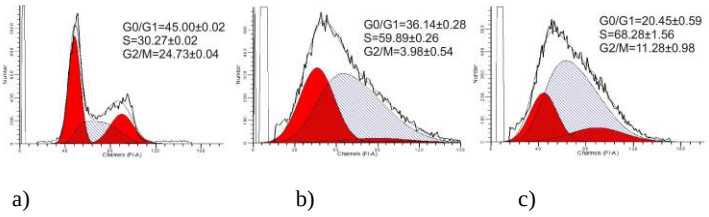

Figure 6. Effect of Cisplatin and *cis*$PtBr_2$ on cell cycle of FLG cells (a=untreated). Effect of cisplatin (b) and of *cis*$PtBr_2$ (c) given at their IC_{50}s on cell cycle of FLG cells after 24h of treatment. The means and SEMs relative to two independent experiments are reported next to each representative panel.

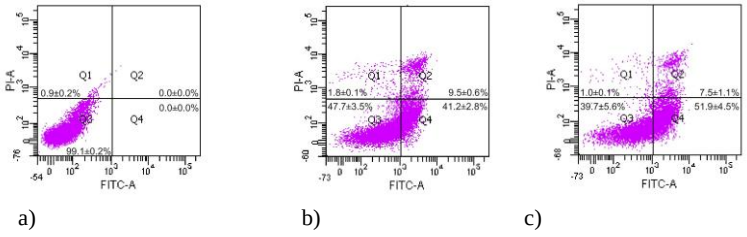

Figure 7. Effect of cisplatin and of *cis*$PtBr_2$ given at their IC_{50}s on the induction of apoptosis on FLG cells after 24h of treatment. The means and SEMs relative to two independent experiments are reported in each representative panel. a=untreated, b=cisplatin, c= *cis*$PtBr_2$

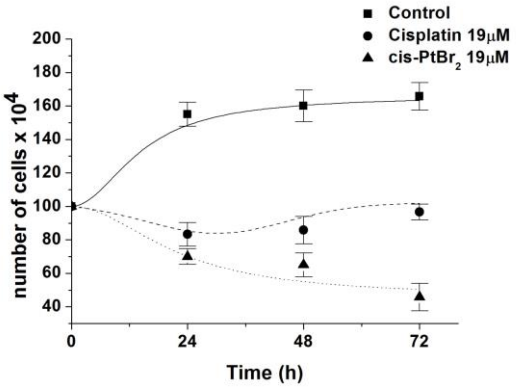

Figure 8: Effects of cisplatin (black dots) and *cis*$PtBr_2$ (black triangles) on FLG 29.1 cell line proliferation during the passage of time given as the number of Trypan Blue negative cells. Drugs were added once a t=0. Data are means ± SD of two independent experiments.

Discussion and Conclusions

Cisplatin is one of the oldest and most used Pt-based anticancer drug with a long success story in the clinics since 1979. Yet, cisplatin shows some severe drawbacks that have triggered a lot of attention in the preparation and identification of new and hopefully more active and less toxic anticancer Pt drugs.

Despite the intense investigations carried out in the field of Pt-based drugs during the last three decades, the closest analogues of cisplatin, *e.g.* Pt complexes differing only in the chemical nature of the two halide ligands, were poorly studied. We have recently analysed the behaviour of the diiodido analogue of cisplatin (Marzo et al. 2015). Very interesting chemical and biological features of this compound emerged. Notably, the diiodido analogue turned out to have strong cytotoxic effects on different cancer cell lines *in vitro*, especially on those more resistant to cisplatin effects. Some peculiarities in the nature of the reaction of *cis*PtI_2 with model proteins were also highlighted (Marzo et al. 2015). Here, we extended our investigation to the dibromido analogue, in order to have a deeper insight and more complete description of this topic.

cis$PtBr_2$ could be prepared quite easily according to the classical synthetic procedure of Dhara, with slight modifications. Its solution chemistry was explored in a standard physiological buffer and a behaviour very close to cisplatin emerged. Indeed, activation *cis*-$PtBr_2(NH_3)_2$ occurs through progressive release of the two bromide ligands.

When tested with a model protein (lysozyme) a platination pattern similar to cisplatin emerged, with protein coordination of $[cis\text{Pt}(NH_3)_2]^{2+}$ fragments. Even the process of platination of standard ctDNA (800 bp) was highly reminiscent of that of cisplatin and CD spectroscopy clearly proved the similar nature of those interactions. One equivalent of ligand (per base pair, 0.5 equiv per base) turned out to achieve full binding. The binding is similar for *cis*-$PtBr_2(NH_3)_2$ and cisplatin. Using our fragmented ctDNA, the effects of cisplatin are slightly different from those reported in the literature for non-sonicated ctDNA (Tamburro et al. 1977; Sristava 1978).

Cellular studies were performed on four different cancer cell lines. Cytotoxic effects were generally comparable to those produced by cisplatin (see LD_{50} values in Table 1), and even slightly higher in FLG 29.1 leukaemia cells. In the latter cells, *cis*$PtBr_2$ turned out to trigger apoptosis, modulate cell cycle distribution and decrease cell proliferation, at the same extent, and even better than cisplatin. All these effects might be at least partially explained by the higher lipophilicity of the dibromido-analogue compared to cisplatin which could ensue in an increased cellular uptake (preliminary data are shown in SI). Also, even the possible small differences in the kinetic of halides release (see SI) may be invoked to play some role in the slight but meaningful differences in the pharmacological effect between cisplatin and its dibromide analogue.

We believe that the findings outlined in this paper might be exploited for further preclinical studies of this analogue and for a full assessment of its pharmacological profile with respect to cisplatin. Indeed, as the two compounds are very similar in their overall chemical and biological profile, the slight differences here highlighted might translate into an effective therapeutic advantage and/or in a somewhat modified spectrum of anticancer actions for *cis*$PtBr_2$ over cisplatin.

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