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High effectiveness of platinum(IV) complex with adamantylamine in overcoming resistance to cisplatin and suppressing proliferation of ovarian cancer cells in vitro

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Abstract

[(*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV)], coded as LA-12, is an octahedral platinum(IV) complex containing a bulky hydrophobic ligand – adamantylamine. The use of bulky hydrophobic amines as non-leaving ligands, may increase uptake of the compound by the cancer cells. Therefore, the effects of LA-12 on sensitive (A2780) and cisplatin resistant (A2780cis) ovarian cancer cell lines were investigated and compared to those of cisplatin. IC_{50} and IC_{90} concentrations of LA-12 were 6- (A2780) or 18-fold (A2780cis) lower than those for cisplatin (MTT assay). Equitoxic concentrations (IC_{50} or IC_{90}) of both compounds caused a significant and similar time- and dose-dependent inhibition of cell proliferation and an increase in the number of floating cells which corresponded to the decrease of total cell viability. A different type and dynamics of cell cycle perturbation after cisplatin and LA-12 treatment were detected. Exposure to LA-12 resulted in transient accumulation of A2780 and A2780cis cells in S phase, while cisplatin caused G_2/M arrest in sensitive and S phase arrest in resistant cells. A relatively low rate of apoptosis after exposure to IC_{50} or IC_{90} of both complexes was observed, markedly higher in resistant A2780cis cells. Western blot analysis indicated a concentration-dependent p53 level increase in both lines (higher after cisplatin treatment). PARP cleavage was observed only in A2780cis cells. In conclusion, LA-12 was found to be significantly more efficient than cisplatin, and it was able to overcome the acquired cisplatin resistance (showing resistance factor 2.84-fold lower than those for cisplatin). In spite of the low rate of apoptosis, LA-12 caused increase of p53 level and cell cycle perturbations in the ovarian cancer cell lines studied.

Keywords: Cisplatin; LA-12; Cytotoxicity; Cell cycle perturbations; Apoptosis; Ovarian cancer

Abbreviations: cis-DDP, *cis*-diamminedichloroplatinum(II); LA-12, (*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV); JM216, (*OC*-6-43)-bis(acetato)amminedichloro(cyclohexylamine)platinum(IV); cisplatin (50 or 90), LA-12 (50 or 90), IC_{50} or IC_{90} drug concentrations that cause 50% or 90% inhibition of cell proliferation; Pt(II), planar and four coordinate platinum complex; Pt(IV), octahedral and six coordinate platinum complex; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4,6-diamidino-2-phenyl-indole dihydrochloride; DABCO, 1,4-diazabicyclo-[2.2.2]octane; PARP, poly(ADP-ribose)polymerase; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis

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1. Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] (Fig. 1) is currently one of the three most widely utilized antitumour drugs, with high efficiency in treating testicular and ovarian cancers [1,2]. Despite its strong antitumour activity, cisplatin therapy can lead to a number of side effects such as nephrotoxicity, neurotoxicity, and emesis. The toxicity of cisplatin limits the dose that can be given to patients. These facts together with the aim to find platinum-based derivatives with higher antitumour activity and overcoming resistance of many tumour types have led many investigators to attempt the synthesis and characterization of new platinum (Pt) analogues. Since the early studies by Rosenberg et al. [3] it has been known that Pt(IV) complexes can display antitumour properties.

Pt(IV) compounds which contain lipophilic non-leaving ligands have generally greater lipophilicity than currently used Pt(II) derivatives [4] which could enable them to overcome cisplatin resistance caused by a decreased Pt accumulation in target cancer cells [5,6]. The lack of cross-resistance with cisplatin reported for some Pt(IV) compounds and the clinical development of the first orally active platinum derivative-JM216 [7,8] have helped to increase the interest in this type of platinum compounds. Pt(IV) complexes are much more inert to ligand substitution reactions than their Pt(II) counterparts [9], and offer some pharmacological advantages in comparison with cisplatin [10,11].

(*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV), coded as LA-12 (Fig. 1), represents an octahedral platinum(IV) complex, containing a bulky non-leaving hydrophobic ligand-adamantylamine. LA-12 was synthesized by the method described previously [12]. The presence of cyclic amines reduces the toxicity of platinum compounds, especially for large rings like cyclopentylamine [13].

With this background Žák et al. [12] designed and synthesized a series of platinum(IV) complexes with amino derivatives of adamantane as one of its six ligands. According to the evaluation of in vitro antitumour activity of adamantylamine Pt(IV) complexes, LA-12 displayed the highest biological effect from the panel of Pt(IV) evaluated complexes with the general formula (*OC*-6-43)-bis(acetato)(alkylamine)amminedichloroplatinum(IV) on the model of ovarian cancer cell lines, parent cisplatin sensitive A2780, and A2780cis (with acquired cisplatin resistance). In addition, LA-12 has shown a higher cytotoxicity than JM216 in the in vitro MTT test for mitochondrial activity on A2780 and A2780cis cells [4]. Therefore, we aimed to study in more detail the cytotoxicity, cell cycle perturbations, and the type of cell death induced by the LA-12 derivative in sensitive and cisplatin resistant ovarian cancer cell lines.

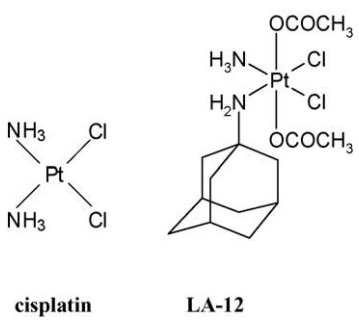


Fig. 1. Chemical structures of cisplatin [*cis*-diamminedichloroplatinum(II)] and LA-12 [(*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV)].

2. Materials and methods

2.1. Materials

Cisplatin (*cis*-DDP, *cis*-diamminedichloroplatinum(II); FW 300.1) (Sigma–Aldrich Corp.) and LA-12 [(*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV); FW 552.4] (PLIVA-Lachema a.s.) were dissolved in DMSO (Sigma–Aldrich Corp.). The stock solutions of cisplatin and LA-12 were freshly prepared before use. The final concentration of DMSO in cell culture medium did not exceed 0.2%.

2.2. Cell lines and culture

The A2780 (parent cisplatin sensitive) and A2780cis (with acquired cisplatin resistance) ovarian carcinoma cell lines, obtained from the European Collection of Animal Culture (ECAC) [14–16], were grown in RPMI 1640 medium (Sigma–Aldrich Corp.) supplemented with gentamycin (50 μg/ml; Serva) and 10% heat inactivated fetal bovine serum (PAN Biotech GmbH). The cells were cultivated in a humidified incubator at 37 °C in a 5% CO_2 atmosphere, and subcultured twice a week with a plating density of 1:4. The acquired resistance of A2780cis cells was maintained by supplementing the medium with 1 μM cisplatin every second passage. For the experiments, the cells were seeded at a density of 30,000 cells/cm^2.

2.3. Cytotoxicity assay

The cells were seeded in 96-well tissue culture plates. After overnight incubation, the cells were treated for 72 h with the derivatives studied. Then 10 μl of MTT (2.5 mg/ml; Sigma–Aldrich Corp.) was added to each well and incubated for 4 h in culture conditions. At the end of the incubation period the medium was removed, the formazan product was dissolved in 50 μl of 10% Triton X-100 in 0.1 M HCl, and optical densities were measured at 570 nm using a microplate spectrophotometer reader (ASYS Hitech GmbH). The reading values were converted to the percentage of control (% cell survival). Cytotoxic

effects were expressed as IC_{50} and IC_{90}, which were the concentrations inducing 50% and 90% inhibition of metabolic activity of the cells treated, respectively.

2.4. Cell number, floating cell quantification, and viability assay

The cells were seeded in 60 mm tissue culture dishes (30,000 cells/cm^2). After overnight incubation, the cells were treated with the calculated IC_{50} and IC_{90} concentration of cisplatin or LA-12 for 24 h, 48 h, and 72 h. The numbers of cells were determined using a Coulter Counter® ZM (Beckman-Coulter). The attached and floating cells were counted separately, and the amount of floating cells was expressed as the percentage of the total cell number. Total cell viability (attached + floating) was analysed using staining with 0.15% eosin via light microscopy.

2.5. Cell cycle analysis

At each time points, floating cells were collected and attached cells were harvested by trypsinization (trypsin/EDTA in PBS; PAN Biotech GmbH), total (floating + attached) cells were washed twice in PBS (4 °C), fixed in 70% ethanol, and stored at 4 °C. The PBS washed cells were subsequently rinsed with 0.2 M phosphate-citrate buffer at pH 7.8 according to Darzynkiewicz et al. [17] – after pelleting the cells were stained with 200 μl staining solution (20 μg PI/ml PBS + ribonuclease A (DNA free; 5 U/ml); both Sigma–Aldrich Corp.) for 30 min at 37 °C in the dark. The DNA content of the cells was analysed using flow cytometry (FACSCalibur™, Becton Dickinson, 488 nm argon laser for excitation). For each sample, 1.5×10^4 cells were acquired, and the percentages of cells in the individual cell cycle phases were analysed using ModFit 2.0 software (Verity Software House). Single cells were identified and gated by pulse-code processing of the area and the width of the fluorescence signal. Cell debris was excluded by appropriate increase of the forward scatter threshold.

2.6. Fluorescence microscopy of apoptotic morphology and DNA fragmentation analysis

At each time point, the samples for apoptotic cell quantification (nuclear morphology analysis) were harvested by trypsinization and collected floating and harvested attached cells were washed twice in PBS. The total cells, fixed in 70% ethanol, were stained with DAPI (Sigma–Aldrich Corp.; final concentration 1 μg/ml) for 30 min at room temperature in the dark. After incubation, the cells were centrifuged, mixed with Mowiol 40–88 solution (for mounting of the cells under coverslips) (Sigma–Aldrich Corp.) with DABCO as an anti-bleaching treatment of the DAPI-stained cells (0.6%, Sigma–Aldrich Corp.) and mounted under coverslips. The incidence of apoptotic bodies was evaluated by fluorescence microscopy (Olympus IX-70; Olympus). The apoptotic index was counted from at least 200 cells.

Subsequently, DNA fragmentation analysis was performed. At 72 h time point, 2×10^6 cells treated with cisplatin or LA-12 with IC_{50} and IC_{90} concentrations were washed twice with PBS (4 °C) and subsequently DNA isolation was performed using the Invisorb Apoptosis Detection Kit I (Invitek; Invitek GmbH). Gel electrophoresis was performed in 1.5% agarose (Sigma–Aldrich Corp.), using 1 kbp DNA ladder as a marker (MBI Fermentas). DNA was stained with SYBR Green I (Molecular Probes, Inc.) and scanned by Storm (Molecular Dynamics, Amersham Biosciences).

2.7. Western blot analysis

At each time point, the treated cells were harvested, washed twice in cold PBS and lysed in lysis buffer (100 mM Tris–HCl, pH 7.4; 1% SDS; 10% glycerol and protease inhibitor cocktail (P2714, Sigma–Aldrich Corp.)) for 10 min on ice. The cell lysates were sonicated (Sonifier® B-12, Branson Ultrasonics Corp.) and centrifuged. The protein concentration of the samples was determined using a detergent-compatible protein assay (Bio-Rad Laboratories, Inc.). The samples were diluted to the same concentration and equal amounts (20 μg proteins) with 0.01% bromphenol blue and 1% 2-mercaptoethanol were separated on 7.5% SDS-polyacrylamide gel, and blotted onto a PVDF membrane (Millipore Corp.) in a transfer buffer containing 192 mM glycine, 25 mM Tris, and 10% methanol. The membranes were blocked overnight in TBS (20 mM Tris–HCl, pH 7.6; 150 mM NaCl) containing 0.05% Tween 20 and 5% non-fat milk at 4 °C. Then the blots were washed with TBS-Tween and incubated with rabbit polyclonal anti-PARP (#sc-7150, 1:1000, Santa Cruz) or mouse monoclonal anti-p53 (DO-1, 1:2000; [18]) primary antibodies for 2 h at room temperature. The membranes were incubated with secondary anti-mouse IgG (#NA931, 1:3000) and anti-rabbit IgG (#NA934, 1:6000; both Amersham Biosciences) antibodies conjugated with horseradish peroxidase for 1 h after washing in TBS-Tween. Detection of the antibody reactivity was performed with an enhanced chemiluminescence detection kit ECL+ (Amersham Biosciences) and visualized on X-ray films (AGFA-Gevaert N.V.). Equal sample loading was verified by immunodetection of β-actin (A5441, 1:10,000, Sigma–Aldrich Corp.) or staining of PVDF membrane with 0.1% amido black (in 1:3:6 (v/v/v) of acetic acid, methanol and water) for non-specific visualization of proteins. Quantification of visualized bands was performed by densitometry using AIDA software (Advanced Image Data Analyzer; Raytest) allowing assessment of increases in the expression of protein levels compared with untreated controls.

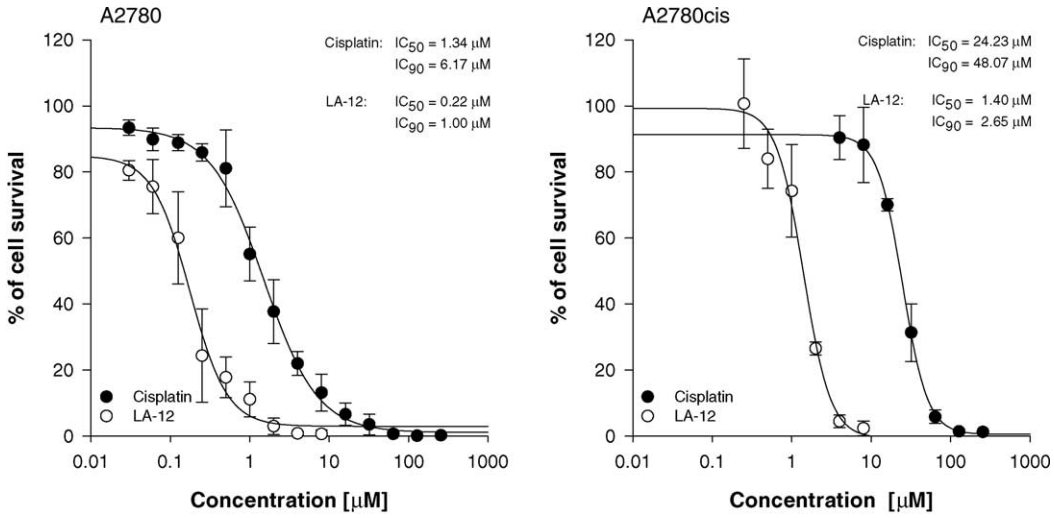


Fig. 2. Time– and dose–response effects on the survival of A2780 and A2780cis cancer cell lines. The effects after 72 h exposure to cisplatin or LA-12 in a concentration range between 0.3 μM and 256 μM were determined by MTT assay. The calculated drug concentrations inhibiting metabolic activity of cells by 50% (IC_{50}) and 90% (IC_{90}) are displayed for both derivatives. The results are expressed as mean ± standard deviations (S.D.) of at least three independent experiments; all concentrations were tested in three replicates.

2.8. Statistical analysis

All the data are expressed as the means ± S.D. of at least three independent experiments. With all statistical analyses, the associated probability (p value) of <5% was considered as significant. Comparisons between the groups were calculated using One-way Analysis of Variance followed by the Tukey range test (*, #). If the variances were non-homogenous, Mann–Whitney U-test analysis was used (×). All statistical analyses were calculated by the software Statistica for Windows, V. 6.1 (StatSoft, Inc.). The dose–response curves were analysed by the software SigmaPlot®, V. 8.0 (SPSS International B.V.).

3. Results

3.1. Cytotoxicity of the drugs

Exposure to the compounds in a concentration range between 0.3 μM and 256 μM resulted in a dose-dependent inhibition of cell survival in both A2780 and A2780cis cell lines (Fig. 2). After 72 h treatment, IC_{50} and IC_{90} values of LA-12 were 6.09-fold and 6.17-fold lower, respectively, in A2780 cells, and 17.31-fold and 18.14-fold lower, respectively, in A2780cis cells, than those of cisplatin. Displayed effects were also characterized by the value of resistance factor which is defined as the ratio of IC_{50} concentration

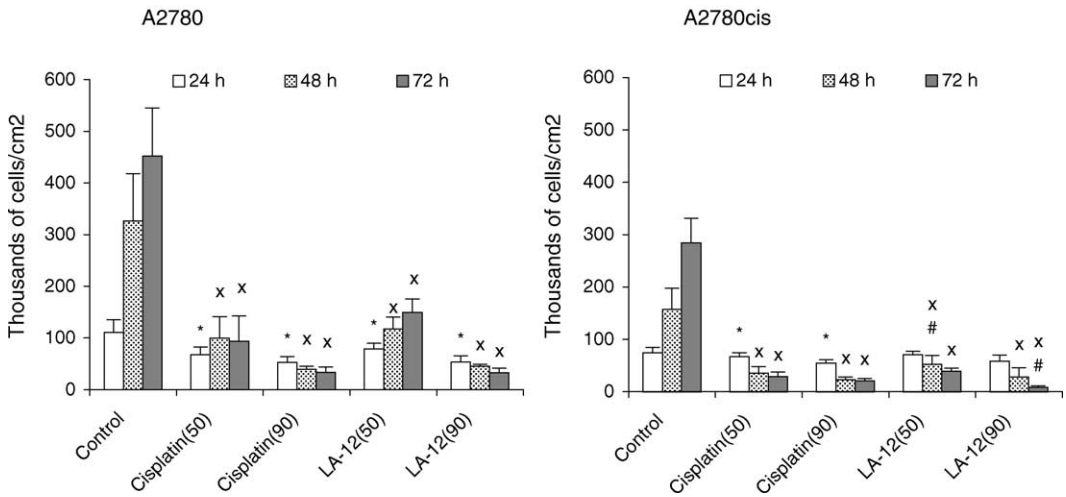


Fig. 3. Effects of equitoxic concentrations of cisplatin or LA-12 on cell growth. The A2780 or A2780cis cells were untreated (control) or sustained treated with IC_{50} and IC_{90} concentrations of cisplatin or LA-12 and harvested at 24 h, 48 h, and 72 h. The cells collected were measured for the number of attached cells per surface area of culture dish (cm^2). The results are expressed as mean ± standard deviations (S.D.) of at least three independent experiments. The symbols (*), (×) denote significant difference ($p < 0.05$) from untreated control; (#) denote significant difference ($p < 0.05$) between equitoxic cisplatin and LA-12 effects.

values of the derivative in resistant A2780cis cells to sensitive parent A2780 cells. Cisplatin showed a resistance factor 18.08 while resistance factor of LA-12 was 6.36. It means that LA-12 overcomes cisplatin resistance in A2780cis cells showing a resistance factor 2.84-fold lower than those for cisplatin.

3.2. Determination of cellular growth and viability

In order to confirm the higher effectiveness of LA-12 found by MTT metabolic assay, the effects of equitoxic concentrations (IC_{50} and IC_{90}) of both compounds on the total cell number, percentage of floating cells and viability were investigated.

As shown in Fig. 3, IC_{50} and IC_{90} concentrations of LA-12 and cisplatin caused a similarly time- and concentration-dependent decrease in the number of attached cells per cm^2 significantly different from the untreated control at all the time points studied in A2780 and A2780cis cells. Parallely, a significant increase in the percentage of floating A2780 and A2780cis cells was detected in all studied groups (Fig. 4a). After 72 h, in cisplatin resistant A2780cis cells the effect of IC_{90} of LA-12 on both parameters was even significantly greater than that of cisplatin. The total cell number (adherent and floating cells together) was also significantly decreased after drug treatment without significant differences between equitoxic concentrations of cisplatin and LA-12 (data not shown).

These results corresponded with the time- and concentration-dependent decrease in cell viability compared with untreated control cells (Fig. 4b), in which no significant differences in cisplatin versus LA-12 effects were recorded, either.

3.3. Cell cycle perturbations

An analysis of cell cycle perturbations was performed in A2780 and A2780cis cells exposed to IC_{50} concentrations

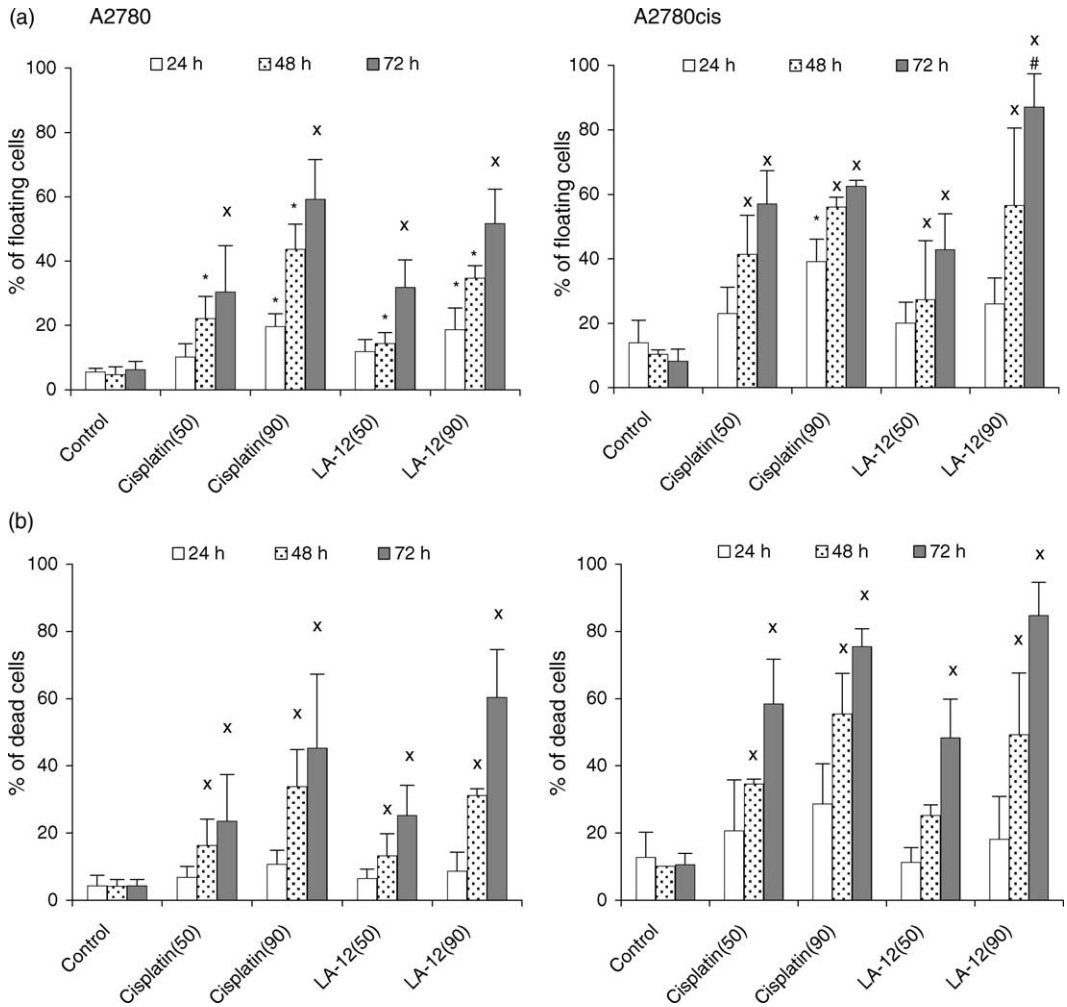


Fig. 4. Effects of equitoxic concentrations of cisplatin or LA-12 on the percentage of floating cells and viability. The A2780 and A2780cis cells were untreated (control) or sustained treated with IC_{50} and IC_{90} concentrations of cisplatin or LA-12 and harvested at 24 h, 48 h, and 72 h. The percentage of floating cells in relation to the total number of cells per dish was determined (a). Viability was measured in the total amount collected (floating + attached) of cells by eosin staining (b). The results are expressed as mean ± standard deviations (S.D.) of at least three independent experiments. The symbols (*), (×) denote significant difference ($p < 0.05$) from untreated control; (#) denote significant difference ($p < 0.05$) between equitoxic cisplatin and LA-12 concentrations.

of cisplatin or LA-12 at 24 h, 48 h, and 72 h of drug exposure. The results of the cell cycle distribution for one representative experiment are shown in Fig. 5. Comparison of the cisplatin versus LA-12 effects in sensitive A2780 cells showed several significant differences. At the 24 h time point, cisplatin caused transient accumulation of cells in the G_2/M-phase of the cell cycle while LA-12 induced accumulation of cells in the S-phase fraction. At the other time points, there were no significant differences compared to controls. On the other hand, resistant A2780cis cells were arrested in the S-phase of the cell cycle after treatment with both cisplatin and LA-12 at the 24 h time interval, cisplatin being more effective (Fig. 5). This block was shifted to G2/M-phase in later time intervals.

3.4. Nuclear morphology

A concentration- and time- dependent increase in the percentage of A2780 and A2780cis cells with apoptotic morphology in comparison to the untreated control cells was observed (Fig. 6). While for cisplatin treated cells the significance of the increase was proved already after 48 h, LA-12 did not significantly increase apoptosis until 72 h. That is why cisplatin showed a significantly higher rate of apoptosis in comparison with LA-12 in the 48 h interval. However, this difference was balanced out after 72 h. Generally, the percentage of apoptotic sensitive A2780 cells was low (about 5–8% maximum) after treatment with both drugs, and it was about two to four times higher (about 15–20%) in resistant A2780cis cells.

3.5. Analysis of DNA fragmentation

In order to investigate whether nuclear fragmentation observed after the treatment of cells was associated with DNA fragmentation typical of apoptosis, the agarose gel electrophoresis of extracted genomic DNA from both control and drug-treated cells (72 h sustained treatment with IC_{50} or IC_{90} concentrations) was performed (Fig. 7). DNA in A2780 and A2780cis cells appeared as a band of high molecular size corresponding to genomic DNA. However, no typical 180-base pair integer oligonucleosome ''DNA ladder'' that would be indicative of intranucleosomal cleavage was detected in any samples. DNA appeared unspecifically degraded as a smudge in both A2780 and A2780cis cells after the 72 h treatment with cisplatin or LA-12.

3.6. PARP and p53 expression

In order to compare the pathway leading to the induction of A2780 and A2780cis cell death in response to DNA damage caused by cisplatin or LA-12, the expression of the tumour suppressor p53 and poly-ADP ribose polymerase (PARP) cleavage was examined by Western blotting.

In A2780 cells, only the full-length PARP (113 kDa) was observed in all tested groups and time points of drug exposure (Fig. 8a, left panel). In contrast, in A2780cis cells (Fig. 8a, right panel) the same treatments were associated with time- and concentration-dependent cleavage of full-length PARP. While the cisplatin effect of IC_{50} was apparent already after 24 h, after LA-12 (IC_{50}) treatment the PARP fragment (89 kDa) was not apparent until 48 h. At the 72 h time point, PARP fragmentation was observed after cisplatin and LA-12 treatment with both IC_{50} and IC_{90} concentrations.

Fig. 8b displays the expression levels of p53 protein after treatment of A2780 (left panel) or A2780cis cells (right panel) with IC_{50} or IC_{90} concentrations of cisplatin or LA-12. In comparison with the untreated control, a concentration-dependent increase in p53 levels was observed in both cell lines studied. However, in comparison to that with LA-12, treatment with cisplatin caused a markedly higher expression of p53 protein levels in both cancer cell lines. Densitometric quantification of visualized bands of p53 level is presented in Fig. 8c. In comparison with untreated control significantly ($p < 0.05$) higher expression of p53 was detected for both derivatives in both cell lines. At the same time significant increase of p53 expression after cisplatin treatment in comparison with LA-12 in both cell lines was detected. In these experiments time- and concentration-dependent results were proved.

4. Discussion

In the present study the ability of the Pt(IV)-complex LA-12 to induce cytotoxicity, cell cycle perturbations, and cell death in ovarian cancer cell lines A2780 (parent cisplatin sensitive) and A2780cis (with acquired cisplatin resistance) has been characterized and compared to the well established anticancer drug – cisplatin. LA-12 exhibited the highest antitumour activity in vitro from the panel of evaluated platinum(II) and (IV) complexes with adamantane in the MTT test of cytotoxicity on both ovarian cancer cell lines tested (our unpublished results). Moreover, LA-12 was able to overcome the acquired resistance to cisplatin in A2780cis cells (showing a resistance factor of 2.84-fold lower than those of cisplatin). The efficacy of the LA-12 complex to produce similar or higher cytotoxicity than cisplatin in lower determined IC_{50} or IC_{90} concentrations was confirmed in several other parameters including decrease in the total and attached cell numbers, increase of the percentage of floating cells, and decrease of cell viability. The ability to overcome the resistance was similar to JM216 and JM221, the first Pt(IV)-containing anticancer agents for oral administration that have been evaluated in a number of intrinsic and acquired cisplatin resistant cell lines. These compounds displayed a lack of cross-resistance with cisplatin, particularly in those cases where reduced platinum accumulation played a dominant

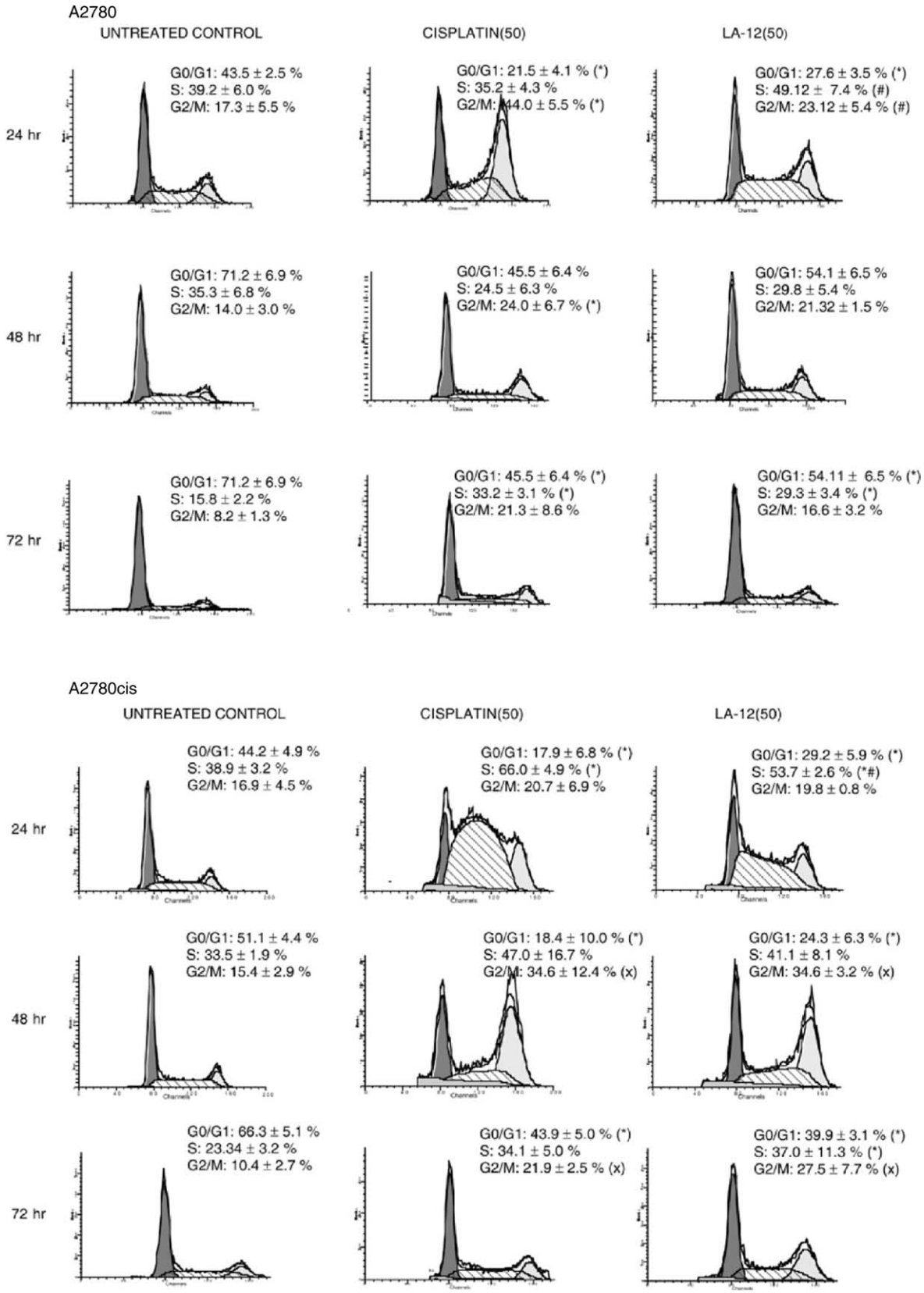


Fig. 5. Effects of IC_{50} concentrations of cisplatin or LA-12 on cell cycle distribution. Untreated (control) cells or cells treated for 24 h, 48 h, and 72 h were harvested, fixed, stained with propidium iodide and assessed for cell cycle distribution by FACS analysis. One representative time course of A2780 and A2780cis cell cycle distribution histograms as analysed by ModFit^{TM} 2.0 software is reported. The estimated percentages of A2780 or A2780cis cells in different phases of the cell cycle are shown. Dark grey color filling represents G0/G1-phase, hatched filling S-phase and light grey color filling G2/M-phase of modeled cell cycle. The results are expressed as mean ± standard deviations (S.D.) of at least three independent experiments. The symbols (*), (×) denote significant difference ($p < 0.05$) from untreated control; (#) denote significant difference ($p < 0.05$) between equitoxic cisplatin and LA-12 concentrations.

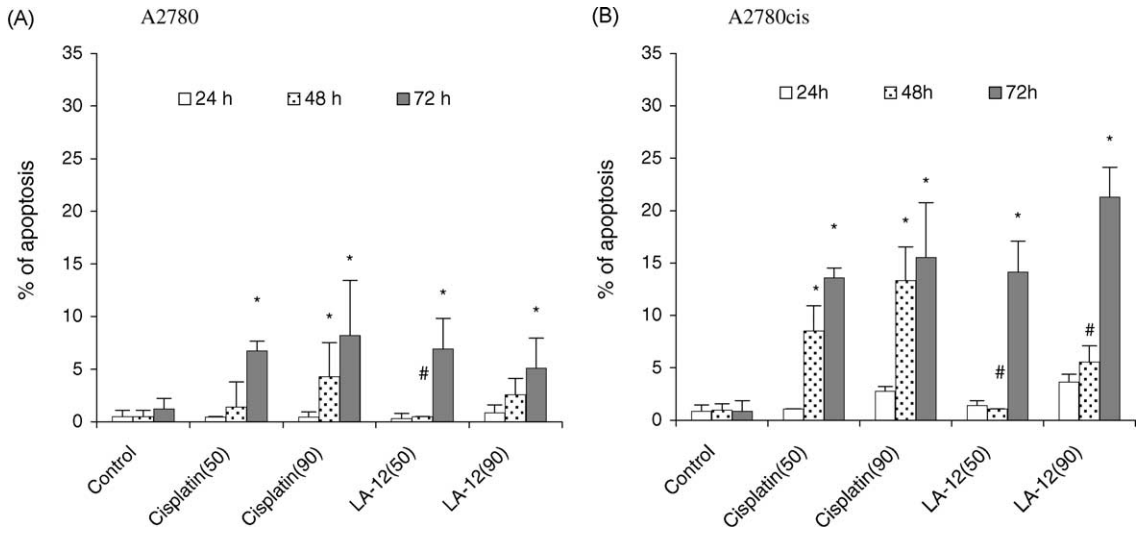


Fig. 6. The percentages of apoptotic cells (apoptotic index) as determined by DAPI staining. The A2780 or A2780cis cells were untreated (control) or treated with IC_{50} and IC_{90} concentrations of cisplatin or LA-12 and harvested at 24 h, 48 h, and 72 h. The results are expressed as mean ± standard deviations (S.D.) of at least three independent experiments. The symbols (*), (×) denote significant difference ($p < 0.05$) from untreated control; (#) denote significant difference ($p < 0.05$) between equitoxic cisplatin and LA-12 concentrations.

role [5,6]. This suggests that the greater lipophilic nature of Pt(IV) compounds [4] might help to overcome the cisplatin resistance that is primarily due to a decreased Pt accumulation.

Activation of cell cycle checkpoints is a general cellular response after exposure to cytotoxic agents. These checkpoints become activated in order to enable some critical cellular functions, such as DNA repair, to be performed before the cell cycle may resume [19]. Previous flow cytometric studies have indicated that cisplatin and other platinum agents predominantly inhibit cell cycle progression at S- and/or G_2/M-phase, independent of drug concentration in a range of (1–5) × IC_{50} [20]. Our results point out possible reasons of differences between cisplatin and LA-12 at the level of cell cycle regulation and cell death induction. We detected a different type and dynamics of cell cycle perturbation of these compounds. Moreover, differences were observed between the response of cisplatin sensitive A2780 and resistant A2780cis cells. While cisplatin blocked sensitive A2780 cells in the G_2/M-phase and resistant A2780cis cells in the S-phase, LA-12 blocked both types of cells in the S-phase only. The cell cycle arrest was found to be transient, which is a typical response to a number of cytotoxic agents, including cisplatin [20,21]. Our findings are consistent with other reports describing transient S- and G_2/M-phase arrests after other platinum-containing agents in a number of cell lines [20,22] or slowed progression through the S-phase following cisplatin treatment [23]. The differences in the extent and duration of G_2/M arrest observed in studies with different cell lines could depend on differences in the cellular capacity for DNA repair [24].

Our previous results showed that a high concentration (10 μM) of LA-12 can induce rapid (4 h) apoptosis in colorectal cancer cells HCT116 [4]. However, the amount of apoptotic ovarian cancer cells after treatment with IC_{50} and IC_{90} doses of LA-12 was low and similar to cisplatin. In general, it was markedly higher in cisplatin resistant A2780cis cells. No detected "DNA ladder" and the appearance of smears of non-specifically degraded DNA confirmed the induction of a low rate of apoptosis in both

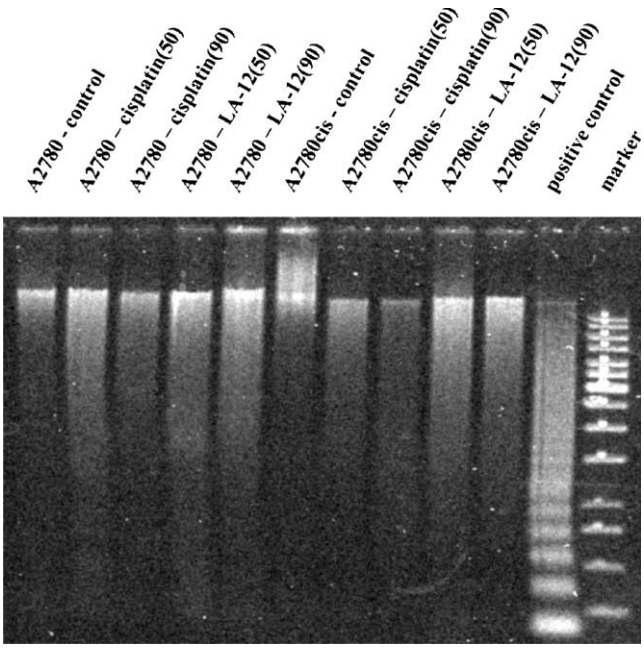
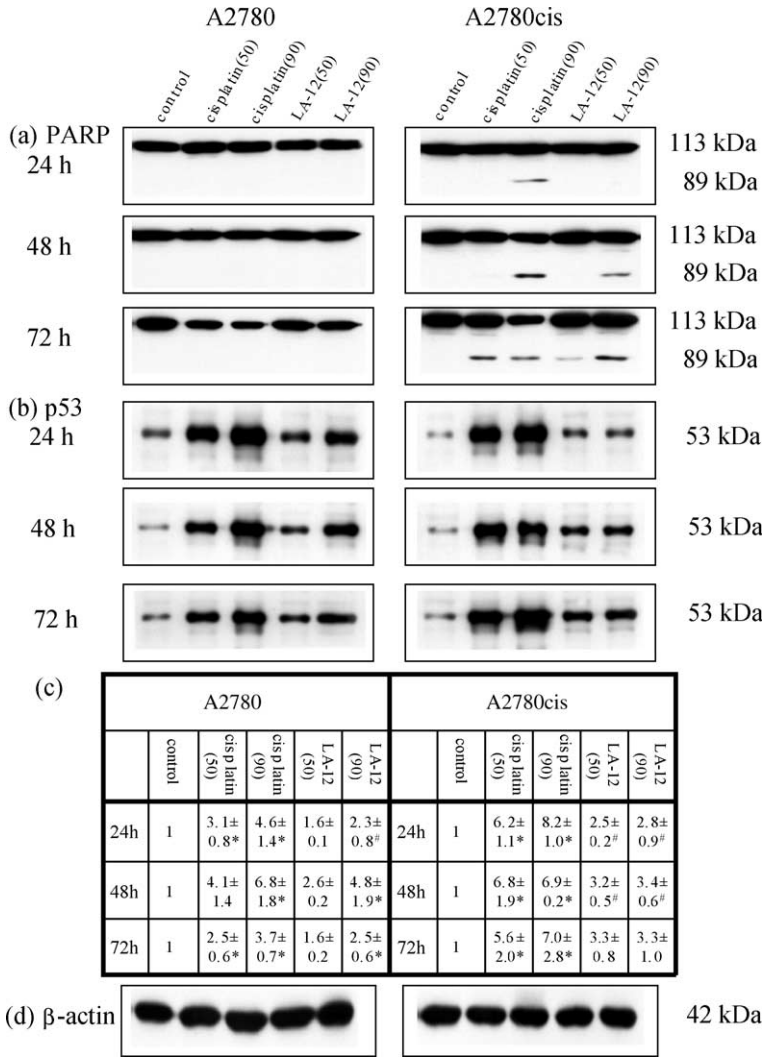


Fig. 7. Agarose gel electrophoresis of genomic DNA isolated from untreated control and cisplatin or LA-12 treated A2780 and A2780cis cells for 72 h with IC_{50} or IC_{90} concentrations of the drugs. The extracted DNA was subjected to gel electrophoresis. One representative experiment out of three is reported. Positive control (HL-60 cells treated with 10 μM etoposide for 4 h).



A2780	control	cis p latin (50)	cis p latin (90)	LA-12 (50)	LA-12 (90)
24h	1	3.1± 0.8*	4.6± 1.4*	1.6± 0.1	2.3± 0.8#
48h	1	4.1± 1.4	6.8± 1.8*	2.6± 0.2	4.8± 1.9*
72h	1	2.5± 0.6*	3.7± 0.7*	1.6± 0.2	2.5± 0.6*

A2780cis	control	cis p latin (50)	cis p latin (90)	LA-12 (50)	LA-12 (90)
24h	1	6.2± 1.1*	8.2± 1.0*	2.5± 0.2#	2.8± 0.9#
48h	1	6.8± 1.9*	6.9± 0.2*	3.2± 0.5#	3.4± 0.6#
72h	1	5.6± 2.0*	7.0± 2.8*	3.3± 0.8	3.3± 1.0

Fig. 8. Western blot analyses of (a) PARP (upper panel) and (b) p53 (middle panel) protein levels in A2780 (left panel) and A2780cis cells (right panel). The cells were not exposed (untreated controls) or exposed to IC_{50} or IC_{90} concentrations of cisplatin or LA-12 and harvested at 24 h, 48 h, and 72 h of sustained drug treatment. One representative experiment of at least three is presented. Table (c) contains values of p53 expression quantified by densitometry (mean ± S.D. of at least three independent experiments). The symbols (*) denote significant difference ($p < 0.05$) from untreated control; (#) denote significant difference ($p < 0.05$) between equitoxic cisplatin and LA-12 effects. Equal loading is documented by detection of (d) β-actin (bottom panel).

studied cell lines after exposure to both drugs. To clarify this type of apoptotic response in more detail, cleavage of PARP and expression of p53 protein were investigated.

PARP, a 113-kDa nuclear protein, has been among the first proteins shown to be specifically cleaved during apoptosis [25]. PARP cleavage to the 89 kDa fragment was observed in diverse models of apoptosis [26–28] and it is considered to be a sensitive parameter of apoptosis even when only limited numbers of cells in the total population undergo apoptosis [29]. In our experiments, time- and concentration-dependent PARP cleavage was detected only in cisplatin resistant A2780cis cells. Moreover, it was detected earlier after cisplatin than after LA-12 treatment.

Our results corresponded to the fact that although A2780 cells are the most sensitive ovarian cell line to cisplatin, they show the lowest percentage of apoptosis (6–14%) [30] and display low levels of DNA degradation [31]. They are also in agreement with Henkels and Turchi [31], who reported different processing of apoptosis between the sensitive A2780 and resistant A2780cis cell lines in response to cisplatin. While in A2780 cells low caspase-3 activation and a very small increase in cytoplasmic cytochrome *c* was detected, in resistant A2780cis cells a dramatic increase of both parameters was observed. Moreover, they have found evidence for an alternative apoptotic pathway in A2780 cells by demonstrating increased FADD expression in response to cisplatin treatment. This supports a model in which cisplatin-induced programmed cell death in the cisplatin-sensitive A2780 and -resistant A2780cis cells proceeds via caspase-3 independent and -dependent pathways, respectively. The lack of DNA fragmentation in our experiments may also be due to the fact that apoptosis may occur in earlier time points. Thus, after 72 h, we

observed mainly secondary necrosis (our unpublished results from PI/Hoechst viable staining and evaluating by fluorescence microscopy). Therefore, no apoptotic DNA could be extractable with a consequence of no intranucleosomal fragmentation with typical DNA laddering on gel.

Since p53 protein is implicated in apoptosis induction and modulation of the cell cycle, as a result of cellular response to DNA damage [32], we analysed whether the cell cycle perturbations and induction of cell death by cisplatin or the LA-12 complex in A2780 and A2780cis cells were associated with changes in the expression levels of this protein. An accumulation of p53 in response to cisplatin that could be connected with its prolonged half-time [33] has been observed in a majority of tumour cells, thus supporting the view that the wild-type p53 protein may inhibit cell growth to allow DNA repair, and in case of irreparable damage, initiate apoptotic cell death [34]. Our data showed that while the A2780 and A2780cis cell lines appear to differ in their p53-dependent apoptotic response to Pt-DNA adducts, transient S or G_2/M arrests in the cell cycle displayed no apparent correlation with p53 induction. The levels of p53 expression remained elevated during the whole interval of cisplatin or LA-12 treatment with IC_{50} as well as IC_{90} concentrations in both A2780 and A2780cis cells. Moreover, higher levels of p53 expression were found in both cell lines after treatment with cisplatin compared to equitoxic concentrations of LA-12. This may be due to the fact that cisplatin caused primarily DNA damage and the biochemical mechanisms of its cytotoxicity involve the binding of the drug mainly to the DNA in the cell nucleus and the subsequent induction of cell death. This may occur not only via apoptosis [35] but in our ovarian cancer cell lines A2780 and A2780cis also via some kind of alternative cell death as seen from results of Henkels and Turchi [31]. They postulated caspase-3-dependent and -independent pathways in cisplatin induced apoptosis for these cells. Both ovarian cancer cell lines (parental and resistant) treated with cisplatin or LA-12 exhibited either signs of apoptosis (DAPI staining) or necrosis and secondary necrosis (PI/Hoechst 33342 viable staining evaluated by fluorescence microscopy, our unpublished results) as demonstrated in our experiments, in selected time-points. On the other hand, the mechanism of the LA-12 effect may predominantly involve the binding of the drug also to non-DNA targets such as cytoplasmic and nuclear proteins producing DNA-protein crosslinks (our unpublished results). Differences in cell cycle perturbations and lower expression of p53 after LA-12 versus cisplatin treatment are very important findings and they are currently under investigation in our laboratory.

In summary, the Pt(IV) adamantane complex LA-12 is characterized by significantly higher cytotoxicity than cisplatin in both parent cisplatin sensitive A2780 and cisplatin resistant A2780cis ovarian cancer cell lines and overcomes the acquired resistance to cisplatin. We conclude that apoptosis is probably not the major type of cell death caused by LA-12 in doses around IC_{50} and IC_{90} in these cell lines, even though this platinum complex strongly increases the expression level of p53 protein. These facts could be associated with cell cycle perturbations. An important finding is the different dynamics of LA-12 effects on cell cycle and apoptosis compared to cisplatin. Based on our results, octahedral platinum(IV) derivative, LA-12, was selected for further evaluation, in which a more detailed analysis of cell cycle modulation and cell death pathways will be realized.

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