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Synthesis, antiproliferative and mitochondrial impairment activities of bis-alkyl-amino transplatinum complexes

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ABSTRACT

A convenient synthetic route and the characterization of complexes *trans*-[$PtCl_2$(L)(PPh_3)] (L = Et_2NH (**2**), $(PhCH_2)_2NH$ (**3**), $(HOCH_2CH_2)_2NH$) (**4**) are reported. The antiproliferative activity was evaluated on three human tumor cell lines. The investigation on the mechanism of action highlighted for the most active complex **4** the capacity to affect mitochondrial functions. In particular, both the induction of the mitochondrial permeability transition phenomenon and an aspecific membrane damage occurred, depending on concentration.

1. Introduction

The discovery of the antiproliferative properties of *cis*-[$PtCl_2(NH_3)_2$] (cisplatin), carried out by Rosenberg in 1965,[1] led to a rapid development of this anticancer agent and, later on, to its introduction in the clinical trials. Nowadays, cisplatin is still widely used in the treatment of tumors, despite its applicability is somehow limited by drug-resistance phenomena[2] and serious collateral effects.[3] Therefore, the search for analogues of cisplatin is a primary task among researchers, with many hundreds of platinum complexes synthesized and tested over the last decades.[4] Although many studies on the mechanism of action of cisplatin have been carried out,[5] basilar questions such as the reason for drug resistance, still remain unsolved[6] and much less is known about the mechanism of action of cisplatin analogues. Among the platinum(II) derivatives showing promising antiproliferative properties, a growing number of them are characterized by the presence of one or more phosphorous-bounded ligands.[7–9] Somewhere a phosphine ligand being *trans* to a secondary amine,[8,9] afforded quite active complexes, even towards cisplatin resistant tumor cells. A different mode of action was suggested; specifically, the hydrophobic phosphine ligand could help the complex go through the cell membrane, enhancing the amount of platinum derivative available for the biological interactions inside the cell.[9]

In this connection, interestingly, in previous papers for the complex bis[1,2-bis(diphenylphosphino)ethane]gold(I) an anti-mitochondrial effect has been proposed, related to an increase in permeability of the inner mitochondrial membrane.[10] Also the bis-chelated Au(I) complex of 1,3-bis(di-2-pyridylphosphino)propane showed a mode of action dependent on mitochondria. Indeed, it demonstrated the ability to induce apoptosis via the mitochondrial pathway, with loss of mitochondrial membrane potential, depletion of glutathione pool and caspase activation.[11] In a more recent study we reported the capacity of the platinum(II) complex [$PtCl(\eta^1\text{-}C_9H_7)L_2$] (where L_2 = 1,2-bis(diphenylphosphino)ethane) to exert its cytotoxicity mainly through the induction of the mitochondrial permeability transition (MPT), a phenomenon strictly related to the activation of the intrinsic apoptotic pathway.[12]

The crucial role played by mitochondria both in vital functions and in cell death machinery has increased the interest toward these organelles inside the field of anticancer therapies[13,14] and particular attention is devoted to the MPT.[15,16] The MPT consists in a sudden increase in permeability of mitochondrial membrane and is regulated by the opening of a high conductance pore, called permeability transition pore, which allows diffusion of solutes with molecular mass up to 1500 Da. Pore opening is accompanied by dissipation of the inner transmembrane potential ($\Delta\Psi$), matrix

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swelling and outer membrane disruption as well as release of pro-apoptotic factors such as cytochrome c (cyt c), which plays a key role in caspase 9 activation, and apoptosis-inducing factor (AIF) which causes nuclear DNA fragmentation independent from caspase activation.[17]

In the course of our studies on platinum(II) derivatives, we have recently developed convenient synthetic procedures for the preparation of complexes $[PtCl_2L(PPh_3)]$,[18,19] starting from easily available $[PtCl_2(NCCH_3)(PPh_3)]$ (**1**).[18] We report here the synthesis and the characterization of complexes *trans*-$[PtCl_2(L)(PPh_3)]$ (L = Et_2NH (**2**),[19] $(PhCH_2)_2NH$ (**3**), $(HOCH_2CH_2)_2NH$) (**4**) (Scheme 1). The antiproliferative activity was evaluated in vitro towards three human tumor cell lines: HeLa (cervix adenocarcinoma), H460 (large cell lung cancer) and A549 (non-small cell lung cancer). The death pathway and the effect on mitochondria in whole cells was investigated by flow cytometry. Finally, the ability to affect mitochondrial functions was studied in depth by using isolated rat liver mitochondria (RLM).

2. Results and discussion

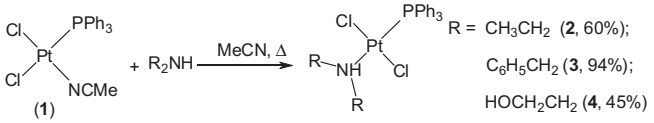
2.1. Chemistry

The synthesis of platinum complexes **2**–**4** is outlined in Scheme 1. *cis*-$[PtCl_2(PPh_3)(NCMe)]$ (**1**), used as starting material, was prepared according to a reported procedure[18] and reacted with the suitable secondary amine in refluxing acetonitrile.

Under these experimental conditions a fast *cis* to *trans* isomerization of the precursor takes place, followed by the substitution of labile coordinated nitrile by the amine.[19] Reactions were followed by ^{31}P NMR spectroscopy, monitoring the disappearance of the precursor signal. In all cases conversion was complete in 3 h and only one new signal was observed, which was ascribed to the desired product. Complexes **2**–**4** were recovered by crystallization and characterized by elemental analysis and spectroscopy. Specifically, in all cases analyses of C, H and N contents were in good agreement with the proposed structures. Moreover, I.R. spectra showed the presence of a stretching $\tilde{\upsilon}_{N-H}$ band near 3200 cm^{-1}, due to the coordinated amine, while in ^1H NMR spectra complicated patterns were observed for CH_2 groups vicinal to coordinated NH, due to non equivalence of diastereotopic hydrogens. *trans* Stereochemistry for complexes **3** and **4** was assumed, because of the already cited[19] known reactivity of the system and by the comparison of measured $^1J_{Pt\text{-}P}$ coupling constants with $^1J_{Pt\text{-}P}$ coupling constant observed in complex **2**, for which the *trans* stereochemistry had already been determined by X-ray diffraction studies.[19] Anyway, in all cases a unique product was observed, which was stable in solution.

2.2. Antiproliferative activity

To evaluate the antiproliferative effect of complexes **2**–**4**, an in vitro assay was performed on three human tumor cell lines, HeLa (cervix adenocarcinoma), H460 (large cell lung cancer) and A549 (non-small cell lung cancer). The results, expressed as GI_{50} values, that is the concentration of compound able to induce 50% cell death with respect to the control culture, are shown in Table 1. The well known drug cisplatin was used as reference compound.



Scheme 1. Synthesis of *trans*-$[PtCl_2(PPh_3)(R_2NH)]$ complexes.

Table 1
Cell growth inhibition values in the presence of examined compounds and cisplatin taken as reference drug

Complex	Cell line GI_{50}[a] (μM)		
	HeLa	H460	A549
2	5.1 ± 1.5	6.8 ± 0.6	8.4 ± 0.4
3	3.3 ± 1.7	6.6 ± 1.4	7.0 ± 1.2
4	0.42 ± 0.06	1.1 ± 0.3	2.3 ± 0.7
Cisplatin	1.5 ± 0.6	0.76 ± 0.11	1.6 ± 0.7

[a] Values are the mean ± SD of at least three independent experiments.

The obtained results highlighted for all complexes an interesting antiproliferative effect, with GI_{50} values ranging from 0.42 to 8.4 μM. In detail, the most active complex appears **4**, having a cytotoxic effect comparable to that of cisplatin on all cell lines taken into consideration. As regard the bis(ethyl)amino (**2**) and the bis(-benzyl)amino (**3**) derivatives, they exert a quite lower cellular effect, with GI_{50} values from about 3 to 12 times higher with respect to those obtained with **4**. Moreover, the cytotoxicity induced by **2** is comparable to that exerted by **3** on all cell lines taken into consideration.

Overall, these results suggest for the bis(2-hydroxyethyl)amino substituent and in particular, for the hydroxyl group a crucial role in inducing the antiproliferative effect.

2.3. Determination of apoptosis

The interesting biological activity of **4** prompted us to investigate its effects at cellular level and in particular, the mechanism responsible of cell death. The apoptosis or programmed cell death is a tightly regulated process characterized by several morphological and biochemical features, including changes in the kinetics of phosphatidylserine exposure on the outer leaflet of plasma membrane, changes in mitochondrial membrane permeability leading to the release of proapoptotic proteins, activation of caspases and internucleosomal cleavage of nuclear DNA.[20,21] Apoptosis induction on HeLa cells was evaluated by using Annexin V-FITC and DNA-specific dye propidium iodide labeling and the results are shown in Figure 1. By increasing concentrations of **4** up to 20 μM, a dose-dependent decrease in viable cells is observed, along with a concurrent increase in apoptotic cells. At higher concentration (30 μM), the percentage of apoptotic cells decreases significantly with respect to that observed at 20 μM concentration, while the percentage of necrotic cells increases noticeably reaching about 50%. This result suggests that in this latter condition cell death may occur mainly by necrosis (Fig. 1A). Otherwise, for the drug cisplatin the percentage of apoptotic cells increases in a dose-dependent manner up to 60 μM concentration with apoptotic cells representing more than 60% and the percentage of both viable and necrotic cells close to 20% (Fig. 1B). Thus, for this drug the apoptosis seems the major pathway of cell death.

These results suggest that **4** may induce cell death through two different mechanisms of action depending on its dose, the first occurs at lower concentrations (10–20 μM), while the second becomes appreciable at higher concentration (30 μM). Furthermore, the different behavior between **4** and cisplatin could indicate a different molecular mechanism of action and then, different intracellular target(s).

2.4. Evaluation of mitochondrial membrane potential on whole cells

Inside the process of apoptosis, a prominent role is played by mitochondria with the induction of the MPT, a process that leads to the opening of a large conductance non-selective pore. This

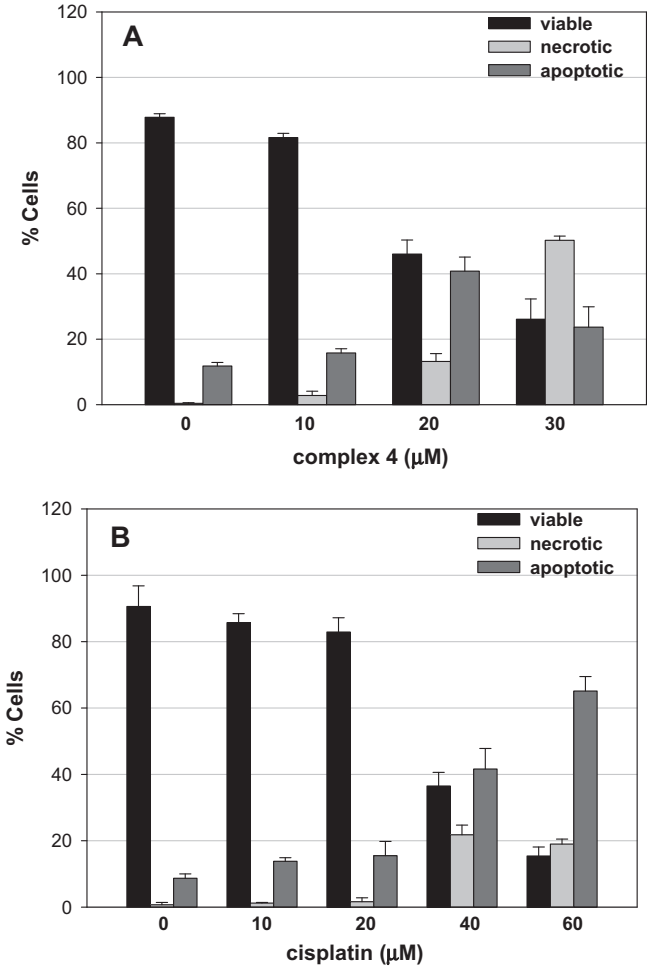


Figure 1. Flow cytometry analysis of cell death induced by **4** (A) or cisplatin (B). Flow cytometry analysis was performed on HeLa cells stained with Annexin V-FITC/propidium iodide after 18 h of treatment with different concentrations of compounds. Values are the mean ± SD of four independent experiments.

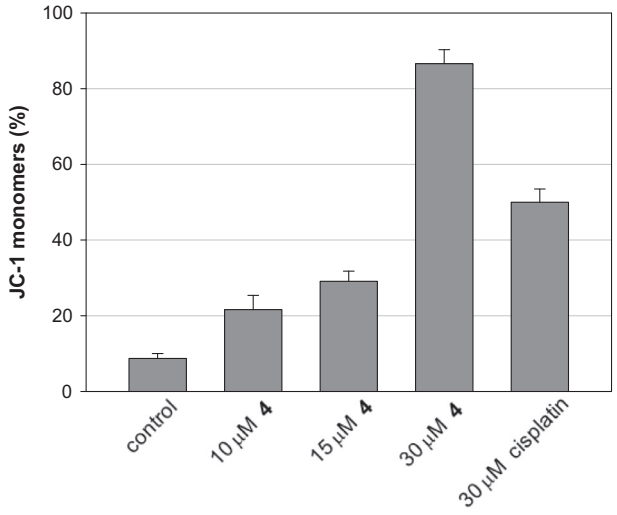


Figure 2. Mitochondrial membrane potential assessed by flow cytometry on whole cells. HeLa cells were treated for 18 h with compound **4** or cisplatin at the indicated concentrations, and stained with JC-1. Results are represented as percentage of cells with depolarized mitochondrial membrane (JC-1 monomers). Values are the mean ± SD of four independent experiments.

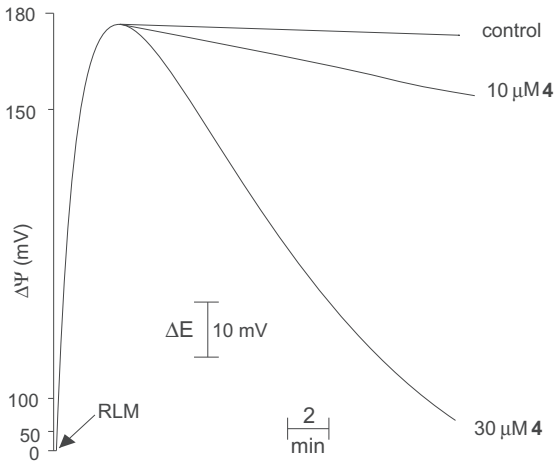


Figure 3. Effect of **4** on mitochondrial membrane potential ($\Delta\Psi$). RLM were incubated for 15 min in standard medium as indicated in Section 4. Compound **4** was present at the indicated concentrations. TPP^+ (1 μM) was added in the medium for $\Delta\Psi$ measurement. ΔE: electrode potential. Six additional experiments exhibited the same trend.

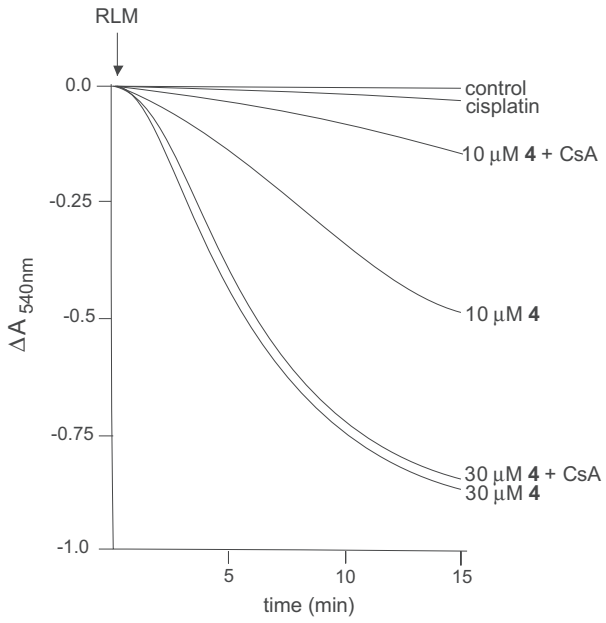


Figure 4. Mitochondrial swelling induced by **4** and cisplatin. Effect of CsA. RLM were incubated for 15 min in standard medium as indicated in Section 4. Compound **4** was present at the indicated concentrations. When present, 1 μM CsA and 10 μM cisplatin. Downward deflection: mitochondrial swelling. Data are representative of six similar experiments.

latter phenomenon causes the dissipation of $\Delta\Psi$, matrix swelling and outer membrane disruption with the consequent release in the cytosol of some proapoptotic factors, such as cyt c and AIF. These factors trigger the caspase-dependent and caspase-independent pathway, respectively, leading to the apoptotic phenotype. In previous studies we indicated for some antiproliferative heterocyclic compounds,[22–24] and for a platinum chloride indenyl complex[12] the capacity to affect mitochondrial functions and in particular, to induce MPT. Interestingly, for all these compounds the MPT appeared as the molecular event mainly responsible for the cytotoxicity.

In this connection, it appeared of interest to investigate the possible effect of **4** on mitochondria and in the first place, to evaluate

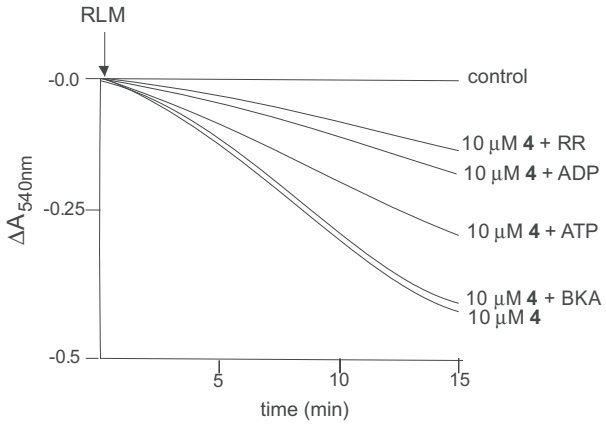


Figure 5. Mitochondrial swelling induced by **4**. Effect of typical MPT inhibitors. Incubation conditions and experimental details as in Figure 4. When present, 10 μM **4**, 1 μM RR, 500 μM ADP, 500 μM ATP, 10 μM BKA. Data are representative of five similar experiments.

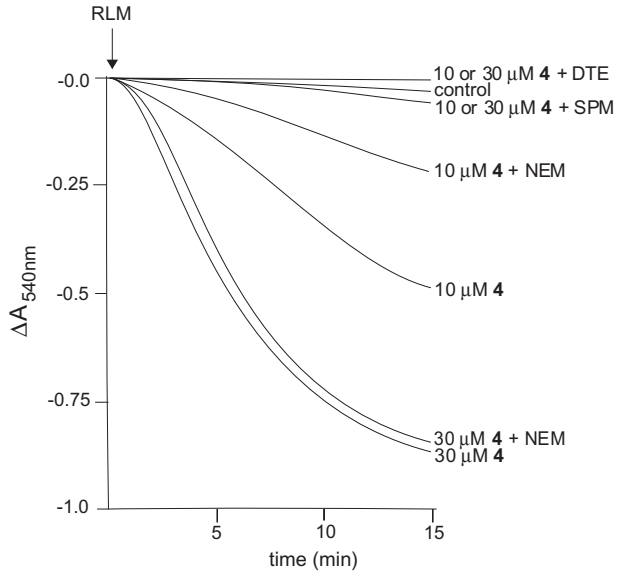


Figure 6. Mitochondrial swelling induced by **4**. Effect of antioxidant agents. Incubation condition and experimental details as in Figure 4. Compound **4** was present at the indicated concentrations. When present, 3 mM DTE, 10 μM NEM, 100 μM SPM. Data are representative of five similar experiments.

the mitochondrial functionality of whole cells. For this purpose, flow cytometry analysis in the presence of the green fluorescent monomeric dye JC-1 was performed. This probe enters selectively into mitochondria driven by mitochondrial membrane potential, negative inside. The uptake increases the concentration of JC-1 in the matrix leading to the formation of aggregates that show high level of red fluorescence emission. In the presence of a compound that affects mitochondria by collapsing mitochondrial transmembrane potential, JC-1 leaks out from the organelles to cytoplasm as monomers, resulting in a decrease of red fluorescence. Figure 2 shows the effect of **4** and cisplatin, taken as reference, on mitochondrial transmembrane potential in HeLa cells. The obtained results highlight for the new complex a noticeable capacity to collapse mitochondrial potential, as assessed by the dose-dependent increase in the percentage of JC-1 monomers in treated cells. Indeed, at 30 μM concentration about 90% of cells treated with **4** shows depolarized mitochondria. Interestingly, in the presence of cisplatin in the same experimental condition, a significant, but considerably lower percentage of cells showing mitochondrial membrane depolarization (about 50%) is observed (Fig. 2).

2.5. Effects on isolated mitochondria

For having a solid validation that the process of cell death induced by **4** takes place following either the extrinsic or intrinsic apoptotic pathway, the effect of this complex on RLM has been studied. In this connection, two fundamental mitochondrial parameters, namely $\Delta\Psi$ and the establishment of mitochondrial swelling, were investigated. In particular, complex **4** at 30 μM concentration completely collapses $\Delta\Psi$ of RLM incubated in standard medium and this effect appears dose-dependent because at 10 μM only a drop in $\Delta\Psi$ of about 15–20 mV occurs (Fig. 3). These results suggest that the complex can act as a protonophore, causing an alteration of the insulating properties of the membrane or, even, an aspecific damage of the membrane. Moreover, **4** causes a dose-dependent decrease of the apparent absorbance of the mitochondrial suspension at 540 nm (Fig. 4). The observed decrease is

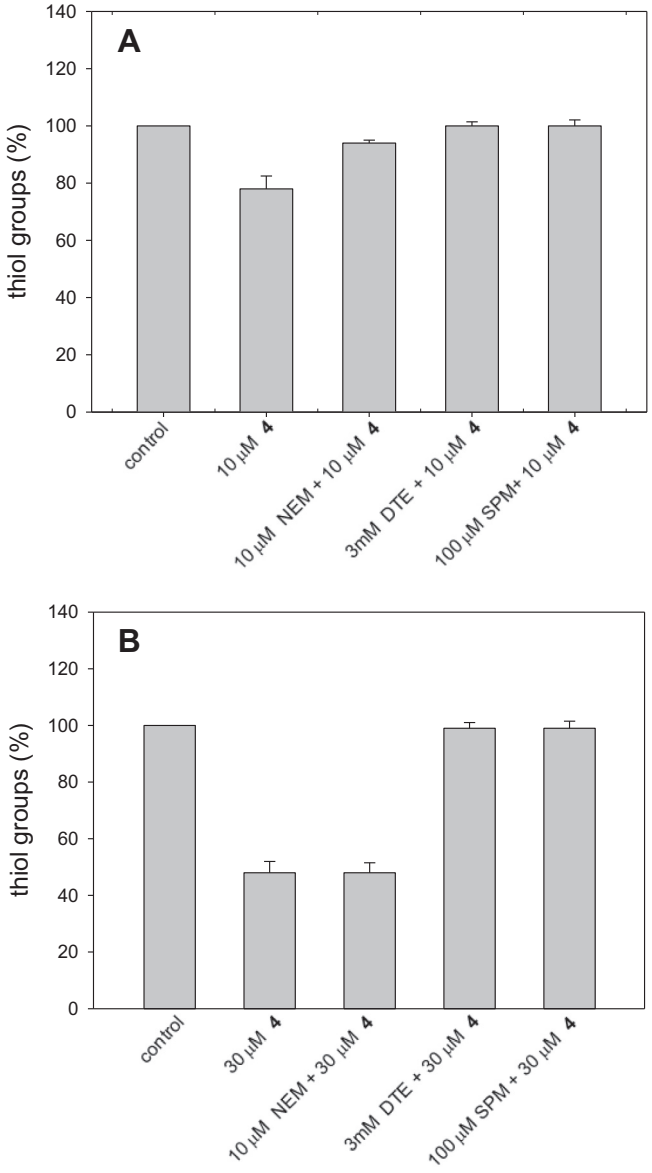


Figure 7. Changes in the redox state of mitochondrial thiol groups induced by **4** at 10 μM (A) or 30 μM (B) concentration. RLM were incubated for 15 min in standard conditions as described in Section 4. When present, 3 mM DTE, 10 μM NEM, 100 μM SPM. Values are mean value of five experiments ± SD.

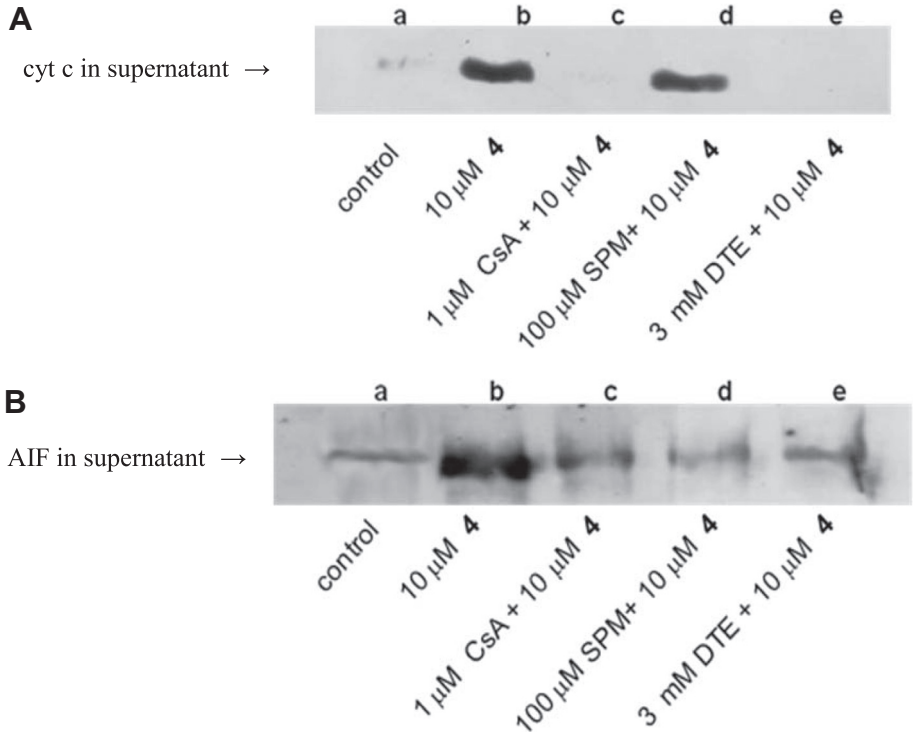


Figure 8. Release of cyt c (A) and AIF (B) induced by **4** at 10 μM concentration. Effect of CsA and antioxidant agents. RLM were incubated for 15 min in standard conditions as indicated in Section 4. Results of Western blotting of supernatant are shown. When present, 10 μM **4**, 1 μM CsA, 100 μM SPM, 3 mM DTE. Three other experiments gave almost identical results.

indicative of a colloid-osmotic phenomenon detectable as a swelling of mitochondria. The observations that **4** causes a $\Delta\Psi$ collapse accompanied by mitochondrial swelling suggest that the complex could be an inducer of MPT phenomenon. It is noteworthy that in the same experimental conditions, the well known drug cisplatin is ineffective (Fig. 4), thus implying a different target from **4**.

2.6. MPT and aspecific membrane permeabilization induction

To further demonstrate the involvement of MPT, RLM treated with the complex were undergone to typical MPT inhibitors. In this regard, the immunosuppressant cyclosporin A (CsA), a specific inhibitor of MPT due to its interaction with cyclophilin D, appears ineffective on mitochondrial swelling induced by 4 at 30 μM, while it exhibits a significant, even if partial, inhibition when 10 μM is used (Fig. 4). Similar results were obtained in the presence of other MPT inhibitors, such as ADP and ATP, while bongkrekic acid (BKA) remains ineffective on both concentrations (Fig. 5, data not shown for 30 μM concentration).

As the phenomenon of MPT is inducible in the presence of Ca^{2+}, and during an oxidative stress,[25] further experiments were performed to elucidate these points.

In particular, the Ca^{2+}-transport inhibitor ruthenium red (RR), exhibits a partial protection on mitochondrial swelling induced by 10 μM **4**, of the same extent as that of CsA (Fig. 5). Because in all the above experiments, Ca^{2+} was not added to the incubation medium, this result proves that the effect of **4** is related to the mitochondrial endogenous Ca^{2+} (about 5 μM) and to the Ca^{2+} present as contaminant in the medium (2–3 μM). This pool of Ca^{2+}, by cycling across the mitochondrial membrane, contributes, most likely together with an oxidative stress, to the induction of MPT and indeed the block of Ca^{2+} cycling by RR prevents, although not completely, the phenomenon (Fig. 5).

In order to determine the involvement of an eventual oxidative stress, the effects of some antioxidant agents were evaluated. In detail, the reductant DTE and the reactive oxygen species (ROS) scavenger, spermine (SPM), completely prevent mitochondrial swelling induced by 10 μM **4**. Otherwise, the alkylant NEM exhibits only a partial protection (Fig. 6). DTE and SPM also completely protect the swelling induced by 30 μM **4**, while, in this case, NEM is ineffective (Fig. 6).

The establishment of an oxidative stress leads to the generation of ROS. The ROS induce the oxidation of critical thiols to disulphide groups on the pore structures, most likely on the adenine nucleotide translocase (AdNT), leading to pore opening. Complex **4** lowers the level of reduced sulphydryl groups of about 20% and 50% at 10 and 30 μM, respectively (Fig. 7A and B), thus indicating their corresponding oxidation. DTE, as well as SPM, completely prevents these oxidations, while NEM exhibits a partial protection against 10 μM **4** (Fig. 7A), while is ineffective with 30 μM **4** (Fig. 7B).

Overall, the partial protection exhibited by CsA and adenine nucleotides, and the ineffectiveness of BKA, indicate that **4** at 10 μM is able to induce both the phenomenon of MPT and, at least in part, an aspecific damage of the membrane. At higher concentration (30 μM) the prominent effect is the aspecific damage, due to the probable formation of leaks, and indeed the MPT inhibitors do not exhibit any effect, apart from the antioxidants DTE and SPM. However, it is to underline that the effectiveness of these antioxidants could come from their capacity to maintain reduced thiols whose oxidation is most likely responsible for leak formation. Concentrations lower than 10 μM are ineffective in inducing any effect (results not reported).

2.7. Release of pro-apoptotic factors

As it is well-known, the induction of MPT is strictly correlated with the triggering of the intrinsic pro-apoptotic pathway (for a review see Susin et al.[26]). To evaluate the possible involvement of **4** in inducing the intrinsic apoptosis it was established if the complex is able to induce the release of some pro-apoptotic factors, such as

cyt c and AIF during MPT. Figure 8A shows the Western Blot analysis on the supernatant of mitochondrial suspension undergone to MPT induction by 10 μM **4**. After 15 min of incubation, cyt c is significantly released (lane b), if compared with the control (lane a), thus suggesting that complex **4** is potentially an intrinsic apoptosis inducer.

It is noteworthy, that in the presence of CsA or DTE, cyt c release is strongly prevented (lanes c and e), while SPM is practically ineffective (lane d). This result, apparently in contrast with the protection exhibited by SPM on thiol oxidation (Fig. 7) and swelling induction (Fig. 6), is explainable, as previously reported,[27] by the electrostatic interaction of the polyamine with the binding sites of cyt c to the inner membrane. This interaction detaches cyt c in the reduced form, as demonstrated by preliminary results, thus permitting its release. This observation permits also to state that, by considering the 'in vivo' conditions, reduced cyt c is not able to form the apoptosome.[28,29]

The results shown in Figure 8B demonstrate that complex **4** induces also a consistent release of AIF (lane b) that is inhibited by CsA (lane c), by SPM (lane d) and by DTE (lane e).

These results permit to state that the characteristic of a potential apoptosis inducer demonstrated by complex **4** is strongly correlated to MPT induction and to an oxidative stress, the mechanism of which, at present, is not known. It is also of large interest that MPT induction by complex **4** does not require addition of exogenous Ca^{2+} but utilizes only the endogenous amount of the cation. This would mean that the complex is able to enhance the affinity of Ca^{2+} for its critical binding sites.

3. Conclusions

The synthesis of some bis-alkyl-amino transplatinum complexes carrying the hydrophobic phosphine ligand is described. The evaluation of the antiproliferative activity showed an appreciable cytotoxicity for all complexes and, interestingly, for **4** the GI_{50} values are even comparable to those of cisplatin on the three cell lines taken into consideration. The investigation on the cellular mechanism of action accountable for cytotoxicity suggests the involvement of mitochondria. Indeed, **4** at lower concentration (10 μM) induces mainly the MPT phenomenon. The opening of the mitochondrial transition pore and the release of proapoptotic factors triggers the instrinsic apoptotic pathway, thus leading to cell death. Nevertheless, a contribution to the mitochondrial damage comes also from an aspecific membrane permeabilization, likely due to the formation of leaks. This latter effect, clearly detectable at higher concentration (30 μM), could be ascribed to the high hydrophobic phosphine ligand.

The peculiarity of the mechanism of action of **4**, along with the differences in behavior with respect to cisplatin, renders these complexes a class of platinum-based compounds worthy to be further developed and studied. Indeed, this feature could allow the attainment of new drugs able to overcome the shortcomings of the current platinum-based drugs.

4. Experimental section

4.1. General experimental conditions

All manipulations were performed under a dinitrogen atmosphere, if not otherwise stated. Solvents and liquid reagents were dried according to reported procedures.[30]

^{1}H, ^{13}C, ^{31}P and ^{195}Pt NMR spectra were recorded with a Bruker 'Avance DRX400' spectrometer, in $CDCl_3$ solution if not otherwise stated. Chemical shifts were measured in ppm (δ) from TMS by residual solvent peaks for ^{1}H and ^{13}C, from aqueous (D_2O) H_3PO_4 (85%) for ^{31}P and from aqueous (D_2O) hexachloroplatinic acid for ^{195}Pt. A sealed capillary containing C_6D_6 was introduced in the NMR tube to lock the spectrometer to the deuterium signal when non-deuterated solvents were used. FTIR spectra in solid phase were recorded with a Perkin–Elmer 'Spectrum One' spectrometer, equipped with an ATR accessory. Elemental analyses (C, H, N) were performed at Dipartimento di Scienze e Tecnologie Chimiche, Università di Udine. *cis*-[$PtCl_2$(NCMe)(PPh_3)] (**1**) was prepared according to a reported procedure.[18]

4.2. Synthesis of *trans*-[$PtCl_2(PPh_3)(R_2NH)$] (2–4)

In a round-bottomed flask equipped with magnetic stirrer, condenser and dropping funnel, a suspension of *cis*-[$PtCl_2(CH_3CN)(PPh_3)$] (**1**) in 10 mL of CH_3CN was refluxed (83 °C) until an homogeneous solution was obtained. The mixture was then treated with a solution containing the secondary amine R_2NH in 2.0 mL of the same solvent (*cis*-[$PtCl_2(CH_3CN)(PPh_3)$]/[R_2NH] = 1/1.2 molar ratio). The progress of the reaction was followed by ^{31}P NMR spectroscopy until the complete conversion of the precursor into the desired product was observed (3 h). Solvent was eliminated under vacuum (0.01 mmHg), the residue was dissolved in 1,2-DCE (5 mL) and treated with heptane (10 mL). Solid products precipitated were recovered by filtration and dried under vacuum (0.01 mmHg).

For each of the *trans*-[$PtCl_2(PPh_3)(R_2NH)$] complexes obtained the amine used, the isolated % yield and the spectroscopic characterization are indicated.

4.2.1. Synthesis of *trans*-[$PtCl_2(PPh_3)(Et_2NH)$] (2)[19]

[19]Diethylamine; 0.2617 g (60%). Anal. Calcd for $C_{22}H_{26}Cl_2NPPt$: C, 43.90; H, 4.40; N, 2.30. Found: C, 43.90; H, 4.40; N, 2.40. Spectroscopic data were in good agreement with previously obtained results.

4.2.2. Synthesis of *trans*-[$PtCl_2(PPh_3)(Bz_2NH)$](3)

Dibenzylamine; 0.4148 g (94%). Anal. Calcd for $C_{32}H_{30}Cl_2NPPt$: C, 52.97; H, 4.17; N, 1.93. Found: C, 52.90; H, 4.20; N, 1.80. I.R. (ATR): 3204 cm^{-1}. ^{1}H NMR: 7.50–7.29 (m, 25H, H_{arom}); 4.50–4.45 (m, 2H, NHC<u>H</u>HPh); 4.35–4.30 (m, 1H, NH); 3.93–3.86 (m, 2H, NHCH<u>H</u>Ph). ^{13}C NMR: 135.8, 134.8 (d, J = 9 Hz), 130.7, 130.2, 128.8, 128.8 (d, J = 64 Hz), 128.3, 127.8 (d, J = 10 Hz), 54.8. ^{31}P NMR: 4.13 ppm ($^{1}J_{P-Pt}$ = 3633 Hz). ^{195}Pt NMR −3631 ppm (d, $^{1}J_{Pt-P}$ = 3633 Hz).

4.2.3. Synthesis of *trans*-[$PtCl_2(PPh_3)\{(HOCH_2CH_2)_2NH\}$](4)

Diethanolamine [$(HOCH_2CH_2)_2NH$]; 0.0939 g (45%). Anal. Calcd for $C_{22}H_{26}Cl_2NO_2PPt$: C, 41.72; H, 4.14; N, 2.21. Found: C, 41.80; H, 4.20; N, 3.10. I.R. (ATR): 3227 cm^{-1}. ^{1}H NMR: 7.67–7.38 (m, 15H, H_{arom}); 4.80 (m, 2H); 4.62 (br s, 1H, N*H*); 3.85 (m, 2H); 3.72 (br s, 2H); 3.34 (m, 2H); 2.85 (m, 2H). ^{13}C NMR: 134.6 (d, J = 8 Hz), 131.6 (d, J = 40 Hz), 131.1, 128.1 (d, J = 10 Hz), 60.4, 54.5. ^{31}P NMR: 4.33 ($^{1}J_{P-Pt}$ = 3532 Hz) ^{195}Pt NMR: −3605 ppm ($^{1}J_{Pt-P}$ = 3532 Hz).

4.3. Inhibition growth assay

HeLa (human cervix adenocarcinoma cells), H460 (large cell lung carcinoma) and A549 (non-small cell lung cancer) were grown in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.), RPMI 1640 (Sigma Chemical Co.) supplemented with 2.38 g/L Hepes, 0.11 g/L pyruvate sodium, 2.5 g/L glucose and Nutrient Mixture F-12-K (Sigma Chemical Co.), respectively. 10% Heat-inactivated fetal calf serum (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma Chemical Co.) were added to the media. The cells were cultured at 37 °C in a moist

atmosphere of 5% carbon dioxide in air. H460 and A549 cells (3–4 × 10^4) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added to the complete medium and incubated for a further 72 h. HeLa (3–4 × 10^4) cells were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of fresh medium, and various concentrations of the test agents were added. The cells were then incubated in standard conditions for a further 72 h. A Trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as GI_{50} values, that is, the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

4.4. Evaluation of apoptotic cell death by Annexin V-FITC and propidium iodide staining

To detect phosphatidylserine translocation from the inner face to the outer surface of plasma membrane a FITC Annexin V Apoptosis Detection Kit I (BD Pharmigen) was used.

HeLa cells (2.5 × 10^5) were seeded into each cell culture plate. After incubation for 24 h the test agents were added to the complete medium at the indicated concentrations and cells were incubated for a further 18 h. After treatment, cells were centrifuged and resuspended at 10^6 cells/mL in binding buffer. Cell suspensions (100 μL) were added with Annexin V-FITC and propidium iodide (PI) as indicated by the supplier's instructions, and incubated for 15 min at room temperature in the dark. The populations of Annexin V-positive/PI-negative cells (early apoptosis) and Annexin V-positive/PI-positive cells (late apoptosis) were evaluated by FACSCanto II flow cytometer (Becton–Dickinson, Mountain View, CA).[31] The reported values are the mean of four independent experiments.

4.5. Determination of mitochondrial membrane potential on whole cells

The mitochondrial membrane potential was evaluated by using the BD™ MitoScreen Kit (BD Pharmigen) containing the membrane-permeable lipophilic cationic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), according to Cossarizza et al.[32] HeLa cells (2.5 × 10^5) were seeded into each cell culture plate. After incubation for 24 h the test agents were added to the complete medium at the indicated concentrations and cells were incubated for a further 18 h. After treatment, cells were centrifuged, resuspended in JC-1 Working Solution and incubated for 30 min at 37 °C in CO_2 incubator. Following incubation, cells are washed twice, resuspended in Assay Buffer and analyzed by a FACSCanto II flow cytometer (Becton–Dickinson, Mountain View, CA). Results are presented as percentage of cells with depolarized mitochondrial membrane (JC-1 monomers) and the reported values are the mean of four independent experiments.

4.6. Mitochondrial isolation and standard incubation procedures

Rat liver mitochondria were isolated by conventional differential centrifugation in a buffer containing 250 mM sucrose, 5 mM Hepes (pH 7.4), and 1 mM EGTA.[33] EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with BSA as a standard.[34]

Mitochondria (1 mg protein/mL) were incubated in a water jacketed cell at 20 °C. The standard medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 1.25 μM rotenone (Sigma–Aldrich), and 1 mM sodium phosphate. Variations and/or other additions are given with each experiment.

4.7. Determination of mitochondrial functions

$\Delta\Psi$ was calculated on the basis of distribution of the lipid-soluble cation tetraphenylphosphonium (TPP^+) measured across the inner membrane using a TPP^+-selective electrode.[35] The redox state of endogenous pyridine nucleotides was followed fluorometrically in a Shimadzu RF-5000 spectrofluorometer, with excitation at 354 nm and emission at 462 nm.

Mitochondrial swelling was determined by measuring the apparent absorbance change of mitochondrial suspensions at 540 nm in a Kontron Uvikon model 922 spectrophotometer equipped with thermostatic control.

The protein sulfydryl oxidation assay was performed as in Santos et al.[36]

4.8. Detection of cyt c and AIF release

The mitochondria (1 mg protein/mL) were incubated for 15 min at 20 °C in standard medium with the appropriate additions. The reaction mixtures were then centrifuged at 13,000*g* for 10 min at 4 °C to obtain mitochondrial pellets. The supernatant fractions were concentrated using a PAGEprep protein cleanup and enrichment kit (Pierce, Rockford, IL, USA). Aliquots of 20 μL of the concentrated supernatants were subjected to 15% and 10% SDS–PAGE for cyt c and AIF, respectively, and analyzed by Western blotting using mouse anti-cyt c and rabbit anti-AIF antibodies (Pharmingen, San Diego, CA, USA).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.09.025.

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