Higher anti-tumour efficacy of platinum(IV) complex LA-12 is associated with its ability to bypass M-phase entry block induced in oxaliplatin-treated human colon cancer cells

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Abstract

Objectives: Therapeutic potential of conventionally used platinum-based drugs in treatment of colorectal tumours has been limited due to high incidence of tumour resistance to them and to their severe side effects. This evokes a search for more suitable anti-cancer drugs. We have compared ability of oxaliplatin and a novel platinum(IV) complex, LA-12, to modulate the cell cycle and induce apoptosis in human colon adenocarcinoma HCT116 wt and p53/p21 null cells, and have investigated molecular mechanisms involved.

Materials and methods: Cell cycle-related changes were analysed by flow cytometry (bromodeoxyuridine/propidium iodide staining, histone H3 phosphorylation). Apoptosis was detected using flow cytometry (assays monitoring caspase activity) and fluorescence microscopy (nuclear morphology). Changes in levels of genes/proteins involved in cell cycle and apoptosis regulation were examined by RT-PCR and western blotting.

Results: Our results highlight the outstanding ability of LA-12 to induce effective elimination of colon cancer cells independently of p53/p21, and in significantly lower doses compared to oxaliplatin. While oxaliplatin induced p53- and p21-dependent G₂-phase arrest associated with downregulation of cyclin B1 and Cdk1, LA-12 allowed cells to enter

M-phase of the cell cycle regardless of p53/p21 status.

Conclusions: Higher malignant cell toxicity and ability to bypass cell cycle arrest important for the cell damage repair suggest LA-12 to be a more effective candidate for elimination of colon tumours from a variety of genetic backgrounds, compared with oxaliplatin.

Introduction

Platinum-based drugs have been used for treatment of various types of solid tumours for more than four decades. Cisplatin was the first platinum complex introduced in clinical practice and nowadays, it is still indicated for therapy of tumours such as those of the testis and ovary (1). However, its application can be complicated due to severe side effects to healthy tissue and to tumour cell resistance. Thus, other platinum complexes have been included in anti-tumour therapy and the search for novel more suitable candidates is still ongoing. Oxaliplatin can exert its toxicity on various cisplatin-resistant cancer cells and is preferentially used for first-line therapy for advanced gastrointestinal malignancies, especially colorectal tumours (in combination regimens with 5-fluorouracil/leucovorin or capecitabine) (2,3). Vast numbers of other platinum derivatives have been synthesized and tested for anti-cancer effects over the years, but few have been preclinically studied and reached clinical trials (1). Satraplatin (JM-216), a platinum(IV) complex, had anti-neoplastic activity, good oral availability and weaker side effects, but failed to improve overall survival of hormone refractory prostate cancer in clinical trials (4). LA-12 is a platinum(IV) complex with non-leaving bulky hydrophobic ligand

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adamantylamine (5). It has been shown to overcome both acquired and intrinsic resistance to cisplatin in a panel of cancer cell lines, especially in ovarian A2780cis (6), SK-OV-3 (7) and colon HT-29 (8) cells. LA-12 also displayed superior cytotoxicity to cisplatin and satraplatin in mouse xenograft models (9,10). Its outstanding *in vitro* and *in vivo* anti-tumour action may be based on its good bioavailability after oral administration, high tissue concentration (11,12), enhanced cellular accumulation (13,14) and DNA-protein cross-linking, stronger inhibition of DNA polymerization and lower DNA repair (13) compared to cisplatin. However, detailed molecular mechanisms involved in regulation of LA-12 action have still needed to be elucidated.

Formation of DNA crosslinks (intrastrand and inter-strand) represents a widely accepted mechanism of cytotoxicity of platinum-based drugs (15). They hamper normal progression of DNA replication and transcription, and activate DNA damage response pathways, in which various kinases are implicated, in particular ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), Chk1 and Chk2 (checkpoint kinase 1, 2) (16). Activation of these pathways leads to halting progression of the cell cycle in G₁, S or G₂ phases, providing time for DNA repair. If damage is extensive and the cell is not able to further proliferate, senescence or cell death are induced. p53 protein is a key component of DNA damage response pathways, being involved in cell cycle control, DNA repair and cell death by modulation of gene expression (16,17) or by direct protein–protein interactions (18). Action of p53 is driven by numerous post-translational modifications (19). The transcriptional target of p53, p21 protein, is an important regulator of cell cycle arrest *via* inhibition of Rb phosphorylation and complexes of cyclins and cyclin-dependent kinases (Cdks), and by impairing DNA replication through interaction with proliferating cell nuclear antigen (PCNA) (20,21). Furthermore, p53 can also slow the cell cycle down by repression of transcription of necessary components of cell cycle machinery (22–25).

Here, we have examined and compared anti-cancer effects of conventionally used oxaliplatin and the novel platinum(IV) complex LA-12, using model human colon adenocarcinoma HCT116 cell line, to discover molecular differences in their regulation of the cell cycle and cell death. Compared to oxaliplatin, LA-12 exerted its cytotoxic effects in significantly lower doses, regardless of cells' p53 or p21 status. Moreover, LA-12-induced toxicity was not associated with p53- and p21-dependent G₂-phase arrest and block in M phase entry, observed in oxaliplatin-treated cells. These properties make LA-12 an interesting candidate for more effective therapy of

colorectal tumours from a variety of genetic backgrounds.

Materials and methods

Cell culture and reagents

Human colon adenocarcinoma cell line HCT116 wt (p53 and p21 wild-type), p53^{-/-}, p21^{-/-} [obtained from Prof. B. Vogelstein, Johns Hopkins University, Baltimore, MD, USA (22, 26)] were maintained in McCoy's 5A modified medium with 1.5 mM L-glutamine (Sigma–Aldrich, St. Louis, MO, USA), supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (both Duchefa Biochemie, Haarlem, The Netherlands), sodium bicarbonate (1.5 g/l; Serva, Heidelberg, Germany), and 10% heat-inactivated foetal bovine serum (PAA, Pasching, Austria). Cells were incubated in a humidified incubator at 37 °C, 5% CO₂ atmosphere, and passaged twice a week by EDTA/PBS washing and trypsinization. Stock solutions of oxaliplatin (L-OHP) [(R,R)-1,2-diaminocyclohexane(ethanedioate-O,O)platinum(II)]; FW 379.29 (PLIVA-Lachema a.s., Brno, Czech Republic); and LA-12 [(OC-6-43)-bis(acetato)(1-adamantylamine) ammine dichloroplatinum(IV)]; FW 552.4 (Platinum Pharmaceuticals, a.s., Brno, Czech Republic) were always prepared freshly before use.

Cell number and viability assay

Cells were seeded (5000; 7500 or 10 000 per cm² for HCT116 wt, p53^{-/-} or p21^{-/-}, respectively) in 40 mm tissue culture dishes (TPP). After overnight incubation, they were treated with oxaliplatin (0.1–5 µM) or LA-12 (0.1–5 µM) for 48 h. Numbers of cells were determined using a Coulter Counter ZM (Beckman-Coulter, Coulter Electronics, Luton, UK). Attached and floating cells were counted separately. Total cell viability (attached + floating) was assessed using propidium iodide (PI) staining and subsequent flow cytometry analysis (FACSCalibur; Becton Dickinson, San Jose, CA, USA). CELLQuestPro software was used for data acquisition and analysis. Ten thousand cells were analysed per sample; PI negative cells were gated as viable.

Flow cytometry detection of M30 CytoDEATH/propidium iodide double-staining

M30 CytoDeath antibody detects specific cleavage of cytokeratin 18 (CK18) by activated effector caspases, a characteristic marker of epithelial cell apoptosis. Cells were harvested, fixed in ice-cold pure methanol at –20 °C for 30 min, washed twice in washing buffer

containing 0.1% Tween 20 in PBS, and incubated in 1% BSA, 0.1% Tween 20 in PBS with M30 CytoDeath fluorescein-conjugated antibody (1:250, #12156857001; La-Roche, Basel, Switzerland), for 1 h RT. Cells were then washed twice in washing buffer and resuspended in PBS containing propidium iodide (5 µg/ml) and RNase (200 µg/ml), incubated at 37 °C for 30 min and analysed by flow cytometry (FACSCalibur) and CELLQuest-Pro software.

Microscope analysis of apoptotic nuclear morphology

Cells were harvested by trypsinization, and floating and adherent cells were washed twice in PBS, fixed in 70% ethanol, and stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Corp.; final concentration 1 µg/ml) for 30 min at room temperature, in the dark. After incubation, cells were centrifuged, mixed with Mowiol 40–88 solution (Sigma-Aldrich Corp.), with DABCO as anti-bleaching treatment of DAPI-stained cells (0.6%, Sigma-Aldrich Corp.), and mounted beneath coverslips. Percentages of cells with condensation and fragmentation of nuclear chromatin, characteristic for apoptosis, were evaluated using fluorescence microscopy (Olympus IX-70; Olympus, Hamburg, Germany), from at least 200 cells per sample.

Cell cycle analysis

Cells were harvested by trypsinization, washed, fixed in 70% ethanol (4 °C) and stored at 4 °C until analysis. After overnight fixation, they were washed in PBS and resuspended in PBS containing propidium iodide (5 µg/ml) and RNase (200 µg/ml) at 37 °C for 30 min, and analysed by flow cytometry (FACSCalibur); minimum of 15 000 events collected per sample. Data were analysed using ModFit LT version 2.0 software (Verity Software House, Topsham, ME, USA).

Analysis of DNA content and synthesis

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdUrd) – analogue of thymidine – was used for labelling newly synthesized DNA in S phase cells. Bromodeoxyuridine (10 µM) was added 45 min before harvesting of untreated or platinum drug-treated cells. They were fixed in 70% ethanol, washed twice in PBS and DNA denatured in 2 M HCl with 0.5% Triton X-100; specimens were neutralized with 0.1 M sodium borate, pH 8.5. Cells were then washed in PBS and incubated with FITC-conjugated anti-BrdUrd antibody (#556028; BD Bioscience Pharmingen, San Diego, CA, USA) in PBS with 1% BSA and 0.1% Tween 20 at 37 °C in the dark

for 4 h. Incubation was followed by two washing steps in PBS, staining with propidium iodide and RNase, and flow cytometry analysis as described above.

BrdUrd labelled cells pulse chase analysis

HCT116 wt cells were seeded in 60 mm dishes and after 24 h, were incubated in BrdUrd (final concentration 10 µM) for 45 min. They were then washed in PBS, and fresh medium was added. Cells were treated with LA-12 (0.3 µM) and harvested every 3 h (0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 h of treatment). BrdUrd positive cells and their DNA content (propidium iodide labelled) were detected as described above. Transit of the BrdUrd-positive population of cells through the cell cycle was monitored using ModFit LT version 2.0 software.

Western blot analysis

Cell lysis (in 1% SDS buffer), protein quantification, electrophoresis and Western blot analysis were performed as previously described (6). Immunodetection was carried out using the following primary antibodies: anti-cyclin B1 (1:500, #245), anti-Cdk1 (1:500, #8395) and anti-p53 (1:750, DO-1, #126) (all Santa Cruz Biotechnology, Dallas, TX, USA), anti-phospho-p53 (Ser15) (1:500, #9284) and anti-p21 (1:1000, #2947) (both Cell Signaling Technology, Danvers, MA, USA), anti-non-phospho-Cdk1 (Tyr15) (1:1000, #1161-1; Epitomics, Inc., Burlingame, CA, USA), and secondary antibodies: anti-mouse IgG (1:4000, #NA931) and anti-rabbit IgG antibody (1:3000, #NA934; both Amersham Biosciences, Piscataway, NJ, USA). Equal loading of samples was verified using anti-β-actin antibody (1:5000, A5441; Sigma-Aldrich Corp.).

RNA isolation and real-time RT-PCR

Total RNA was isolated using High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Sequences of gene-specific primers for combination with Universal ProbeLibrary probes (Roche Diagnostics GmbH) were: CCNB1 (NM_031966.2), F- CAT GGT GCA CTT TCC TCC TT; R- AGG TAA TGT TGT AGA GTT GGT GTC C. Amplifications of samples were carried out, final volume of 20 µl, in reaction mixture containing 10 µl QuantiTect Probe RT-PCR Master Mix, 0.2 µl of QuantiTect RT Mix (Qiagen, Valencia, CA, USA), 2 µl solution of primers and probe, 5.8 µl water and 2 µl RNA per sample. Final concentration of each primer was 0.4 µM and probe was

0.1 μM . Amplifications were run on RotorGene3000 with RotorGene Real-Time Analysis Software (Corbett Research, Sydney, NSW, Australia), using the following program: 50 °C for 30 min reverse transcription and 95 °C for 15 min denaturation of cDNA, followed by cycling (40 repeats) 94 °C for 15 s and 60 °C for 60 s acquiring fluorescence. All PCR reactions were performed in triplicate and changes in gene expression were calculated using the comparative threshold cycle method (27).

Flow cytometry detection of histone H3 phosphorylation and expression of cyclin B1

Phosphorylation of histone H3 on serine 10 correlates with chromatin condensation during mitosis (28). Cells were harvested by trypsinization and fixed in 2% methanol-free formaldehyde in 37 °C for 10 min. They were then spun down and resuspended in PBS; ice-cold methanol was added. After 30-min fixation on ice, cells were washed twice in 0.5% BSA in PBS and incubated in 0.5% BSA in PBS with rabbit polyclonal anti-phosphohistone H3 (Ser10) (#9701S, 1:50; Cell Signaling Technology) or anti-cyclin B1 (#SC-752, 1:50; Santa Cruz Biotechnology) for 1 h. After washing in 0.5% BSA in PBS, cells were incubated in secondary antibody conjugated with Alexa Fluor 488 (A21206, 1:2000; Molecular Probes, Eugene, OR, USA) for 30 min. Cells were incubated with propidium iodide and RNase, and analysed by flow cytometry. For analysis of phospho-H3 expression, propidium iodide staining was employed as control of DNA content (4) in phospho-H3-positive cells.

Statistical data analysis

Data are expressed as mean $\pm$ SD, analysed by ANOVA followed by Tukey test or by nonparametric Mann–Whitney *U*-test. *P* value of less than 0.05 or 0.01 was considered significant, as indicated in figure legends. All statistical analyses were performed using Statistica for Windows software, V. 6.1 (StatSoft, Inc., Tulsa, OK, USA).

Results

Proliferation and death of LA-12- or oxaliplatin-treated HCT116 cells

Following 48-h treatment, platinum(IV) complex LA-12 (0.1–5 μM) induced a rapid dose-dependent reduction in number of adherent HCT116 human colon cancer cells (Fig. 1a) and reduction in their viability (Fig. 1b). These effects were independent of cells' p53 or p21 status –

similar trends were observed in those expressing or lacking these proteins (Fig. 1a,b). In contrast, platinum(II) complex oxaliplatin, applied over the same concentration range, was significantly less toxic to HCT116 cells and its effects on proliferation were even lower to p53^{−/−} and p21^{−/−} cells (Fig. 1c,d). To confirm that the cell death induced by LA-12 and oxaliplatin occurred by caspase-mediated apoptosis, changes in typical parameters associated with this process were detected. Significant time-dependent caspase-mediated cleavage of cytokeratin 18 (CK18) was observed in cells treated with LA-12 (0.5–5 μM) for 12 and for 24 h. In contrast, oxaliplatin caused substantially lower levels of apoptosis, even after longer (24 and 48 h) time of exposure (Fig. 1e). Bivariate analysis of DNA content and CK18 cleavage revealed that both complexes triggered apoptosis predominantly in G₁ and G₂ phases (Fig. 1e). Furthermore, apoptosis induced by LA-12 (5 μM) was independent of p53 and p21 protein as similar numbers of HCT116 wt, p53^{−/−} and p21^{−/−} cells with typical apoptotic nuclear morphology were observed after 48 h treatment (Fig. 1f).

Cell cycle changes induced by LA-12 or oxaliplatin

Based on evaluation of cytotoxic and cytostatic effects (floating/adherent cell numbers, viability, cytotoxicity tests) of the two platinum-based drugs (Fig. 1 and data not shown), comparable toxic doses of oxaliplatin (5 μM) or LA-12 (0.3 μM) were selected for detailed cell cycle analyses. In addition, effects of the lower concentration of oxaliplatin (0.5 μM) were examined in parallel and compared to the 5- μM dose. We were able to demonstrate significant differences in parental HCT116 cell cycle distribution after treatment with LA-12 (0.3 μM) or oxaliplatin (0.5, 5 μM), and further studied roles of p53 and p21 in the effects observed. We found that oxaliplatin triggered p53 and p21-dependent accumulation of cells in G₀/G₁ (0.5 μM) and/or G₂/M phases (5 μM) (Fig. 2a), accompanied by reduced numbers of cells in S-phase. Treatment with LA-12 also resulted in accumulation of cells in G₂/M, but this was significantly less apparent compared to oxaliplatin treatment. LA-12-mediated increase in G₂/M was independent of cell cycle regulatory proteins p53 and p21, and again was associated with reduction in cell numbers in S phase (Fig. 2a).

We investigated in more detail changes in DNA synthesis and cell cycle distribution after treatment with oxaliplatin and LA-12. Before harvesting, cells were incubated in bromodeoxyuridine (incorporated into newly synthesized DNA during replication in the S phase). We found that oxaliplatin-treated cells (5 μM)

Figure 1. LA-12- but not oxaliplatin-induced suppression of proliferation or stimulation of apoptosis in HCT116 cells is independent of p53 and p21 proteins. (a–d) Quantification of adherent HCT116 wt, p53^{-/-} and p21^{-/-} cells and their viability following treatment (48 h) with LA-12 (a, b) or oxaliplatin (c, d) (0.1–5 μ M). Results are mean $\pm$ SD of 3 independent experiments. (e) Cell-cycle phase specific apoptosis (FITC-conjugated M30 CytoDeath antibody/propidium iodide) in HCT116 wt cells treated with LA-12 (0.5, 1 and 5 μ M; 12, 24 h) or oxaliplatin (0.5, 1 and 5 μ M; 24, 48 h). Dot plots of one representative experiment and mean $\pm$ SD (% of cells with cleaved CK18) from 3 independent experiments are shown. (f) Percentage of HCT116 wt, p53^{-/-} and p21^{-/-} cells with apoptotic nuclear morphology (fluorescence microscopy) following incubation (48 h) with LA-12 (5 μ M). Results are mean $\pm$ SD from 3 independent experiments.

Figure 2. LA-12-induced accumulation in G2/M phase was less pronounced compared to oxaliplatin, and did not require p53 and p21 protein. (a) Percentage of HCT116 wt, p53^{-/-} and p21^{-/-} cells in individual cell cycle phases following incubation (6–48 h) with oxaliplatin (0.5 and 5 μM) or LA-12 (0.3 μM). Results are mean ± SD from 3 independent experiments. Statistically significant differences ($P < 0.01$) compared to control are denoted [*]. (b) Flow cytometry analysis of DNA synthesis (BrdUrd incorporation) and DNA content (PI) in HCT116 wt cells treated (12, 24, 48 h) with oxaliplatin (0.5 and 5 μM) or LA-12 (0.3 μM). Bars represent percentage of the cells (mean ± SD) in G₀/G₁, S (synthesizing DNA) or G₂/M according DNA content and BrdUrd positivity. Statistically significant differences ($P < 0.01$) compared to control are denoted [*]. Dot plots from one of three independent experiments are displayed and population of cells in G₀/G₁, S and G₂/M is indicated. (c) Pulse-chase analysis of cell cycle transit of BrdUrd-incubated HCT116 wt cells after LA-12 (0.3 μM) treatment. Plots show percentage of BrdUrd-positive cells (chased population) in G₀/G₁, S and G₂/M phases of the cell cycle, from one representative experiment.

were arrested in G₁ and G₂ and were not able to replicate their DNA (BrdUrd negative) 24 and 48 h after addition of the drug. In contrast, after 24 h of LA-12 treatment, significant numbers of BrdUrd positive cells comparable to controls were detected (Fig. 2b), suggesting that the cells were capable of replicating DNA, regardless of damage sustained. Similar trends and moderate G₂ accumulation could also be observed following 48 h LA-12 incubation (Fig. 2b). For further description of cell cycle modulation caused by LA-12, BrdUrd pulse chase analysis was performed. Chasing the population of cells with incorporated BrdUrd and observation of their cell cycle transit confirmed our previous findings. The HCT116 cell cycle was not halted until 18 h treat-

ment with LA-12. After this time, it slowed and cells transiently accumulated in G₂/M (Fig. 2c).

Different levels of cell cycle regulatory proteins in LA-12- or oxaliplatin-treated cells

While oxaliplatin induced increase in total and phosphorylated (Ser15) p53 and total p21 levels in HCT116 wt cells, no or only slight changes in these parameters were detected in LA-12-treated cells (Fig. 3a). In p21^{-/-} cells, platinum drug-induced increase in phosphorylated p53 was apparent. We further examined expression of selected proteins involved in G₂/M cell cycle checkpoint regulation. Oxaliplatin treatment resulted in reduction in

Figure 2. (Continued)

total protein levels of cyclin B1 and Cdk1, and active Cdk1 (antibody specific to dephosphorylated tyrosine 15) in HCT116 wt, but not in p53- and p21-deficient cells (Fig. 3a). Importantly, no significant reduction in protein levels of cyclin B1 and active Cdk1 kinase were found in LA-12-treated cells (Fig. 3a). Differences in cyclin B1 protein level between LA-12- and oxaliplatin-treated parental HCT116 cells were also confirmed using flow cytometry. Expression of cyclin B1 in oxaliplatin-treated cells rapidly decreased as early as after 24 h, and after 48 h, there were almost no cells with high cyclin B1 expression. In LA-12-treated cells, we observed only minor shifts from high to low/medium expression after 48 h incubation (Fig. 3b). In addition to protein level, we evaluated changes in cyclin B1 mRNA induced by the platinum-based drugs. Amounts of cyclin B1 mRNA decreased after both LA-12 and especially after oxaliplatin treatment compared to controls, and significant differences in cyclin B1 mRNA level were also detected between LA-12- or oxaliplatin-treated cells (Fig. 3c).

Representation of mitosis following treatment with LA-12 or oxaliplatin

As both platinum drugs examined in this study were able to cause different extents of accumulation of cells in G₂/M phase of the cell cycle (Fig. 2a), we also detected different changes in levels of proteins regulating transit through G₂ to M (Fig. 3a). To distinguish between cells in G₂ from cells undergoing mitosis, we employed a method that combines detection of phosphohistone H3 (serine 10) as specific mitotic marker, and DNA staining by propidium iodide. This bivariate analysis revealed that transition of treated cells from G₂ to mitosis is abrogated in the case of oxaliplatin, while insignificant changes (compared to controls) were observed after LA-12 treatment in HCT116 wt cells (Fig. 4). In HCT116 p53^{-/-} and p21^{-/-} cells, no significant alterations of number of mitotic cells after treatment with either of the two platinum complexes were detected (Fig. 4).

Figure 3. Proteins involved in cell cycle regulation, specially G₂/M-phase transit, are differently affected in HCT116 cells following treatment with oxaliplatin or LA-12. (a) Levels of total and phosphorylated (Ser15) p53, p21, total and non-phosphorylated (Tyr15) Cdk1, and cyclin B1 in HCT116 p53 wt, p53^{-/-} and p21^{-/-} cells incubated (24, 48 h) with oxaliplatin (5 μM) or LA-12 (0.3 μM), detected by western blotting. Results are representative of three independent experiments. (b) Flow cytometry analysis of cyclin B1 expression in HCT116 wt cells after incubation (24, 48 h) with oxaliplatin (5 μM) or LA-12 (0.3 μM). Legends "low, medium or high expression" indicate cell populations according to intensity of cyclin B1 expression. Results are mean ± SD from 3 independent experiments. Statistically significant difference (P < 0.05) compared to control is denoted [*]. (c) qRT-PCR analysis of cyclin B1 mRNA levels in HCT116 wt cells treated with oxaliplatin (5 μM) or LA-12 (0.3 μM) for 0, 6, 12, 24 and 48 h. Results are mean ± SD from 3 independent experiments. Statistically significant difference (P < 0.05) between experimental groups is denoted [*].

Discussion

Success of conventional platinum drug-based therapy for solid tumours is limited by development of acquired or intrinsic cancer cell resistance. This resistance may arise as a consequence of reduced cell uptake of the drug, enhanced detoxification and excretion from the cells, increased DNA repair or tolerance of DNA damage, abnormal cell cycle disturbances, aberrant DNA damage response, or apoptotic pathways. Oxaliplatin was introduced to chemotherapy as an alternative drug for cisplatin- and carboplatin-resistant tumours. Its applica-

tion is restricted mainly to colorectal and gastric cancers (29,30) and often hampered by undesirable side effects such as peripheral neuropathy (31). Moreover, oxaliplatin resistance of p53-deficient cancer cell lines *in vitro* has been described (32–37) and lower efficiency of regimens comprising oxaliplatin has been reported in p53 deficient tumours *in vivo* (38). Thus, introduction and testing of novel platinum derivatives capable of overcoming the above-mentioned limitations are highly desirable for more favourable outcomes to the therapy.

Interestingly, we and others have previously demonstrated that the cytotoxic effects of novel platinum(IV)

Figure 4. LA-12 was able to bypass block to M phase entry observed in oxaliplatin-treated HCT116 cells. Percentage of phosphorylated histone H3 positive HCT116 wt, p53^{-/-} and p21^{-/-} cells after treatment (48 h) with oxaliplatin (5 μ M) or LA-12 (0.3 μ M), assessed by flow cytometry. Results are mean $\pm$ SD from 3 independent experiments, *P* values of statistically significant differences between experimental groups are shown.

complex LA-12 on various types of cancer cells can be independent or only partially dependent on p53 status (7,14,39,40). These findings are in agreement with our present results demonstrating that while p53 deficient HCT116 cells, compared to their wild-type counterparts, are significantly less sensitive to the toxic effects of oxaliplatin, both p53 expressing and null cells succumbed to LA-12-mediated cytotoxicity. In addition to its outstanding ability to exert its cytotoxic action even in cancer cells lacking functional p53, here we show that LA-12 can trigger HCT116 cell death at significantly lower doses compared to oxaliplatin. We have also reported similar trends previously in HT-29 colon cancer cells, together with the fact that LA-12 was able to overcome their confluence-dependent resistance to oxaliplatin or cisplatin (8). We have also detected higher cytotoxic effects of LA-12 compared to oxaliplatin in RKO human colon cancer cells (unpublished data). These properties make LA-12 an attractive candidate for effective elimination of colon cancer cells compared to platinum(II) derivatives, and also support its promising effectiveness in tumours with non-functional p53.

Exposure of cancer cells from a variety of origins to platinum-based chemotherapeutic drugs can result in induction of apoptosis, and activate molecules involved in both intrinsic and extrinsic apoptotic pathways (41). Here, we show that LA-12 is a stronger inducer of cas-

pase-dependent apoptosis of HCT116 cells than is oxaliplatin. Oxaliplatin-induced apoptosis has been reported to be associated with mitochondrial translocation of active Bax protein and cytochrome c release, and targeted inactivation of Bax in HCT116 cells resulted in significant increase in their resistance to oxaliplatin (33). We have also suggested involvement of mitochondria, accompanied by significant reduction in mitochondrial membrane potential in HT-29 cells treated with LA-12 or oxaliplatin (8). Our results obtained from bivariate analysis of CK18 cleavage and DNA content indicated that both LA-12 and oxaliplatin mainly induce apoptosis in G₁ or G₂/M phases of the cell cycle. Association between arrest in particular phases and level of apoptosis induced by oxaliplatin has been reported previously (32,36).

Platinum complexes are known to cause DNA damage and activate cell cycle checkpoints, ensuring time for cells to repair DNA lesions. The G₁/S checkpoint prevents replication of damaged DNA. Activated Chk1 targets phosphatase Cdc25a for degradation, which leads to failure in activation of Cdk2 (42). p53-mediated transcription is often essential for maintaining the arrest, and p21 protein inhibits cyclin E-Cdk2 or cyclin D-Cdk4 complex, thereby suppressing the Rb/E2F pathway (20). The G₂/M checkpoint prevents cells from entry into mitosis through inactivation of cyclin B-Cdk1 complex, by subcellular sequestration or degradation and inhibition of Cdc25 family of phosphatases. Maintenance of this checkpoint may also be orchestrated by p53-dependent mechanisms, for example p21, GADD45 and 14-3-3sigma that retain cyclin B-Cdk1 in its inactive form (43). Detailed analysis of the cell cycle was performed in our experimental model to discover molecular differences between action of oxaliplatin and LA-12 with regard to these parameters, and their association with induction of cell death. We were able to show that low concentration of oxaliplatin (0.5 μ M) induced p53/p21-dependent accumulation of cells in G₁. These results are in agreement with Hata *et al.* who demonstrated that ablation of p21 in p53 wt HCT116 cells blocked G₁ arrest and reduced cell population growth inhibitory effects exerted by oxaliplatin (32). It has also been shown by others that introduction of p21 to colon cancer cells with mutated p53 or p53 null, resulted in increased oxaliplatin toxicity (36). Oxaliplatin-mediated G₁ phase arrest has been reported in cells with functional p53 and p21 (32,34,36,44). On the other hand, we demonstrated that incubation of HCT116 wt cells with higher doses of oxaliplatin (5 μ M) resulted in marked increase in percentage of cells in G₂/M, which was partially blocked in the absence of p53/p21. G₂/M accumulation has also been described by others concerning oxaliplatin-treated cancer cells of different origins, including colon (32,34,36,44–47). Importantly, oxalipla-

tin-induced G₁ or G₂/M phase arrest has been shown to be dependent not only on its dose and duration of treatment, but also on cell type and its genetic background. In contrast, no apparent G₁ arrest was evident in HCT116 wt cells treated with LA-12 (0.3 µM). Although LA-12 induced accumulation of cells in G₂/M, it was much less pronounced compared to oxaliplatin, and moreover, independent of p53 and p21 proteins. Increase in percentage of cells in G₂/M following treatment with LA-12 (0.1–1 µM) has also been reported in H1299 lung cancer cells lacking p53 (39).

We observed that significant concentration- and time-dependent increases in HCT116 cell numbers in G₁ and/or G₂/M phase induced by oxaliplatin were accompanied by their simultaneous reduction in S phase, while no such effects were apparent in LA-12-treated cells until after 48 h incubation. This was further supported by our flow cytometry analysis of BrdUrd incorporation showing significant reduction in cell ability to actively replicate DNA following oxaliplatin, but not after LA-12 treatment. These results are in accordance with Volland *et al.* who showed that oxaliplatin-induced G₁ and G₂ arrest was associated with blockage of DNA replication and mitosis, and simultaneous downregulation of expression of genes involved in regulation of these processes (44). In some cases, oxaliplatin/LA-12-mediated increase in G₂/M could be preceded by transient accumulation of cells in S phase, as we have previously reported using the HT-29 colon adenocarcinoma cell line which expresses mutant p53 (8). We have also demonstrated the ability of LA-12 to induce transient/persistent accumulation of A2780 ovarian cancer cells in S phase (48) previously. These observations suggest that effects of LA-12 may depend on tumour type and/or cell context.

The G₂/M checkpoint ensures that cells do not enter mitosis before being provided with time to repair DNA damage after replication and contact with chemotherapeutic drugs. Inability of cells to halt their cell cycle in G₂ may therefore be fatal and result in death during/after cell division. We speculate that differences in percentage of cells arrested in G₂/M detected by flow cytometry following treatment with oxaliplatin and LA-12 may be related to different degrees of toxicity of the two drugs. To shed light on molecular regulation of entry into M phase of cells treated with oxaliplatin or LA-12, we compared levels/activity of essential proteins involved, such as cyclin B1 and Cdk1, and quantified percentage of mitotic cells expressing phospho-histone H3. Cyclin B1 is a main mitotic cyclin and together with Cdk1 represents a crucial component of MPF (mitosis-promoting factor) complex. Cdk1 is effectively regulated by modulations of its tyrosine-15 residue, being deactivated *via*

phosphorylation by Wee1 kinase, and stimulated by phosphatase Cdc25 (49,50). MPF is activated at the end of G₂ phase and promotes entry into mitosis by phosphorylating multiple proteins needed during this cell cycle phase. Thus, tight regulation of level and activity of essential MPF complex components is a vital prerequisite for effective G₂/M phase transit. We have shown that expression of cyclin B1 and total/active Cdk1 was reduced by oxaliplatin, but only in cells expressing p53 and p21, which supports roles of these proteins in oxaliplatin-induced G₂ arrest. It has been shown that oxaliplatin inhibits MPF by p53-dependent downregulation of cyclin B1 (44,45) and expression (32,44,46) or activating phosphorylation of Cdk1 on threonine 161 (45). On the other hand, LA-12 was unable to induce significant reduction in total cyclin B1 level or induce changes in Cdk1 activity observed in oxaliplatin-treated HCT116 and RKO (data not shown) cells, thus enabling them to enter mitosis. This is in good accordance with our results showing that oxaliplatin, but not LA-12, induced significant reduction of percentage of phospho-histone H3-positive (mitotic) HCT116 wt cells. Taken together, we suggest that lower toxicity of oxaliplatin could be at least partially explained by its ability to halt the cell cycle and provide cells time to repair their damage. Compared to oxaliplatin, LA-12 did not cause any significant cell cycle arrest and cells may have not had enough time to recover before entering mitosis.

Our data highlight the ability of platinum(IV) complex LA-12 to induce effective elimination of colon cancer cells in significantly lower doses compared to conventionally used oxaliplatin, which may potentially reduce undesired side effects and help minimize therapeutic doses, when used in future clinical applications. Moreover, in contrast to oxaliplatin, p53 and p21 are not essentially required for apoptosis induced by LA-12, which provides additional therapeutic benefit for tumours with mutated p53. We have demonstrated differential modulation of cell cycle progression in cells treated with LA-12 or oxaliplatin, with the latter being a more potent inducer of cell cycle arrest. Unlike oxaliplatin, LA-12 action was not associated with reduction in cyclin B1 and active Cdk1 level or block in M phase entry of HCT116 colon cancer cells. We suggest that the ability of LA-12 to bypass the block in cell cycle progression observed in oxaliplatin-treated colon cancer cells is associated with their higher sensitivity to its cytotoxic effects when compared to oxaliplatin. The unique properties of LA-12 to differently affect the colon cancer cell cycle and death regulatory mechanisms, compared to conventionally used oxaliplatin, make it a promising candidate for treatment of otherwise refractory colon tumours.

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Conflict of interest

Authors declare no conflict of interest. Petr Sova is an employee of the company owning intellectual property rights regarding LA-12 and co-author of patents in the field of LA-12.

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