LA-12 Overcomes Confluence-dependent Resistance of HT-29 Colon Cancer Cells to Pt (II) Compounds

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Abstract. *Background: LA-12 is a new platinum (IV) drug with promising cytotoxic effects in a wide range of cancer cell lines. Its confluence-dependent effects were compared with cisplatin (CDDP) and oxaliplatin (L-OHP) in HT-29 cells. Materials and Methods: Cytotoxicity was determined by MTT test, eosin exclusion assay, and cell number quantification. The cell cycle was analysed using propidium iodide DNA staining (flow cytometry), apoptosis by phosphatidylserine externalisation (annexin-V assay), mitochondrial membrane potential by flow cytometry, nuclear morphology by means of fluorescence microscopy, and PARP cleavage by Western blotting. Results: While L-OHP and CDDP were practically inactive in the subconfluent cell population, LA-12 showed a similar toxicity in both subconfluent and growing populations. All compounds induced apoptosis, although with different potentials. Conclusion: LA-12 was able to overcome confluence-dependent resistance of HT-29 cells observed for other platinum compounds, which may have potential therapeutic use in slowly growing tumours.*

The platinum (II) derivatives cisplatin (CDDP) and oxaliplatin (L-OHP) have been used in the therapy of many types of cancer including testicular, ovarian, colon, lung, head and neck tumours (1-4), but their negative side-effects, as well as intrinsic or acquired cell resistance (3), limit their therapeutic application. A new platinum (IV) derivative, LA-12 (OC-6-43-*bis*(acetato) (1 adamantylamine)amminedichloroplatinum (IV)), has the potential to overcome some of these limitations and has been shown to exert a rapid and strong cytotoxic effect, without cross-resistance in a panel of cisplatin-sensitive and -resistant cell lines of different origin (5-7). In preclinical *in vitro* and *in vivo* studies, LA-12 had lower negative side-effects and was more effective against tumour xenografts than satraplatin (8-10).

Apoptosis is particularly disrupted in cancer cells, which are characterized by acquired resistance to apoptosis. In these cells, defects in apoptosis signalling pathways may cause non-responsiveness to anticancer therapy or limited efficacy of different chemotherapeutic agents (11, 12). It has been shown that some adherent cell lines are more resistant to toxic compounds when cell culture density reaches confluence *in vitro* (13, 14). This confluence-dependent resistance can be explained by a decrease in intracellular drug accumulation and/or by a decrease/loss of cell sensitivity due to changes in intracellular signalling pathways (15).

This study aimed to investigate cytotoxic response, cell cycle perturbations, and apoptosis induced by LA-12 in an HT-29 human colon adenocarcinoma cell line at various degrees of confluence, and to compare these effects with those exerted by cisplatin and oxaliplatin.

Materials and Methods

Cells. Human colon adenocarcinoma HT-29 cells (ATCC; Manassas, VA, USA) were cultured in McCoy's 5A medium (Sigma Aldrich Corp., St. Louis, MO, USA) supplemented with gentamycin (50 mg/l; Serva Electrophoresis GmbH; Heidelberg, Germany) and 10 % heat-inactivated foetal calf serum (FCS; PAN Biotech GmbH; Aidenbach, Germany) under standard conditions (37˚C; 5% CO_2; 95% humidity). The cells were passaged twice a week after exposure to trypsin/EDTA solution.

Platinum derivatives. LA-12 (PLIVA-Lachema A.S.; Brno, Czech Republic), CDDP (Sigma Aldrich Corp.), L-OHP – (PLIVA-Lachema A.S.) were dissolved in DMSO (Sigma Aldrich Corp.)

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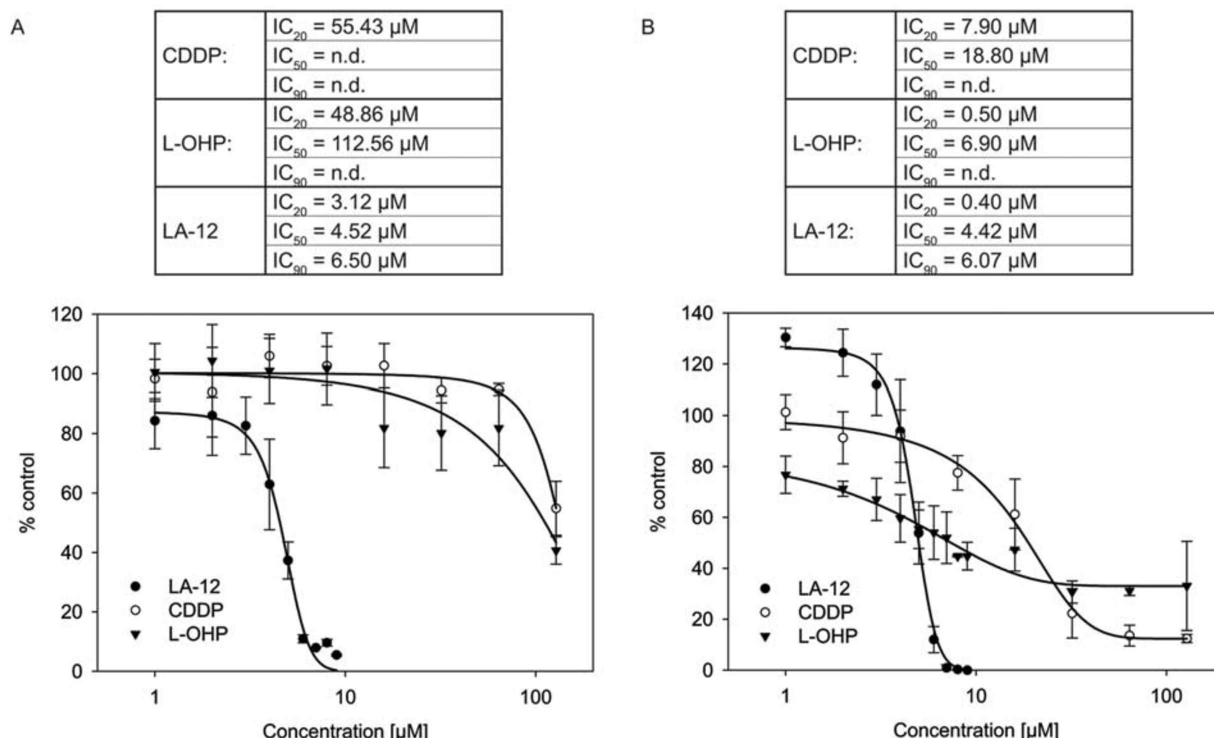

Figure 1. *Time and dose–response effect on the survival of HT-29 cancer cells in different confluence: A: subconfluent population (180×10^3/cm^2); B: growing population (50×10^3/cm^2). The effects after 72 h exposure to CDDP, L-OHP and LA-12 within the concentration range of 1-128 μM were determined by MTT assay. The calculated drug concentrations inhibiting metabolic activity of cells by 20 % (IC_{20}), 50% (IC_{50}), and 90% (IC_{90}) were determined for all derivatives. The results are expressed as the mean±standard deviations (S.D.) of at least three independent experiments. n.d., Not detected.*

freshly before use; the final concentration of DMSO in cell culture medium did not exceed 0.2%.

Cytotoxicity assay. The design of the assay was based on a previously published protocol (6). The cells were seeded at a density of 50×10^3 (the 'growing population') and 180×10^3 cells/cm^2 (the 'subconfluent population'). The concentration range of the drugs used was 1-128 μM. Cytotoxic effects were expressed as IC_{20}, IC_{50} and IC_{90}, relating to 20%, 50% and 90% inhibition of the metabolic activity of the cells, respectively.

Cell number, floating cell quantification and viability assay. Twenty-four hours after seeding (50×10^3 and 180×10^3 cells/cm^2), the cells were treated (24, 48, 72 h) with IC_{20}, IC_{50}, and IC_{90} concentrations of the drugs or vehicle (DMSO). Attached and floating cell numbers were determined separately using a Coulter Counter ZM (Beckman-Coulter; Fullerton, CA, USA). The proportion of floating cells was expressed as percentage of the total cell number. Total cell viability was analysed by light microscopy after staining with 0.15% eosin.

Cell cycle. The cells were seeded at 50×10^3 cells/cm^2 concentration for 24 h, treated with IC_{20} and IC_{50} concentrations of drugs or vehicle, harvested by trypsinisation at each time point, washed with phosphate-buffered saline (PBS), fixed in 70% ethanol at 4˚C, and stored at –20˚C until analysis. Fixed cells were stained by propidium iodide and analysed using flow cytometry (FACSCalibur; Becton Dickinson; San Jose, CA, USA), as described previously (16).

Apoptotic nuclear morphology. For apoptotic nuclear morphology detection by fluorescence microscopy, the cells were fixed in ethanol, stained by 4,6-diamidino-2-phenyl-indole (DAPI; Fluka, Sigma-Aldrich Corp.), and mounted on slides as described previously (5). The results were percentages of the cells with the characteristic apoptotic condensation and fragmentation of nuclear chromatin, evaluated from a minimum of 300 cells per sample.

Western blot analysis. Electrophoresis, Western blot analysis, and immunodetection were performed in accordance with a previously published protocol (6), using primary rabbit polyclonal anti-PARP antibody (#sc-7150, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature (RT), and secondary anti-rabbit antibody (#NA934, 1:6000; GE, Amersham Biosciences, Buckinghamshire, UK) for 1 h at RT.

Detection of mitochondrial membrane potential (MMP). The changes of MMP were analysed by flow cytometry using tetramethylrhodamine, ethyl ester, perchlorate (TMRE; Invitrogen, Carlsbad, CA, USA) in adherent cells treated for 48 h by IC_{50} concentrations of the compounds studied, as previously (17).

Phosphatidylserine (PS) externalisation (annexin-V assay). The cells were treated for 48 h with IC_{50} concentrations of the compounds studied and were washed with PBS. A total of 2×10^5 cells per sample were resuspended in a total volume of 100 μl of the incubation buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl, 5 mM $CaCl_2$). Annexin-V-Fluos (0.5 μl per sample; Roche Diagnostics GmbH, Mannheim, Germany) and propidium iodide (final concentration 20 μg/ml) were added, and the cell suspension was incubated for 20 min in the dark. Fluorescence was then measured using flow cytometry. The data were evaluated using the CellQuest Software (Becton Dickinson) and expressed as percentage of the cells positive for annexin-V and negative for propidium iodide (apoptotic cells).

Statistical analysis. The data were expressed as means±SD and analysed by the Tukey's range test or Student's *t*-test. When the variances were not homogenous, the non-parametric Mann-Whitney *U*-test was used. *P*-values of less than 0.05 were considered significant.

Results

Cytotoxicity of all compounds was determined by MTT assay. Only LA-12 was cytotoxic (IC_{50}=4.52 μM) in the subconfluent population (Figure 1A). CDDP had no effect and the effect of L-OHP was very weak (IC_{50}=112.56 μM). In the growing population (Figure 1B), all compounds elicited toxicity, with LA-12 being the most potent (IC_{50}=4.42 μM). The effects of L-OHP resembled those of LA-12 (IC_{50}=6.90 μM), but at least 30% of the activity of control cells remained unsuppressed. CDDP was less toxic than the other two compounds (IC_{50}=18.80 μM).

For further experiments, the growing population and equitoxic concentrations IC_{20} and IC_{50} were selected to compare the effects of the compounds. All derivatives caused a significant decrease in the numbers of adherent cells (Figure 2A). CDDP and LA-12 were responsible for a strong increase in the percentage of floating cells, in a similar manner at both IC_{20} and IC_{50} concentrations (Figure 2B). A significantly lower effect of L-OHP was apparent compared to the other two agents at both concentrations used.

After 24 h, IC_{20} concentrations of all compounds caused accumulation of cells in the S phase of the cell cycle (about 60% of all cells) (Figure 3). At later time points (48 and 72 h), this effect was still apparent in CDDP- and LA-12-treated cells, and was further accompanied by an increase in the G_2/M phase (about 20% at 48 h and about 30 % at 72 h). On the contrary, the delay in the S-phase caused by L-OHP was only transient (about 30% at 48 h and 72 h) and was followed by an increase in the G_2/M phase (about 30% at both time points).

The effect of IC_{50} of CDDP, L-OHP, and LA-12 was studied on parameters reflecting HT-29 cell apoptosis. The most pronounced effects on PS externalisation and decrease of MMP after 48 h were apparent in CDDP-treated cells compared to those treated with L-OHP or LA-12 alone (Figure 4A, B). Furthermore, a significant cleavage of PARP and apoptotic changes of nuclear morphology were detected after treatment (48 h) with both CDDP and LA-12 (Figure 4C, D), and even more enhanced after 72 h, and were more apparent compared to L-OHP-treated cells (72 h) (Figure 4C, D).

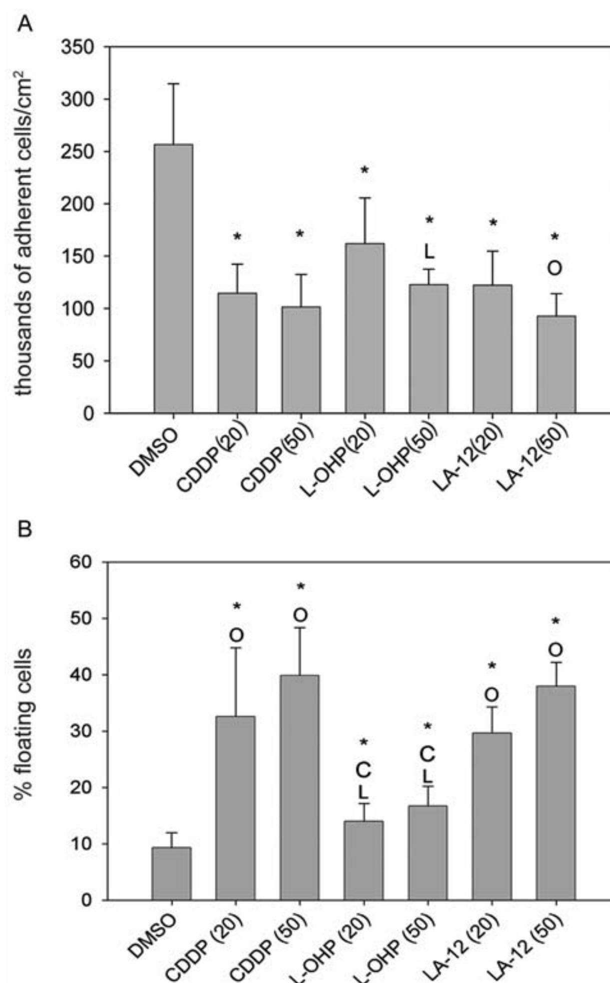

Figure 2. *Effects of equitoxic concentrations of CDDP, L-OHP, and LA-12 on cell numbers (A) and on the percentage of floating cells (B). The cells were treated (72h) with DMSO (vehicle) or IC_{20} and IC_{50} concentrations of platinum derivatives. The results are expressed as a mean±standard deviations (S.D.) of at least three independent experiments. The symbols denote significant difference (p<0.05): * from untreated control (DMSO); C, from equitoxic concentration of CDDP; O, from L-OHP and L, from LA-12.*

Discussion

In the present study, the effects of LA-12 on proliferation and death of HT-29 cells were studied and compared to those of CDDP and L-OHP. The toxicity of all compounds was determined in two different populations, which were

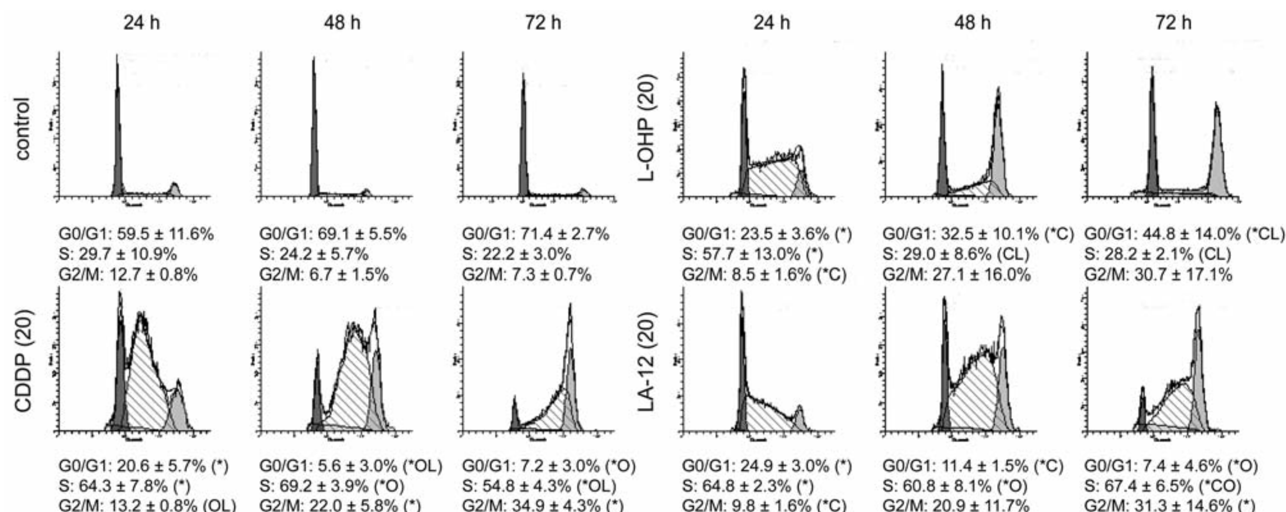

Figure 3. *Effects of IC_{20} concentrations of CDDP, L-OHP, and LA-12 on HT-29 cell cycle distribution detected (flow cytometry) after 24, 48, and 72 h of treatment. Representative histograms (G_0/G_1-phase, dark grey; S-phase, shaded filling; and G_2/M-phase, light grey) are presented. The results 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 (DMSO); C, from equitoxic concentration of CDDP; O, from L-OHP and L, from LA-12.*

designed to mimic miscellaneous cell subpopulations in a solid tumour in different phases of growth: the exponential phase of growth with a high ratio of cells in the S-phase, and the near to plateau phase of growth with cells mainly in the G_0/G_1 phase of the cell cycle. Solid tumours are characterized by heterogeneous composition, arising from not only different cell types presented in such tumours, but also a different cytokinetic state (18). It is known that cells with different growth rates are diversely sensitive to toxic compounds (13, 14). Moreover, HT-29 cells exhibit confluence-dependent resistance to many drugs, including CDDP (15). This study showed that LA-12 was the only drug exerting cytotoxicity in the subconfluent population, and the most effective compound towards growing cells. Due to the ineffectiveness of CDDP and L-OHP in the subconfluent population, the subsequent experiments were performed using the growing cell population.

A similar decrease of adherent cell numbers and increase in the percentage of floating cells was observed after treatment with equitoxic concentrations of CDDP and LA-12, whereas the effect of L-OHP was significantly weaker. This may indicate a different mechanism of action of L-OHP in HT-29 cells. In a previously published study with ovarian cancer cells, equitoxic concentrations caused similar effects on cell numbers (6).

DNA damage usually blocks progression through the cell cycle in different cell types. The checkpoints in the G_1, S and/or G_2/M phases are activated to allow DNA repair or to induce apoptosis (19, 20). Platinum derivatives are known to cause transient or stable blocks of cell cycle in the S and/or G_2/M phases depending on dose and time (5, 6, 21, 22). In this study, IC_{20} of CDDP and LA-12 blocked the cell cycle in the S phase, whereas L-OHP caused only a transient delay in the S phase turning to higher percentage in the G_2/M and G_0/G_1 phases, which is in accordance with the results of Arnould *et al.* (23).

This study showed that LA-12, CDDP and L-OHP were capable of inducing apoptosis in HT-29 cells, though with different intensities. CDDP was shown to be the most potent inducer of apoptosis, as demonstrated by the increase of the number of cells with PS externalisation, and decreased MMP, relatively early markers of this type of cell death. However, changes of the parameters reflecting the later phases of apoptosis, such as specific PARP cleavage and nuclear chromatin fragmentation, were similar in both CDDP- or LA-12-treated cells after 48 and 72 hours. It had been previously shown that LA-12 induced apoptosis in a different manner than did CDDP in ARN-8 and MCF-7 cells, and LA-12 caused massive apoptosis in shorter times and lower concentrations than CDDP. It was associated with a different induction of *p53* and $p21^{WAF1}$ and no significant phosphorylation of p53 at Ser 15 and Ser 392 in contrast to CDDP (24). Furthermore, a partially different gene expression profile was observed after treatment with LA-12 compared to CDDP (25). Previously published data also showed differences between LA-12 and CDDP effects in the ovarian CDDP-sensitive/-resistant cell system (5, 6). These findings indicate that LA-12 may cause apoptosis through different pathway(s) than CDDP, and the effects seem to be cell type-specific. In all cases, L-OHP exerted the lowest apoptotic effects compared to LA-12 and CDDP.

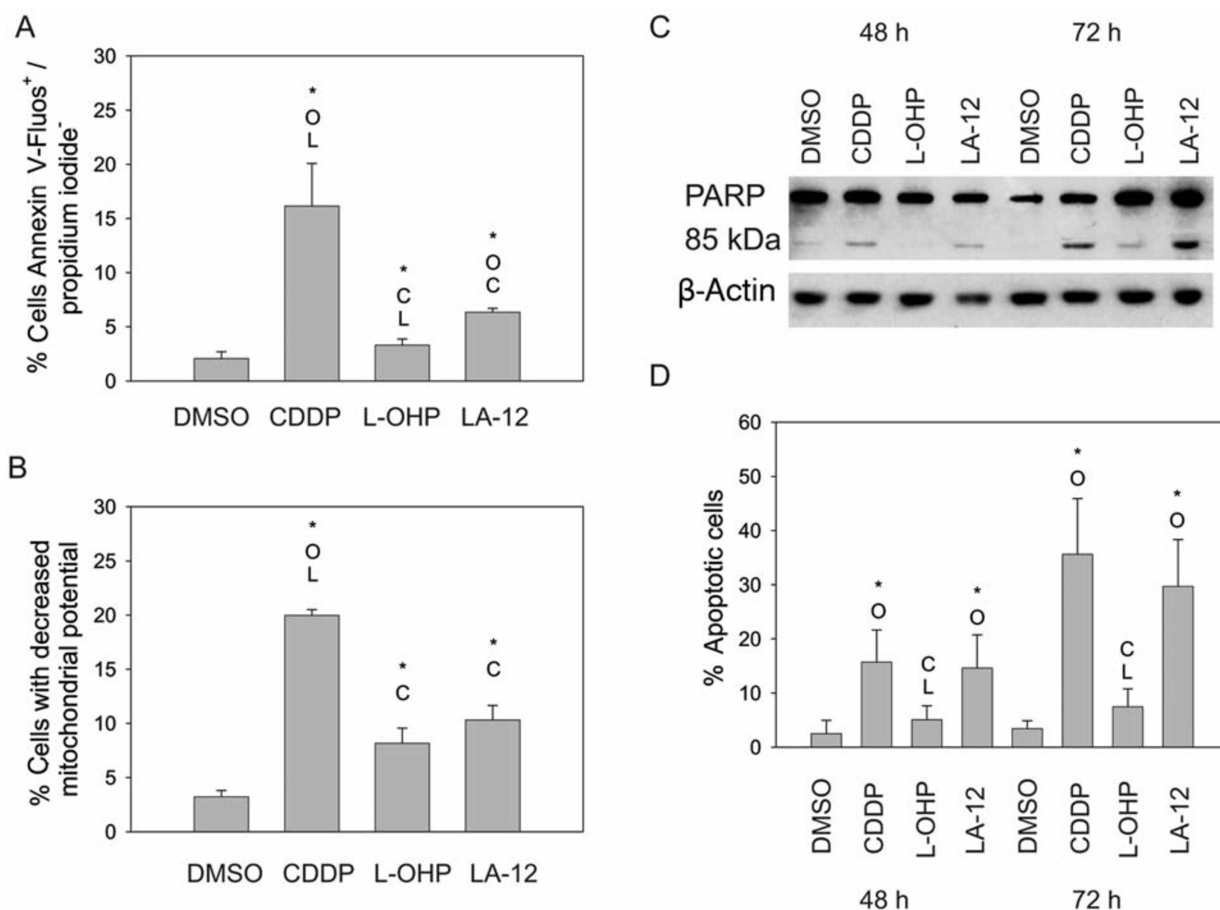

Figure 4. *Effects of IC_{50} concentrations of CDDP, L-OHP, and LA-12 on HT-29 cell apoptosis. Percentages of apoptotic cells with PS externalisation (A) and decreased MMP (B) after 48 h treatment. The results are expressed as mean±standard deviations (S.D.) of at least three independent experiments. C: PARP cleavage after 48 and 72 h treatment. Equal loading was verified using anti-beta-actin antibody. One representative experiment of at least three performed is presented. D: Percentage of cells with apoptotic nuclear morphology after 48 and 72 h treatment. The results are expressed as a mean±S.D. of at least three independent experiments. The symbols denote significant difference ($p<0.05$): * from untreated control (DMSO); C, from equitoxic concentration of CDDP; O, from L-OHP and L, from LA-12.*

In conclusion, LA-12 overcame confluence-dependent HT-29 cell resistance to CDDP and L-OHP, which may have potential therapeutic use in slowly growing solid tumours. Data in this study also supported previous observations showing that LA-12 is able to cause apoptosis in many cell types but in a manner different from that of CDDP. Understanding the mechanisms of LA-12 action is beneficial to the development of new cancer treatment strategies, especially in the case of otherwise resistant cancer cells.

Acknowledgements

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