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New platinum(II) complexes containing both an O,O′-chelated acetylacetonate ligand and a sulfur ligand in the platinum coordination sphere induce apoptosis in HeLa cervical carcinoma cells

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ABSTRACT

We report the cytotoxic effects obtained in HeLa cells of three newly synthesized platinum complexes containing both an O,O′-chelated acetylacetonate ligand and a sulfur ligand in the platinum coordination sphere, which show, by ^{1}H NMR, negligible reactivity with purine bases. These compounds induce cell death with [Pt(O,O′-acac)(γ-acac)(DMS)] being the most effective (IC_{50} = 0.98 ± 0.056 and 1.82 ± 0.023 μM for [Pt(O,O′-acac)(γ-acac)(DMS)] and *cisplatin*, respectively). About 50% of cells died after 5 h treatment with 100 μM [Pt(O,O′-acac)(γ-acac)(DMS)] whilst a 16 h incubation was required to get the same results using 100 μM *cisplatin*. Cellular accumulation measurements, after treatment with equimolar drug concentrations, indicated the major lipophilicity and cellular uptake of the new compounds. While the cytotoxicity of *cisplatin* was due to both intracellular accumulation and DNA binding, that of [Pt(O,O′-acac)(γ-acac)(DMS)] was associated with intracellular Pt accumulation only, since it has low reactivity to DNA in intact cells and *in vitro*. The reaction of the new complexes with guanosine and 5′-GMP was negligible, whereas the L-methionine instantly reacted with the initial Pt complexes. Both *cisplatin* and [Pt(O,O′-acac)(γ-acac)(DMS)] induced apoptosis in HeLa cells. [Pt(O,O′-acac)(γ-acac)(DMS)] provoked the early signs of apoptosis induction (cleavage of PARP and activation of caspases-9, -3 and -7) only 1 h after addition of the drug. However, in *cisplatin*-treated cells, cleavage of PARP was seen after 9 h with activation of caspases also proceeding more slowly. In conclusion, these results indicate that the newly synthesized platinum(II) complexes have high and rapid cytotoxic activity *in vitro*, and suggest that DNA may not be their primary target.

1. Introduction

Cisplatin (*cis*-[$Pt^{II}Cl_2(NH_3)_2$]) is one of the most potent anticancer agents, displaying significant clinical activity against a variety of solid tumors. Unfortunately, the development of resistance is a hurdle in the use of this drug. The molecular mechanisms that underlie this chemoresistance are largely unknown. Possible mechanisms of acquired resistance to *cisplatin* include reduced intracellular accumulation of *cisplatin*, enhanced drug inactivation by metallothionine

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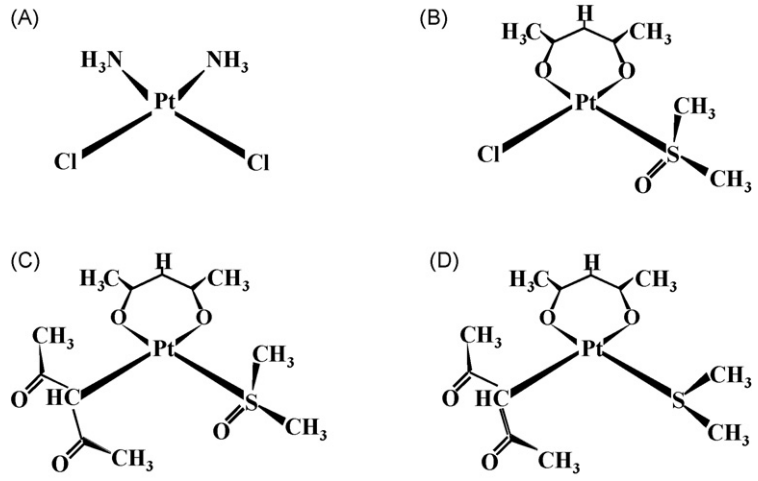

Fig. 1 – Chemical structures of $[PtCl_2(NH_3)_2]$ *cisplatin* (A), [PtCl(O,O′-acac)(DMSO)]] (B), [Pt(O,O′-acac)(γ-acac)(DMSO) (C) and [Pt(O,O′-acac)(γ-acac)(DMS)] (D).

and glutathione, increased repair activity of DNA damage, and altered expression of oncogenes and regulatory proteins [1–3]. It is generally accepted that cytotoxicity of *cisplatin* is mediated through induction of apoptosis and arrest of the cell cycle resulting from its interaction with DNA, such as the formation of *cisplatin*–DNA adducts, which activates multiple signaling pathways, including those involving p53, members of the Bcl-2 family, caspases, cyclins, cyclin-dependent kinases (CDKs), pRb, PKC, MAPK and PI3K/Akt [4]. Increased expression of anti-apoptotic genes and mutations in the intrinsic apoptotic pathway may contribute to the inability of cells to detect DNA damage or to induce apoptosis [4].

For a long time there has been a keen interest in obtaining agents that have less toxicity and more favourable therapeutic indices than *cisplatin*. In this context, with the aim to find platinum-based derivatives with higher anti-tumor activity and overcoming resistance of many tumor types, we reported recently the synthesis of new β-diketonate complexes [5]. These are novel platinum(II) complexes containing both acetylacetonate (acac) and dimethylsulfoxide (DMSO) in the coordination sphere. More precisely, [PtCl(*O*,*O*′-acac)(DMSO)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] contain a single chelated acetylacetonate (*O*,*O*′-acac), and one chelated and one σ-bonded (γ-acac) respectively. In the presence of dimethylsulfide (DMS), these platinum(II) complexes selectively undergo substitution of the sulfur ligand to give the analogous complexes [PtCl(*O*,*O*′-acac)(DMS)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] (see Sections 2.3 and 2.4).

The water solubility of the new platinum(II) complexes ([PtCl(*O*,*O*′-acac)(DMSO)], [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] (Fig. 1) allowed us to characterise their ability to produce cytotoxicity in human cervical carcinoma HeLa cells. However, [PtCl(*O*,*O*′-acac)(DMS)] was only sparingly water soluble and was therefore not used. In this paper we also compared the efficacy of these compounds to their ''parent'' Pt(II)-analogue and the established anticancer drug, *cisplatin*. In addition, we also show the mechanism underlying their activity in HeLa cells.

2. Materials and methods

2.1. Physical measurements

^{1}H NMR spectra were recorded on a Bruker Avance DPX 400, using CD_3OD, $CDCl_3$, and D_2O as solvent. Chemical shifts in CD_3OD and $CDCl_3$ were referred to TMS by using the residual protic solvent peaks as internal references; chemical shifts in D_2O were referenced to TSP (2,2,3,3-*d*(4)-3-(trimethyl-silyl)-propionic acid sodium salt), δ(H) = 0 ppm, as an external reference. Microanalyses were performed with Carlo Erba Elemental Analyser Mod. 1106 instrument.

2.2. Starting materials

Commercial reagent grade dimethysulfoxide (DMSO) and dimethylsulfide (DMS) were supplied by Aldrich. Complexes, [PtCl(*O*,*O*′-acac)(DMSO)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)], were synthesized according to the previously reported procedures [5].

2.3. Synthesis of [PtCl(O,O′-acac)(DMS)]

To a solution of [PtCl(*O*,*O*′-acac)(DMSO)] (0.1 g, 0.24 mmol) in chloroform (3 mL) an excess of DMS (0.149 g, 2.24 mmol) was added and the reaction was left to stir at room temperature, overnight. The resulting yellow solution was added of pentane (10 mL) and kept at 5 °C overnight. The crystals of [PtCl(*O*,*O*′-acac)(DMS)] that formed were isolated as yellow needles, washed with pentane, and dried under vacuum (yield 0.73 g, 78%). Anal.Calcd for $C_7H_{13}ClO_2PtS$(391.773): C 21.46; H 3.34. Found: C 21.27; H 3.20. ^{1}H NMR in ($CDCl_3$, 298 K): δ 1.97s [3H, CH_3(*O*,*O*′-acac)], 1.88s [3H, CH_3(*O*,*O*′-acac)], 2.33s [1H, CH(*O*,*O*′-acac)], 2.33s [6H, CH_3(DMS), $^3J_{H-Pt}$ 46 Hz]. Due to its low water solubility, this compound was not tested.

2.4. Synthesis of [Pt(O,O′-acac)(γ-acac)(DMS)]

To a solution of [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] (0.1 g, 0.21 mmol) in chloroform (3 mL) an excess of DMS (0.130 g, 2.10 mmol) was

added and the mixture was left to stir at room temperature overnight. The resulting pale yellow solution was mixed with pentane (10 mL) and kept at 5 °C overnight. The pale yellow crystals of [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] that formed were isolated, washed with pentane, and dried under vacuum (yield 0.070 g, 73%). Anal. Calcd for $C_7H_{13}ClO_2PtS$(455.428): C 31.65; H 4.43. Found: C 31.72; H 4.56. ^{1}H NMR in ($CDCl_3$, 298 K): δ 1.90s [3H, CH_3(*O*,*O*′-acac)], 1.95s [3H, CH_3(*O*,*O*′-acac)], 2.21s [6H, CH_3(γ-acac)], 2.29s [6H, CH_3(DMS), $^3J_{H–Pt}$ 48 Hz], 4.88s [1H, CH(γ-acac), $^2J_{H–Pt}$ 125 Hz], 5.47s [1H, CH(*O*,*O*′-acac)].

2.5. Reaction of [PtCl(*O*,*O*′-acac)(DMSO)], [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] with guanosine (Guo), 5′-GMP and L-methionine

A solution containing the platinum complex (approximately 2×10^{-3} mmol) and an excess of Guo, 5′-GMP or L-methionine (1.6×10^{-2} mmol) dissolved in D_2O (1 mL), was placed in an NMR tube and the reaction monitored by ^{1}H NMR spectroscopy. For all complexes tested, the reaction with Guo and 5′-GMP was negligible after 24 h, whereas the L-methionine instantly reacted with the initial Pt complexes.

2.6. Compound stability

The overall loss of the water soluble platinum compounds under investigation ([PtCl(*O*,*O*′-acac)(DMSO)], [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMS)]) was measured after 24, 48 and 72 h (evaluated by ^{1}H NMR monitoring) and was always found to be negligible in both D_2O, and the solutions used in cell culture (0.1% NaCl, phosphate-buffered saline (PBS) and Dulbecco's modified eagle medium (DMEM) with and without a physiological concentration of albumin (40 g/L) or 10% foetal bovine serum).

2.7. Cell culture

The human cervical carcinoma cell line (HeLa), was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum. Cells were grown to 70% confluence and then treated with Pt compounds at various concentrations and for different incubation periods.

2.8. Cytotoxicity assay

Cells at 70–80% confluency were trypsinised (0.25% trypsin with 1 mM EDTA), washed and resuspended in growth medium. One hundred microlitres of a cell suspension (10^4 cells/mL) was added to each well of a 96-well plate. After overnight incubation, cells were treated with specific reagents for 24–72 h.

The conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide) by cells was used as an indicator of cell number as described by Mosmann [6], with some modification [7]. This method measures the reduction of MTT by active mitochondria, which results in a colorimetric change measured at OD 550 nm. Experiments were performed to define the linear range of the assay. A good correlation was observed up to 50,000 cells per well (data not shown). Increasing concentrations of heat inactivated cells per well (killed by incubating at 70 °C for 15 min) caused no significant change in the absorbance; thus, this spectrophotometric method was shown to be a valid technique for measuring the number of viable cells. All subsequent experiments performed were within the linear range of the assay.

The percentage cell survival was calculated as the absorbance ratio of treated to untreated cells. The data presented are means ± standard deviation (S.D.) from eight replicate wells per microtitre plate, repeated four times.

2.9. Sulforhodamine B (SRB) assay

The SRB assay was carried out as previously described [8]. Briefly, 70 μL 0.4% (w/v) sulforhodamine B in 1% acetic acid solution was added to each well and left at room temperature for 20 min. SRB was removed and the plates washed five times with 1% acetic acid before air drying. Bound SRB was solubilized with 200 μL of 10 mM unbuffered Tris-base solution and plates were left on a plate shaker for at least 10 min. Absorbance was measured in a 96-well plate reader at 492 nm. The test optical density (OD) value was defined as the absorbance of each individual well, minus the blank value ('blank' is the mean optical density of the background control wells, $n = 8$). The percentage survival was calculated as the absorbance ratio of treated to untreated cells. The data presented are means ± standard deviation (S.D.) from eight replicate wells per microtitre plate, repeated four times.

2.10. Cell count and floating cell quantification

The cells were seeded into 60 mm tissue culture dishes (100,000 cells/mL). After overnight incubation, the cells were treated with the concentrations of *cisplatin* or [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] which were found to produce an IC_{50} and IC_{90}, for 12, 24, 48, and 72 h. Cell viability was estimated using the trypan blue exclusion assay and light microscopy. Adherent cells and cells in suspension were counted separately, and the estimate of cells in suspension was expressed as the percentage of the total cell number.

2.11. Preparation of cytosolic and nuclear extracts

To obtain protein cell extracts, cells were washed twice in ice-cold PBS and harvested in 1 mL of PBS. The samples were centrifuged for 30 s at 10,000 × *g*, and cell pellets were resuspended in the following buffer (mM): 20 Tris–HCl, pH 8, containing 420 NaCl, 2 EDTA, 2 Na_3VO_4, and 0.2% Nonidet P-40 10% glycerol, supplemented with a cocktail of protease inhibitors. After a 10 min incubation on ice, cells were passed several times through a 20 gauge syringe and then centrifuged at 13,000 × *g* for 10 min at 4 °C. Other samples were centrifuged (200,000 × *g*) for 20 min at 4 °C. The resultant supernatant is referred to as the cytosolic fraction.

Nuclei were pelleted by centrifugation at 2,000 × *g* for 15 min at 4 °C and resuspended in high salt buffer (20 mM Tris–HCl, pH 7.9, 420 mM NaCl, 10 mM KCl, 0.1 mM NaVO4, 1 mM EDTA, 1 mM EGTA, 20% glycerol, supplemented with a cocktail of protease inhibitors) and sonicated until no nuclei remained intact. Samples were then centrifuged at 13,000 × *g* for 10 min (4 °C), and the resultant supernatant was used as the nuclear

extract. The integrity of the cytosolic and nuclear fractions was assessed using G-6-PDH activity as a specific marker for the cytosol and NucP62 was used to evaluate the degree of enrichment and/or contamination of nuclear components in these different fractions.

2.12. DNA extraction

After drug incubation, cells were washed with PBS and the and lysed in a buffer containing 150 mM Tris–HCl, pH 8.0, 100 mM EDTA, and 100 mM NaCl, incubated for 15 min at 4 °C and centrifuged at 18,000 × *g* for 15 min. Supernatants were treated for 3 h at 37 °C with 20 μg/mL of proteinase K (Sigma, Milano) and then further incubated for 16 h at 37 °C with 4 μL of RNase A of 100 μg/mL (Sigma, Milano). Finally, DNA was extracted with a volume of phenol/chloroform/isoamyl alcohol (50:49:1), precipitated with 2.5 volumes of cold ethanol, and 0.1 volumes of 3 M sodium acetate, washed with 75% of ethanol, dried, and then resuspended in 1 mL of water. The DNA content in each sample was measured by UV spectrophotometry at 260 nm.

2.13. Platinum determination

To determine total platinum content, cells were washed twice in 0.9% NaCl solution, after counting viable cells for determination of cell survival, and then digested in 0.5 mL of 65% nitric acid at 37 °C for at least 2 days to obtain a clear solution.

To determine platinum content in distinct subcellular fractions, cytosolic, nuclear and DNA extracts were digested in 65% nitric acid solution by incubation at 37 °C for at least 2 days to obtain a clear solution.

Platinum content in the samples was determined by atomic absorption spectrometry on a Varian SpectrAA-880Z spectrometer (Varian, Inc. Vacuum Technologies, CA, USA). The reproducibility of measurements, linearity of calibration curve, and low limit of detection demonstrated in preliminary studies proved that this method is both sensitive and accurate and therefore suitable for determination of platinum content in cells. All measurements were performed in triplicate and included three quality controls according to standard operating procedures. The cellular Pt levels were expressed as ng of Pt/mg of protein or as pg of Pt/μg of DNA. The data were obtained from four independent experiments using duplicate cultures.

2.14. Nucleolar staining

Nucleolar RNA was stained with toluidine blue at pH 5 without previous fixation to visualise the RNA-containing structures. All chemicals were purchased from Sigma (USA).

2.15. Apoptosis analysis

For 4,6-diammine-2-phenylindol (DAPI) staining, cells treated with *cisplatin* or [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] were fixed with 3% formalin and stained with 1 mg/mL DAPI in PBS for 10 min. Cells were mounted on glass slides, covered, and analysed using fluorescence microscopy. For statistical analysis of each experiment, 5–10 fields (magnification 400×) were counted (between 400 and 700 cells in total). The mean ± S.D. was calculated and displayed as a bar graph.

2.16. Western blot analysis

Proteins in homogenates and cellular fractions were determined using the Bio-Rad protein assay kit 1 (Milan, Italy). Lyophilised bovine serum albumin was used as a standard.

Total cell proteins or proteins of the distinct subcellular fractions were dissolved in SDS sample buffer and separated on 10% or 15% SDS gels. Separated proteins were transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF) (Amersham International). Equal protein loading was confirmed by Ponceau S staining. Blots were incubated with specific primary antibodies and the immune complexes were detected using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescent detection reagent ECL (Amersham International). In some cases, blots were stripped and used for several sequential incubations.

2.17. Statistical analysis

Experimental points represent means ± standard deviation (S.D.) of three to six replicates. Statistical analysis was carried out using ANOVA. When indicated, post hoc tests (Bonferroni/Dunn) were also performed. A p value less than 0.05 was considered to indicated statistical significance.

3. Results

3.1. Synthesis of platinum complexes

[PtCl(*O*,*O*′-acac)(DMSO)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] containing a single chelate acetylacetonate (*O*,*O*′-acac), and one chelated and one σ-bonded (γ-acac), respectively, were readily synthesised using methods previously described [5]. In the presence of dimethylsulfide (DMS), these platinum(II) DMSO complexes selectively undergo substitution of the sulfur ligand to give the analogous DMS complexes [PtCl(*O*,*O*′-acac)(DMS)] and [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] (see Sections 2.3 and 2.4). Interestingly, the substitution reaction appears to be very selective not only for [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)], where DMSO is the only expected exchangeable ligand, but also for [PtCl(*O*,*O*′-acac)(DMSO)], where, in principle, the chloro ligand was also able to undergo substitution with DMS.

Other reactions with soft biological nucleofiles, such as L-methionine, rapidly give the same selective substitution of DMSO (Fig. 2A and B). Moreover, in the reactions with biological nitrogen ligands (L), such as purines (Guo, 5′-GMP), both PtCl(*O*,*O*′-acac)(DMSO) and [Pt(*O*,*O*′-acac)(γ-acac)(DMSO)] show little reactivity even after several hours incubation (Fig. 2A′ and B′). The same selective reactivity in the substitution of the soft sulfur ligand is observed in the reaction of the water soluble [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] with L-methionine, Guo, and 5′-GMP. The poor water solubility of [PtCl(*O*,*O*′-acac) (DMS)] prevents further investigation on its reactivity and biological behaviour.

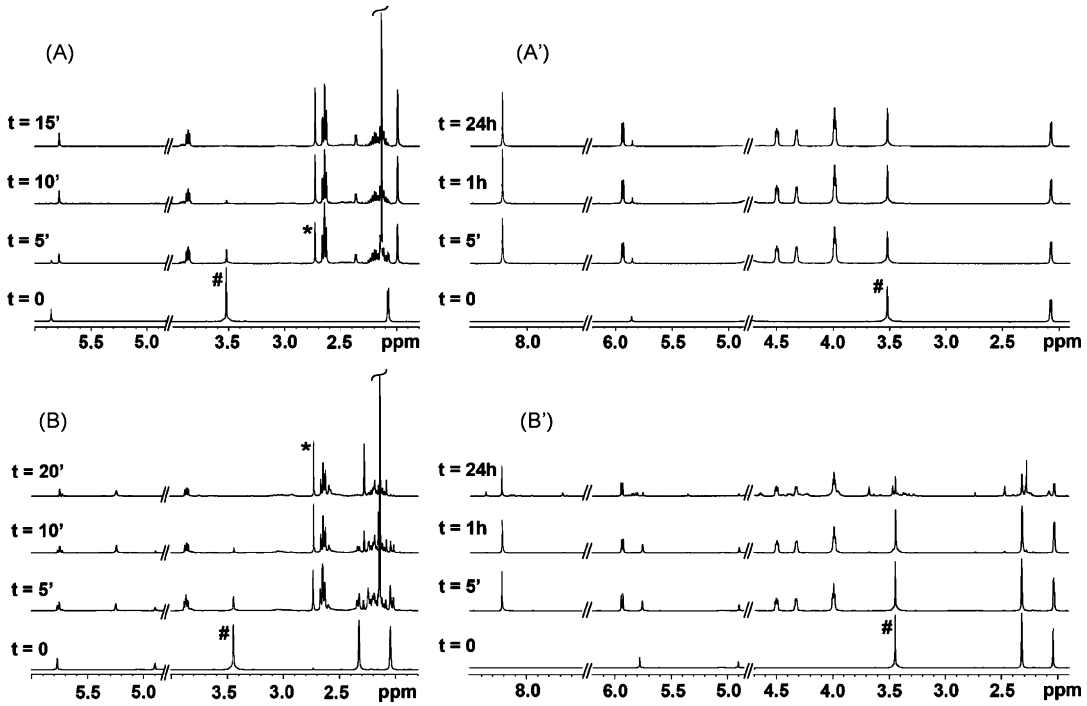

Fig. 2 – ^{1}H NMR spectra in D_2O (400.13 MHz, standard TSP) of [PtCl(O,O′-acac)(DMSO)] (A and A′) and [Pt(O,O′-acac)(γ-acac)(DMSO)] (B and B′) with excess of L-methionine (A and B) and 5′-GMP (A′ and B′) at different reaction times. For both complexes rapid reaction with methionine (decreasing coordinated (#) and increasing free DMSO (*) signals) and negligible or very slow reaction with 5′-GMP was observed.

Such behaviour is very peculiar and suggests a possible selectivity in the substitution reaction at the metal centre in these systems ruled by the hard–soft characteristics of the leaving and incoming ligands. This selectivity could be also operating when the substitution at the metal involves biological sulfur ligands such as thiols or thioethers attached to proteins.

3.2. *Cytotoxicity of the drugs*

The cytotoxicity data shown here were obtained by MTT metabolic assay and confirmed by SRB assay to rule out potential effects of Pt compounds on mitochondrial enzymes. Indeed, comparable results were obtained when cell number was directly determined by cell counting.

Exposure of the HeLa cells to the Pt compounds at concentrations ranging from 1 to 200 μM resulted in dose-dependent inhibition of cell survival (MTT assay) (Fig. 2). IC_{50} and IC_{90} values of Pt(II)-analogues after 72 h of treatment are presented in Table 1.

Table 1 – The IC_{50} and IC_{90} values (concentration required for 50% and 90% growth inhibition, respectively) after 72 h *cisplatin* and new Pt-compounds exposure are indicated

Pt-compounds	IC_{50} (μM)	IC_{90} (μM)
Cisplatin	1.82 ± 0.023	57.84 ± 0. 65
[Pt(O,O′-acac)(γ-acac)(DMSO)]	4.21 ± 0.5	n.d.
[PtCI(O,O′-acac)(DMSO)]	1.22 ± 0.09	73.85 ± 1.0
[Pt(O,O′-acac)(γ-acac)(DMS)]	0.98 ± 0.056	36.12 ± 2.1

[Pt(O,O′-acac)(γ-acac)(DMS)] (IC_{50} 0.98 ± 0.056 μM) and [PtCl(O,O′-acac)(DMSO)] (IC_{50} 1.22 ± 0.09 μM) showed potencies approximately 2-fold and 1.5-fold greater than those observed for *cisplatin* (IC_{50} 1.82 ± 0.023 μM). Conversely, [Pt(O,O′-acac)(γ-acac)(DMSO)] displayed a significantly lower cytotoxicity than the other drugs (IC_{50} 4.21 ± 0.5 μM).

Interestingly, about 50% of cells died after only 5 h treatment with 100 μM [Pt(O,O′-acac)(γ-acac)(DMS)] while it was necessary to incubate for 10–16 h to get the same effect using 100 μM [PtCl(O,O′-acac)(DMSO)] and 100 μM *cisplatin*, respectively (Fig. 3).

IC_{50} and IC_{90} concentrations of [Pt(O,O′-acac)(γ-acac)(DMS)] and *cisplatin* caused a time- and dose-dependent decrease in cell number with a parallel increase in the percentage of floating cells (data not shown).

3.3. *Accumulation of new platinum(II) compounds and cisplatin in HeLa cells*

As has previously been reported for *cisplatin* [9], cellular accumulation measurements showed that the novel Pt(II) compounds accumulated in a linear fashion that was correlated with the drug concentration up to the highest concentration tested of 100 μM. This indicates that there is no saturation of the uptake of either drug at concentrations in this range. After treatment with equimolar drug concentrations the rate of platinum accumulation was [Pt(O,O′-acac)(γ-acac)(DMSO)] > [Pt(O,O′-acac)(γ-acac)(DMS)] > [PtCl(O,O′-acac)(DMSO)] > *cisplatin* (Fig. 3B). The kinetic of [Pt(O,O′-acac)(γ-acac)(DMS)] uptake was different compared to *cisplatin*;

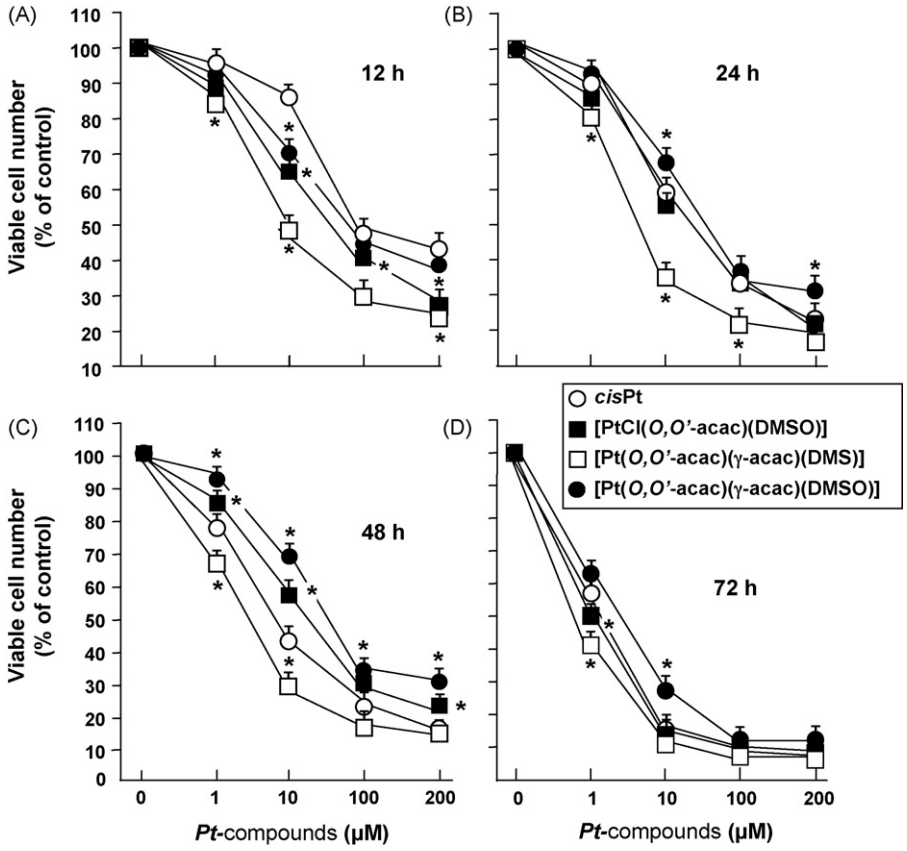

Fig. 3 – The sensitivity of HeLa cells to Platinum(II) complexes. Cells were treated with and without increasing concentrations of *cisplatin* and novel Pt-compounds; viable cell number was determined 12 h (A), 24 h (B), 48 h (C) and 72 h (D) later by MTT assay. The data are means ± S.D. of four different experiments run in eight replicates and are presented as percent of control. Asterisks indicate values that are significantly different ($p < 0.05$) than *cisplatin* alone at the same concentration and the same time point.

[Pt(O,O′-acac)(γ-acac)(DMS)] concentration in HeLa cells increased rapidly and reached the maximum level after 6 h, at which point its cellular accumulation was approximately six times higher than that of *cisplatin* ($p < 0.001$) (Fig. 4B).

After 24 h incubation of HeLa cells with 100 μM *cisplatin* the total platinum content was 1.12 ± 0.07 μg Pt/mg protein; an equivalent Pt cellular content was obtained by using 17 μM [Pt(O,O′-acac)((-acac)(DMS)]. These concentrations (100 and 17 μM) caused a similar time-dependent Pt uptake by whole cells for both *cisplatin* and [Pt(O,O′-acac)((-acac)(DMS)] (Fig. 5A and B). However, the Pt content in DNA bases was considerably higher for *cisplatin* than [Pt(O,O′-acac)(γ-acac)(DMS)], thus excluding DNA as a target of [Pt(O,O′-acac)(γ-acac)(DMS)] (Fig. 5C). Since the higher cytotoxicity of [Pt(O,O′-acac)(γ-acac)(DMS)] could be due to its increased intracellular uptake, concentrations of 100 and 17 μM for *cisplatin* and [Pt(O,O′-acac)(γ-acac)(DMS)] respectively, were also used in subsequent experiments to study and compare the characteristics of apoptosis provoked by both Pt compounds (Fig. 5D).

3.4. *Nucleolar structure*

Normal HeLa cells contain intact nuclei with no condensation of cytoplasmic and nuclear content. Cells without nuclei, and multinucleated cells were not considered. Intact HeLa cells had generally two or three compact nucleoli (nucleolar RNA) with an average of three satellites localized in the central portion of the nucleus (Fig. 6A). Five hours treatment with 100 μM *cisplatin* led to an increase in the number of nucleoli from 2.27 ± 0.05 (control cells) to 3.11 ± 0.1 (treated cells, $n = 5$) which increased the toluidine blue staining (Fig. 6B), whilst the nucleolar RNA of cells treated with 17 μM [Pt(O,O′-acac)(γ-acac)(DMS)] showed the same effect after 1 h (from 2.27 ± 0.04 to 3.18 ± 0.09, $n = 5$) and also increased the toluidine blue staining (Fig. 6C). Nucleolar RNA was disassembled 12 and 6 h after *cisplatin* and [Pt(O,O′-acac)(γ-acac)(DMS)] treatment respectively, which may be a direct consequence of apoptotic cell death.

Fig. 6D–F shows the results of a DAPI (known to form fluorescent complexes with natural double-stranded DNA) staining; nuclei are considered to have the normal phenotype when glowing bright and homogenously (Fig. 6D). Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. Between 400 and 700 cells in 5–10 fields (magnification 400×) were counted and the percentage of apoptotic nuclei determined after varying incubation times. The data obtained is shown in Fig. 6G (17 μM

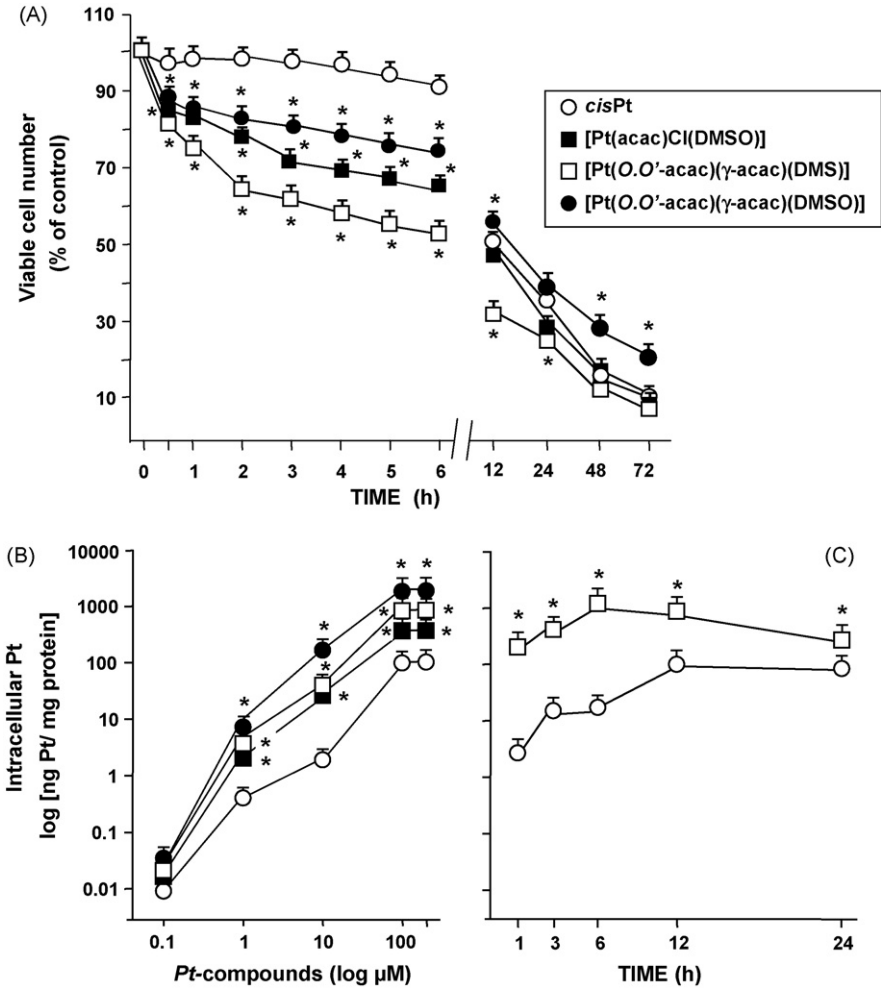

Fig. 4 – Different dynamics of Pt(II)-analogue effects on HeLa cells. (A) Cells were continuously exposed to 100 μM of *cisplatin* or to Pt(II)-analogues and cell viability was monitored by MTT assay over a period of 72 h. (B) Cells were exposed to increasing concentrations (0–200 μM) of Pt(II)-analogues and intracellular accumulation of these analogues was determined by atomic absorption spectrometry after 6 h of exposure. Each point represents the means ± S.D. of three different experiments. (C) Cells were continuously exposed to 100 μM of *cisplatin* or of [Pt(O,O′-acac)(γ-acac)(DMS)] for the indicated period. Intracellular accumulation was determined by atomic absorption spectrometry. Each point represents the means ± S.D. of four different experiments run in eight replicates and are presented as percent of control. Asterisks indicate values that are significantly different ($p < 0.05$) from *cisplatin* alone at the same concentration and the same time point.

of [Pt(O,O′-acac)(γ-acac)(DMS)] and Fig. 6H (100 μM of *cisplatin*). The time course of the nuclear changes revealed that HeLa cells reacted differently to Pt compounds inasmuch as 50% of apoptotic cells were seen after 6 h treatment with [Pt(O,O′-acac)(γ-acac)(DMS)] and after 12 h treatment with *cisplatin*.

3.5. *Induction of apoptosis by [Pt(O,O′-acac)(γ-acac)(DMS)]*

In order to compare the pathway leading to the induction of cell death in response to DNA damage caused by *cisplatin* and the new platinum(II) complex, the cleavage patterns of caspases-3, -7 and -9, and poly(ADP-ribose) polymerase (PARP) were analysed by Western blotting. PARP, a 113-kDa nuclear protein, has been shown to be one of the first proteins specifically cleaved by caspase-3 and -7 during apoptosis [10]. Therefore, PARP cleavage was examined by Western blotting of proteins obtained from isolated nuclei. As shown in Fig. 7 both *cisplatin* (100 μM) and [Pt(O,O′-acac)(γ-acac)(DMS)] (17 μM) significantly increased the amounts of cleaved PARP, thus confirming the early signs of apoptosis induction. PARP degradation was evident after 9 h of *cisplatin* treatment while [Pt(O,O′-acac)(γ-acac)(DMS)] provoked the fragmentation of PARP (89 kDa fragment) after 1 h, and this hydrolysis proceeded so slowly that intact PARP was still present after 12 h treatment. Sequential incubation of the blot with anti-actin antibodies confirmed equal protein loading.

Procaspase-3 was found exclusively in the cytoplasm of untreated cells and moved into the nucleus in response to 100 μM *cisplatin* or to 17 μM [Pt(O,O′-acac)(γ-acac)(DMS)] treatments. As shown in Fig. 7 a substantial portion of procaspase-3 was detected in isolated nuclei 1 h post [Pt(O,O′-acac)

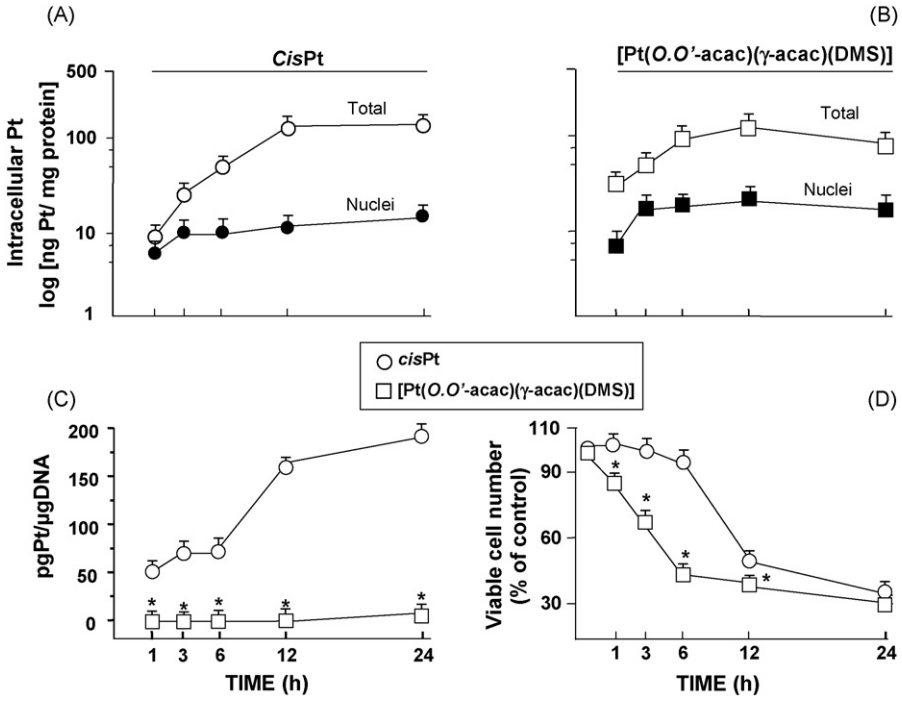

Fig. 5 – Distribution of platinum in HeLa cells. Cells were treated with or without 100 μM *cisplatin* or 17 μM [Pt(O,O′-acac)(γ-acac)(DMS)] for the indicated period. Total cellular and nuclear platinum in HeLa cells treated with *cisplatin* (A) or [Pt(O,O′-acac)(γ-acac)(DMS)] (B) was determined by atomic absorption spectrometry. (C) Platinum bound to DNA in HeLa cells treated with *cisplatin* (open circles) or [Pt(O,O′-acac)(γ-acac)(DMS)] (open squares) determined by atomic absorption spectrometry. Each point represents the means ± S.D. of three different experiments. (D) Viable cell number was determined by MTT assay and the data are means ± S.D. of four different experiments run in eight replicates and are presented as percent of control. Asterisks indicate values that are significantly different ($p < 0.05$) than *cisplatin* alone at the same concentration and the same time point.

(γ-acac)(DMS)] treatment but only after 9 h post *cisplatin* treatment. The conversion of procaspase-3 into the activated forms of low molecular mass p20/p17 was observed in *cisplatin*-treated cells (Fig. 7) but not in [Pt(O,O′-acac)(γ-acac)(DMS)]-treated cells. Both [Pt(O,O′-acac)(γ-acac)(DMS)] and *cisplatin* caused the proteolysis of procaspase-7; this cleavage occurred faster in [Pt(O,O′-acac)(γ-acac)(DMS)]-treated cells. Procaspase-7 increased in isolated nuclei 6 h post [Pt(O,O′-acac)(γ-acac)(DMS)] treatment, but was not observed in *cisplatin*-treated cells.

Finally we examined caspase-9, known to predominantly activate caspase-3 and -7, although some alternative pathways have also been described [11]. In cytosolic fractions obtained from [Pt(O,O′-acac)(γ-acac)(DMS)]-treated HeLa cells the earliest generation of the activated heterodimers occurred after 0.5 h (after 1 h in *cisplatin*-treated cells) and preceded the activation of caspase-3 and -7 (Fig. 7). In addition procaspase-9, found exclusively in the cytoplasm of untreated cells, moved into the nucleus in response to *cisplatin* or to [Pt(O,O′-acac)(γ-acac)(DMS)] treatments with differing kinetics (Fig. 7).

3.6. *Time course of [Pt(O,O′-acac)(γ-acac)(DMS)] and cisplatin phosphorylation of ERK1/2*

The time course of ERK1/2 phosphorylation stimulated by [Pt(O,O′-acac)(γ-acac)(DMS)] and *cisplatin* are illustrated in Fig. 8. The effect of 100 μM *cisplatin* on the phosphorylation state of ERK1/2 was time-dependent: there was a threshold increase at 6 h and a maximal effect at 12 h. In HeLa cells, an increase in phosphorylation of ERK1/2 was observed with 17 μM [Pt(O,O′-acac)(γ-acac)(DMS)] within 0.5 h and remained high for up to 24 h.

[Pt(O,O′-acac)(γ-acac)(DMS)] and *cisplatin* did not have an effect on the total ERK1/2 levels in HeLa cells.

3.7. *The mechanism of [Pt(O,O′-acac)(γ-acac)(DMS)]-induced ERK1/2 phosphorylation*

PD98059, a specific inhibitor of MAPK/ERK kinases 1 (MEK1), was used in order to determine whether MEK was involved in [Pt(O,O′-acac)(γ-acac)(DMS)]- and *cisplatin*-induced phosphorylation of ERK1/2. The pre-treatment of cells with 15 and 30 μM PD98059 for 30 min did not alter the basal phosphorylation state of ERK1/2 (Fig. 8B), HeLa cells pre-treated with PD98059 showed a dose-dependent inhibition of [Pt(O,O′-acac)(γ-acac)(DMS)]-induced phosphorylation of ERK1/2 (Fig. 8B). We examined whether a phosphorylated ERK1/2 is required for the [Pt(O,O′-acac)(γ-acac)(DMS)] cytotoxicity. Pre-treatment with PD98059 resulted in a reduction in [Pt(O,O′-acac)(γ-acac)(DMS)] sensitivity (Fig. 8D); and similar to previous reports, inhibition of ERK1/2 phosphorylation resulted in *cisplatin* resistance (data no shown).

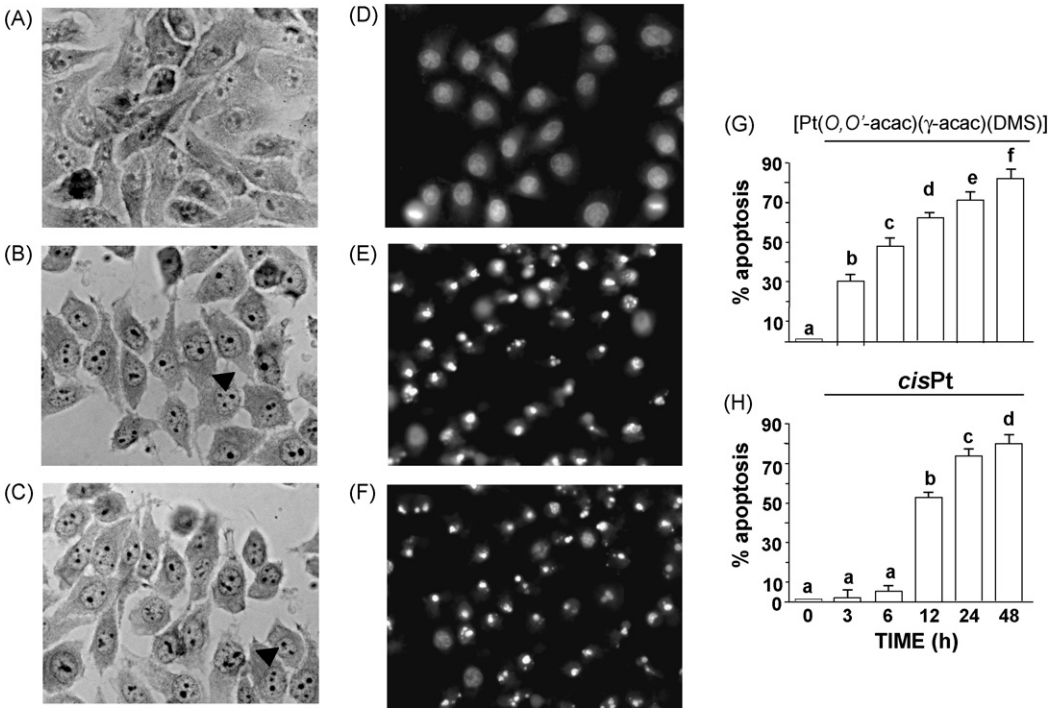

Fig. 6 – [Pt(O,O′-acac)(γ-acac)(DMS)]- and cisplatin-provoked apoptosis. Visualization of nucleolar RNA in HeLa cells using toluidine blue: (A) Untreated HeLa cells, (B) [Pt(O,O′-acac)(γ-acac)(DMS)]- and (C) *cisplatin*-treated cells after 6 and 12 h, respectively. (D–F) HeLa cells were treated or not (control, D) with [Pt(O,O′-acac)(γ-acac)(DMS)] (E) and *cisplatin* (F) for 6 h and then stained with DAPI; the representative fields of one of four independent experiments are shown (magnification 400×). (G and H) Quantification of the percentage of apoptotic nuclei obtained from cells stained with DAPI (mean ± S.D.; $n = 4$), after treatment for different time with 17 μM [Pt(O,O′-acac)(γ-acac)(DMS)] and 100 μM *cisplatin*. Values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

It was previously shown that the PKC signal transduction pathway influences sensitivity of HeLa cells to *cisplatin* [12]. We, therefore, examined if the PKC signal transduction pathway is also involved in [Pt(O,O′-acac)(γ-acac)(DMS)] sensitivity. The PKC inhibitor GF109203X (1 and 10 μM) was used; when HeLa cells were pre-incubated for 30 min with GF109203X, the effect of [Pt(O,O′-acac)(γ-acac)(DMS)] on ERK1/2 phosphorylation was inhibited (Fig. 8C). Ten micromolars of GF109203X also completely reversed the [Pt(O,O′-acac)(γ-acac)(DMS)]-mediated cell death (Fig. 8E).

4. Discussion

Cisplatin is widely used for the treatment of solid tumors including cervix and ovarian cancer [13,14] but drug resistance has become a notable challenge. Therefore, the search for new metal-based drugs is one of the main topics in experimental oncology. In recent years new platinum complexes have been identified as a very promising class of anti-tumor active compounds. They showed a different toxicity profile than *cisplatin* and a slightly different mode of action. Despite the large number of compounds synthesised and tested, few of these have advanced to the late stages of clinical development. We have synthesised a group of new *platinum* complexes for anti-tumor evaluation, containing both an O,O′-chelated acetylacetonate ligand and a sulfoxide in the platinum coordination sphere [5], which show low reactivity in a model reaction with purine bases monitored by ^{1}H NMR. The ability of these new platinum(II) compounds (Fig. 1) to induce cell death in human cervical carcinoma HeLa cells has been characterized and compared to the well established anticancer drug, *cisplatin*. [Pt(O,O′-acac)(γ-acac)(DMS)] exhibited the highest *in vitro* activity from the panel of evaluated platinum(II) complexes in both the MTT and SRB tests. The efficacy of the [Pt(O,O′-acac)(γ-acac)(DMS)] complex to produce similar or higher cytotoxicity than *cisplatin* using lower IC_{50} or IC_{90} concentrations was confirmed by the decrease in total cell numbers and the increase of floating cells. Of particular interest was the different dynamics of the effects of the new platinum(II) complexes on cell apoptosis compared to *cisplatin*. Apoptosis induced by *cisplatin* is generally considered to be generated by formation of covalent DNA adducts, which block replication and transcription, as well as reactive oxygen intermediates [15–17]. *Cisplatin* and its derivatives bind mainly to the N(7) of purine bases, forming stable inter- and intra-strand crosslinks [15,16]. The development of new *platinum* compounds is based on the hypothesis that these structurally dissimilar analogues of *cisplatin* may have, by virtue of formation of different types of Pt–DNA adducts, a spectrum of anti-tumor activity complementary to that of *cisplatin*. Interestingly, the reduced reactivity of these novel compounds with nucleobases suggests that their cytotoxic activity may not necessarily require reaction with DNA. In fact, the specific

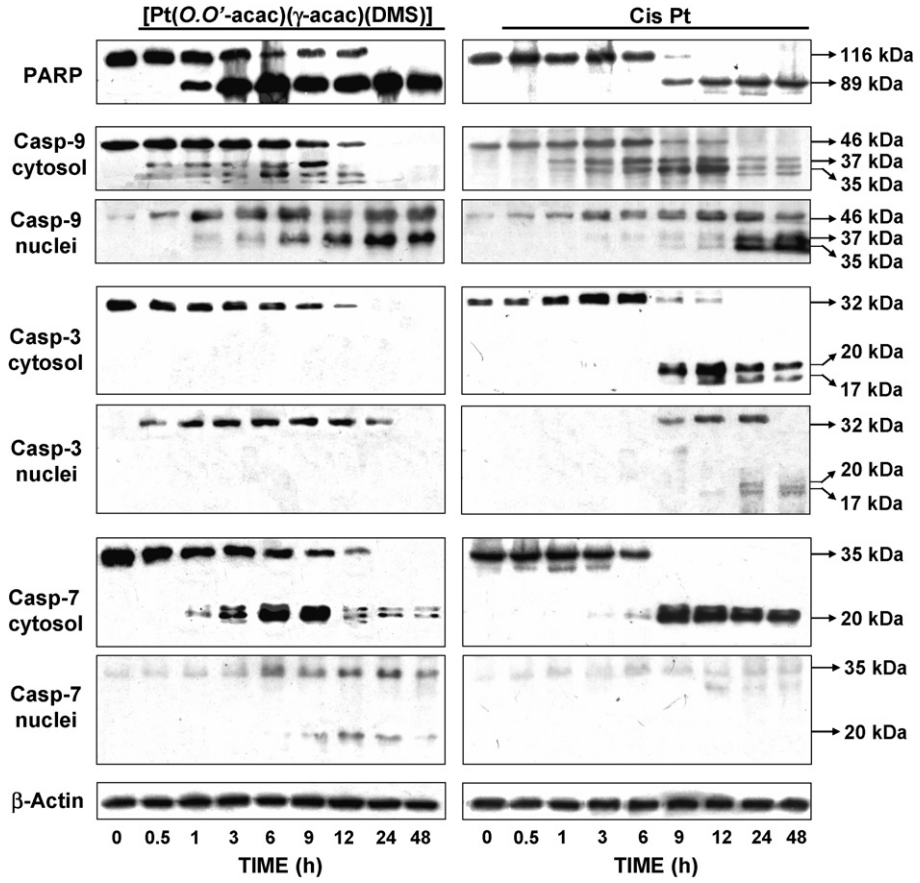

Fig. 7 – Effect of *cisplatin* and [Pt(O,O′-acac)(γ-acac)(DMS)] on the activation of caspase-3, caspase-7, and caspase-9 and the cleavage of PARP. Cytosolic and nuclear proteins were obtained from HeLa cells, treated or not with 17 μM [Pt(O,O′-acac)(γ-acac)(DMS)] (left) or 100 μM *cisplatin* (right). Samples were dissolved in SDS sample buffer and separated on SDS gel. Immunoblotting was performed using monoclonal anti-PARP and anti-caspases-3, -7 and -9. Sequential incubation with anti-actin confirmed the equal protein loading. The figures are representative of four independent experiments.

reactivity with sulphur ligands which allowed the synthesis of [PtCl(O,O′-acac)(DMS)] and [Pt(O,O′-acac)(γ-acac)(DMS)] from [PtCl(O,O′-acac)(DMSO)] and [Pt(O,O′-acac)(γ-acac)(DMSO)] by selective substitution of the sulfoxide with the sulfide ligand, strongly suggests protein thiols or thioethers are preferred targets for these molecules. Indeed, as expected, almost instantaneous *in vitro* substitution of sulfoxide with methionine lead to the production of [Pt(O,O′-acac)(γ-acac)(methionin)] from [Pt(O,O′-acac)(γ-acac)(DMSO)]. The reactivity of the tested compounds could therefore be ascribed to their tendency to give sulfur ligand exchange at the platinum centre. It is certainly true that DNA is not the primary target of other metal complexes, such as the ruthenium NAMI and its analog (ImH)*trans*-[Ru(Im)(Me_2SO)Cl_4] (NAMI-A) [18,19] and the Au(I) complexes with 1,2-bis(diphenylphosphino)ethane, with 1,2-bis(dipyridylphosphino)ethane ligands [20] and with monophosphine and diphosphine ligands [21].

[Pt(O,O′-acac)(γ-acac)(DMS)] entered the cells quickly and its cellular accumulation was approximately six times higher than that of *cisplatin*; this enabled us to use a 6-fold lower concentration of [Pt(O,O′-acac)(γ-acac)(DMS)] in further experiments. Despite this, the cytotoxic effects of 17 μM [Pt(O,O′-acac)(γ-acac)(DMS)] were greater and more rapid than the effects elicited by 100 μM *cisplatin*. Another important point was the cellular distribution of *cisplatin* and [Pt(O,O′-acac)(γ-acac)(DMS)], which were indeed different such that, while both gave approximately the same nuclear content, [Pt(O,O′-acac)(γ-acac)(DMS)] was rarely bound to DNA (approximately 25-fold lower than *cisplatin*).

The biological evaluation showed that ot the compounds examined [Pt(O,O′-acac)(γ-acac)(DMS)] displayed higher cytotoxicity and was therefore selected for further evaluation, i.e. a more detailed analysis of the cell death pathways. The proteolytic enzymes, caspases, have a critical role in the execution of apoptosis. It is suggested that *cisplatin* may induce apoptosis through both caspase-3-dependent and independent pathways [22,23]. The ubiquitous nuclear enzyme PARP-1 (poly(ADP-ribose) polymerase-1), a unique sensor of DNA breaks [24] can be cleaved by both caspase-3 and -7. PARP-1 cleavage separates the DNA-binding domain of these enzymes (essential for its activity) and the catalytic domain, and prevents necrosis during apoptosis, avoiding inflammation. PARP cleavage to the 89 kDa fragment has been observed in diverse models of apoptosis and is therefore considered to be a sensitive parameter of apoptosis [25,26]. In our experiments, PARP cleavage was detected after 1 h of

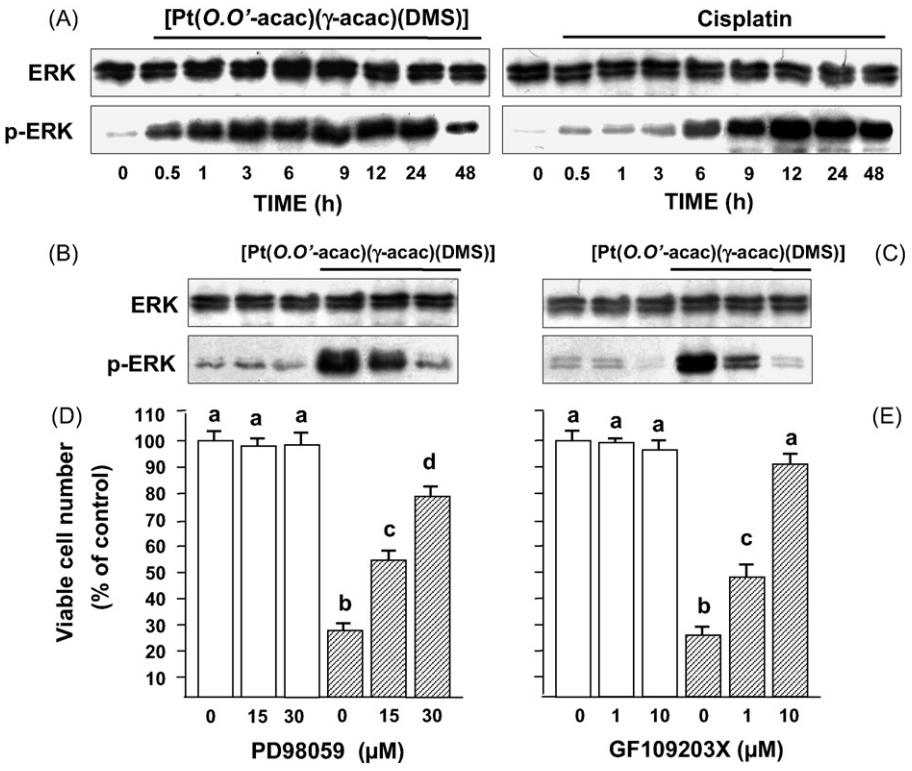

Fig. 8 – Activation of ERK1/2 by *cisplatin* and [Pt(O,O′-acac)(γ-acac)(DMS)] in HeLa cells. (A) Cells were treated with or without 17 μM of [Pt(O,O′-acac)(γ-acac)(DMS)] (left) or 100 μM *cisplatin* (right), for the indicated times. Cells were pre-treated in the with or without various concentrations of PD98059 (B and D) or GF109203X (C and E) for 30 min and then with or without [Pt(O,O′-acac)(γ-acac)(DMS)], for 16 h. (A, B and C) Cell lysates were electrophoresed through 10% SDS-PAGE and analysed by Western blotting using the antibody against the active (dually phosphorylated) ERK1/2 (p-ERK1/2) or the anti-total ERK1/2 antibody. The figure is representative of four independent experiments. (D and E) Viable cell numbers assessed by MTT assay as described. The data are means ± S.D. of four different experiments run in eight replicates and are presented as percent of control. Values with shared letters are not significantly different according to Bonferroni/Dunn post.

[Pt(O,O′-acac)(γ-acac)(DMS)] treatment, in accordance with the rapid effect on HeLa cell survival. Usually, when cells undergo apoptosis, the 32 kDa pro-enzyme caspase-3 is proteolytically transformed into the active heterodimeric complexes composed of a 20 kDa (p20) and an 11 kDa (p11) subunit [27,28]. In our study, these p20 and p11 forms of caspase-3 were detected in *cisplatin*- but not in [Pt(O,O′-acac)(γ-acac)(DMS)]-treated cells, perhaps because [Pt(O,O′-acac)(γ-acac)(DMS)]-treated cells clear them rapidly, as observed in other studies [28]. Nevertheless, [Pt(O,O′-acac)(γ-acac)(DMS)] caused a significant decrease of cytosolic procaspase-3 level and a coincident translocation into the nucleus. HeLa cells also express caspase-7, which is closely related to caspase-3 [29,30]. Recent studies suggested that caspase-7 has an important, non-redundant, role in normal physiology and apoptosis [31], thus suggesting that caspase-7 and -3 have complementary but not completely overlapping roles. Caspase-7 cleavage pattern in both cytosol and nuclear fractions, was detected earlier in [Pt(O,O′-acac)(γ-acac)(DMS)]-treated cells compared to *cisplatin* treatment, and matched the cleavage of PARP, thus supporting the study showing that caspase-7 is responsible for PARP cleavage [32].

Caspase-9 can activate caspases-3 and -7 [10]. Consistent with other results [33], in both *cisplatin* and [Pt(O,O′-acac)(γ-acac)(DMS)] treated HeLa cells, the procaspase-9 activation proceeded together with the generation of the mature caspase-7 form, indicating the involvement of the intrinsic pathway. Thus, apoptosis is the major pathway of cell death caused by [Pt(O,O′-acac)(γ-acac)(DMS)] and occurs through events involving caspase activation. The evaluation of nucleolar morphology, shows an association of caspase-induced degradation of PARP-1 with an alteration of nucleolar structure: we observed a rapid onset of nucleolar segregation as well as micronucleoli, after the treatment with [Pt(O,O′-acac)(γ-acac)(DMS)]. Similarly, we detected a remarkable shrinkage of nuclei by 35% within 3 h of [Pt(O,O′-acac)(γ-acac)(DMS)] treatment. A possibility is that disruption of nucleoli observed in these treated HeLa cells is a direct consequence of apoptotic cell death, indeed this has been observed in *cisplatin*-treated HeLa cells [33].

Previous studies have described the link between induction of ERK1/2 activation and apoptosis in response to *cisplatin* treatment in HeLa cells and in other cell types [34–36]. Consistent with a role for the ERK1/2 pathway in *cisplatin*-induced HeLa cell death [34], we here provided evidence that the activation of ERK1/2 is important for the induction of [Pt(O,O′-acac)(γ-acac)(DMS)] apoptosis. In fact, [Pt(O,O′-acac)(γ-acac)(DMS)] treatment also resulted in high and sustained

activation of ERK1/2 and inhibition of ERK1/2 activation by the MEK inhibitor, PD98059, decreased the [Pt(O,O′-acac)(γ-acac)(DMS)]-mediated cell death.

With regard to upstream mediators implicated in the [Pt(O,O′-acac)(γ-acac)(DMS)]-induced ERK1/2 activation, PKCs are crucial elements in the pathway linking [Pt(O,O′-acac)(γ-acac)(DMS)] to the ERK1/2 cascade as demonstrated when inhibition (with GF109203X), of PKCs had a significant effect on the [Pt(O,O′-acac)(γ-acac)(DMS)]-evoked ERK1/2 phosphorylation. We and others have previously shown that the PKC signal transduction pathway regulates cell death by *cisplatin* [36,12]. In the present study, we have used the PKC inhibitor GF109203X, to establish the involvement of a PKC isozyme in the regulation of cell death by [Pt(O,O′-acac)(γ-acac)(DMS)]. GF109203X completely reversed [Pt(O,O′-acac)(γ-acac)(DMS)] cytotoxicity, suggesting that inhibition of PKCs was responsible for protection against [Pt(O,O′-acac)(γ-acac)(DMS)] cytotoxicity.

In conclusion, the results presented here indicate that these novel synthesized Platinum(II) complexes have high cytotoxic activity *in vitro*, and that in contrast to *cisplatin*, DNA may not be their primary target. Studies aimed at understanding the precise mechanism responsible for apoptosis and identifying the molecules targeted by these complexes are in progress.

Acknowledgments

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