The novel platinum(IV) complex LA-12 induces p53 and p53/47 responses that differ from the related drug, cisplatin

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The platinum(II)-based complex cisplatin is one of the most frequently used antitumour agents; however, a high incidence of harmful side effects and the frequent emergence of acquired resistance are the major clinical problems. The novel platinum(IV)-based complex LA-12 exhibits a high efficacy against cancer cell lines, including cisplatin-insensitive cells, but the mechanisms by which LA-12 perturbs cell growth are unclear. We tested the effects of LA-12 on the p53 response and demonstrate that LA-12 induces unique changes in the profile of gene expression compared with cisplatin and doxorubicin. Furthermore, the ability of LA-12 to disrupt cellular proliferation is greatly enhanced by the expression of p53 and p53/47 indicating both p53-dependent and p53-independent effects of LA-12. Exposure of the human cancer cell lines H1299, A2780, BT549 and BT474 to LA-12 alters the expression of p53 and p53/47 in both a time-dependent and dose-dependent manner. Treatment of cells with a low concentration of the drug results in accumulation of p53 and p53/47 concomitant with their posttranslational modification, whereas a high dose results in the disappearance of both the forms of p53. The distinct p53 activation profile of LA-12 compared with cisplatin and doxorubicin provides a molecular explanation for the ability of this drug to treat cisplatin-resistant cells and indicates its potential usefulness as an alternative antitumour agent in first-line therapy or as a second-line therapy in patients with acquired cisplatin resistance. *Anti-Cancer Drugs* 19:369–379 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The square planar, platinum(II)-based complex cisplatin [*cis*-diamminedichloroplatinum(II)] is a potent antitumour agent commonly used in the treatment of testicular and ovarian cancers [1]. The frequent emergence of cisplatin resistance in patients, coupled with the abundance of severe side effects, including nerve and kidney damage [2], has, however, led many investigators to search for analogues and derivatives of the drug. By testing the efficiency of such analogues on cisplatin-resistant cell lines, a series of novel platinum(IV) complexes was reported [3]. One of these candidates (*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV), coded as LA-12, displayed a high efficacy towards many types of cancer cell lines *in vitro* [4,5] and favourable pharmacokinetics in early animal trials [6,7]. Clinical evaluation of this promising antitumour drug is in progress.

The cytotoxicity of platinum-based compounds such as cisplatin has been attributed to their ability to interact with and damage DNA through the formation of DNA–DNA intrastrand and interstrand adducts [2]. Such lesions are detected by DNA damage response proteins, which in turn initiate a series of events that lead to a blockade in cellular proliferation and the induction of apoptosis. As the intermediate pathways linking DNA lesions to the induction of cell death remain to be fully defined, there is a mounting evidence for a role of the tumour suppressor p53, and large-scale studies have demonstrated that the efficacy of the cisplatin-based compounds is closely associated with the presence of functional p53 [8,9].

p53 is a ubiquitously expressed, sequence-specific transcription activator that functions to maintain cellular integrity. Under normal conditions, p53 is present at low cellular levels in a nonactive state; however, in response to cellular insults and DNA damage, p53 is stabilized and its DNA binding is activated through posttranslational covalent modifications, including phosphorylation and acetylation [10]. These result in the transactivation of numerous response genes whose products promote cell cycle arrest or apoptosis [11]. Genes transactivated by

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p53 after exposure to cisplatin include those associated with promoting cell cycle arrest (e.g. $p21^{WAF1}$), DNA repair (GADD45) and apoptosis (BAX) [12]. Additionally, p53 can also transactivate mouse double minute homologue 2 (MDM2), which has been shown to bifunctionally regulate p53 levels by targeting the p53 protein for degradation as well as promoting the synthesis of p53 and the alternatively initiated p53/47 [13]. This novel truncated isoform of p53 lacks the most N-terminal p53 transactivating domain (aa 1–40) and the MDM2 binding site (aa 17–23), but retains the second transactivation domain (aa 43–63), the complete central DNA binding domain and the C-terminal tetramerization domain of its larger sibling [13]. Our earlier work has demonstrated that in addition to promoting apoptosis independent of p53, this isoform can interact with p53, becoming incorporated in the tetramers formed by p53 in its active state, and that an increasing ratio of p53/47 to p53 modulates the transactivation of p53 response genes [13,14]. It has been reported that small changes in the expression of p53/47 could be responsible for large changes in p53-directed activity [15,16], adding to the notion that p53/47 plays an important role in the p53 stress response.

Recent studies have demonstrated that LA-12 is a potent cytotoxic agent that promotes cell death and apoptosis in a variety of cultured cancer cell lines. Additionally, these studies have shown that LA-12 induces different dynamics of cell cycle perturbation compared with cisplatin and other platinum derivatives, and induces cell death in cisplatin-resistant cell lines [4,17–19] indicating that it acts via a different cellular target than other platinum compounds, and/or activates a different subset of stress response pathways. These studies also revealed that the induction of cell death and apoptosis in LA-12-treated cells was preceded by an increase in the expression of p53; however, the role played by p53 as a mediator of the cytotoxic effects of the drug is yet unknown.

Materials and methods

Chemicals

LA-12 was synthesized and supplied by PLIVA-Lachema (Brno, Czech Republic).

Culture and constructs

H1299 (p53-negative human lung carcinoma cells) and A2780 (human ovarian carcinoma cells) were cultured in RPMI 1640 medium. BT474 and BT549 (human breast carcinoma cell lines) were cultured in Dulbecco's modified Eagle's medium in the presence of 10% foetal calf serum and penicillin/streptomycin. All constructs were generated according to standard protocols using the pcDNA3 vector. H1299 cells were transfected with 0.5 μg DNA per 3×10^5 cells with 3 μl Genejuice (Merck Biosciences, Nottingham, UK) per microgram of DNA and the total amount of DNA was kept constant by adding empty vector.

SDS–PAGE, immunoblotting and two-dimensional gels

Cell lysates were prepared by washing the cells twice in cold phosphate-buffered saline (PBS) before using a rubber policeman to detach the cells, and the cell pellets were collected by brief centrifugation. The cells were lysed in 1% Nonidet P-40, 150 mmol/l NaCl, 20 mmol/l Tris (pH 7.4) in the presence of complete protease inhibitor cocktail (Roche, Nutley, New Jersey, USA). Protein concentrations were measured using Bradford assay (Bio-Rad, Hercules, California, USA). A total of 20 μg of protein was separated by SDS–PAGE on 10% gels and transferred onto nitrocellulose membranes in a Bio-Rad Trans-Blot SD semidry transfer cell for 1 h by applying 200 mA in transfer buffer (240 mmol/l Tris, 190 mmol/l glycine and 20% methanol). Prestained molecular weight markers (Bio-Rad) were run in parallel. The blotted membranes were blocked in 5% milk and 0.1% Tween 20 in PBS for 2 h at room temperature and probed overnight with specific monoclonal antibodies or rabbit polyclonal sera. After washing three times in PBS plus 0.1% Tween 20, peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum or swine antirabbit immunoglobulin antiserum (DAKO, Glostrup, Denmark) diluted to 1 : 1000 in 5% milk and 0.1% Tween 20 in PBS was used as the secondary antibody. To visualize peroxidase activity, enhanced chemiluminescence reagents from Amersham-Pharmacia (Little Chalfont, UK) were used according to the manufacturer's instructions.

The two-dimensional (2-D) gel analysis was performed using ZOOMIPGRunnersystem (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. We have used nonlinear ZOOM strips with a pH range 3–10 for isoelectric focusing and 1 mm NuPAGE 4–12% Bis-Tris ZOOM gels for analysis of proteins after isoelectric focusing.

Antibodies

The antibodies used in this study are listed below. (i) CM1 rabbit polyclonal antibody, which recognizes the human p53 protein. (ii) DO-1 monoclonal antibody directed towards the epitope 20-SDLWKL-25 within the p53 N-terminal region. (iii) ACMDD rabbit polyclonal antibody raised against the peptide (MDDLM LSPDDIEQC) recognizing p53/47. (iv) 2A9 monoclonal antibody, which detects MDM2 [20]. (v) AC-40 monoclonal antibody, which recognizes actin (Sigma-Aldrich, St Louis, Missouri, USA).

Macroarray analysis

To identify p53-related genes that are dysregulated by LA-12, we used GEArray Q series Human p53 Signalling

Array HS-027 macroarray membranes (Superarray Bioscience Corporation, Bethesda, Maryland, USA). H1299 cells were transfected with wild-type (wt) p53 cDNA or mock transfected with empty pcDNA3 vector, before treatment with LA-12 (10 μmol/l) for 2 h, or before treatment with LA-12 (1.0 μmol/l, 48 h), cisplatin (3.3 μmol/l, 48 h) or doxorubicin (0.1 μmol/l, 24 h). Total RNA was prepared by TRIzol Reagent (Invitrogen). An aliquot of 4 μg of total RNA was reverse transcribed (Promega, Madison, Wisconsin, USA) and the probe was simultaneously labelled using the GEA labelling buffer mix (Biotin-16-dUTP; Roche Diagnostics, Nutley, New Jersey, USA) according to the instructions of the manufacturer. The membrane was prehybridized with sheared salmon sperm DNA in GEAhyb hybridization solution followed by hybridization at 68°C overnight with continuous agitation. The membrane was washed at 68°C twice with 2 × standard saline citrate, 1% SDS and then twice with 0.1 × standard saline citrate, 0.5% SDS. For detection of chemiluminescence, the membrane was blocked using the alkaline phosphatase-conjugated streptavidin method in combination with CDP-Star solution (SuperArray Bioscience Corporation, Bethesda, Maryland, USA). Signals were captured and expression of genes was quantified using a cooled CCD camera and associated software (5000 series camera and Bio-1D software; Vilber Lourimat, Torcy, France). The raw signal intensities were corrected for background by subtracting the signal intensity of the average of the three negative controls (pUC18 cDNA) and the four blanks and were expressed as fold changes given as the relative expression ratio: gene/housekeeping gene. Any signal whose raw intensity was less than 150% of the background was treated as a background signal and thus interpreted to be not detectable in the sample.

Flow cytometry

Propidium iodide staining of cells was performed as described earlier [21]. Cells were treated with indicated cytotoxic drug and cultured in standard growth conditions. Subsequently both floating and adherent cells were pelleted before fixation and permeabilization with ice-cold 70% ethanol. The cells were then washed twice in PBS before incubation with RNase at 37°C for 30 min. Propidium iodide (50 μg/ml; Sigma) was then added, and the propidium iodide fluorescence emission was analyzed with LSR flow cytometer (Becton-Dickinson, San Jose, California, USA). Events that fell within the hypodiploid region (sub-G_0/G_1) were accounted as apoptotic events and enumerated using the CellQuest software (Becton-Dickinson), the results were expressed as percentage of the total events.

Results

Regulation of p53 and p53/47

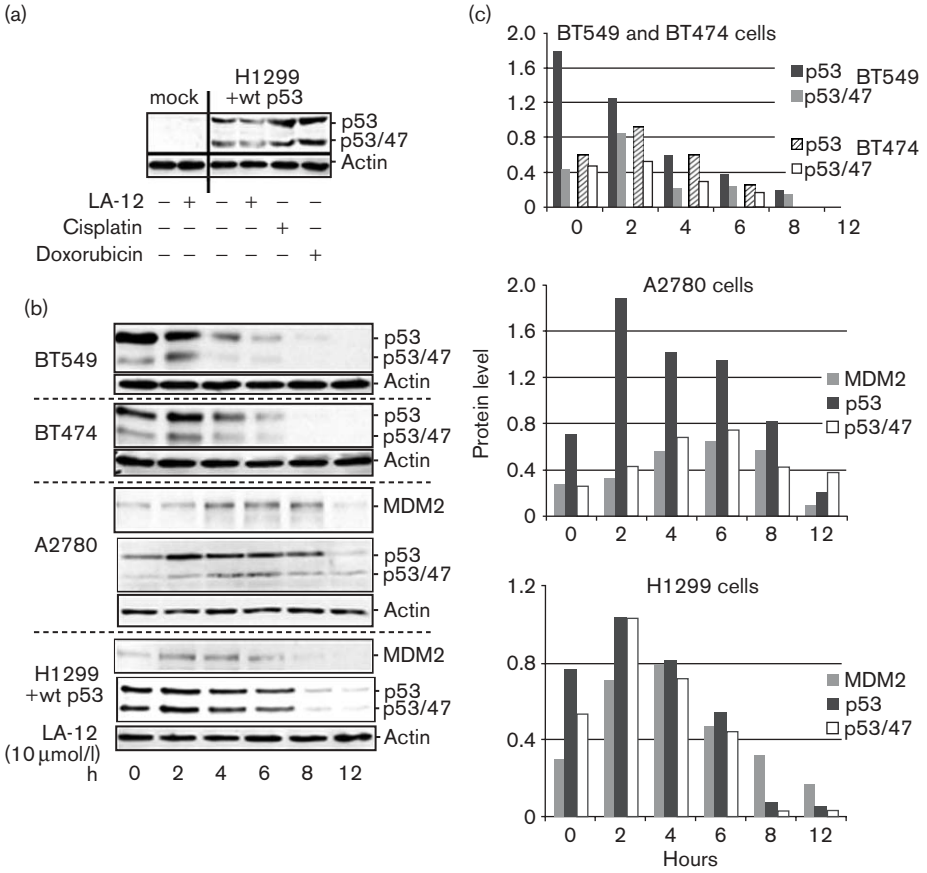
We compared the effects of LA-12 on the expression of p53 and p53/47 with those of cisplatin and doxorubicin using human H1299 p53-null cells transfected with wt p53 vector. As shown in Fig. 1a, exposure of cells to either cisplatin or doxorubicin increases the expression of both p53 and p53/47 within 16 h. In contrast, treatment with 10 μmol/l LA-12 induced a specific loss in the expression of both the p53 proteins, indicating that LA-12 may exert a different effect on the cells than other DNA damaging agents, such as cisplatin and doxorubicin.

To investigate the reduced expression of p53 induced by 10 μmol/l LA-12, we compared the effects of the drug on p53-transfected H1299 cells with those on human breast cancer-derived cell lines BT474 and BT549, which express endogenous mutant p53 and on human ovarian cancer-derived A2780 cells, which express wt p53 (Fig. 1b). After a time course of LA-12 treatment, we found that the compound induces near complete loss of p53 and p53/47 after 12 h in all cells, although with different kinetics. Interestingly, it is the p53/47 isoform that seems to be highly responsive to LA-12 treatment, thus resulting in a change in the ratio of p53 and p53/47. MDM2 was elevated in a pattern that mirrored the transient accumulation of p53/47 in both H1299 and A2780 cells (Fig. 1b). As MDM2 has been shown to positively feedback on the rate of p53/47 synthesis [13], the induction of MDM2 via p53 may offer an explanation for the changes in p53 and p53/47 in LA-12-treated cells. To test this hypothesis, we confirmed that LA-12 does not affect MDM2 levels in untransfected (p53-null) H1299 cells (data not shown). Taken together, our results indicate that LA-12 promotes transient transcriptional activity of p53 that in turn transactivates MDM2, leading to enhanced synthesis of p53/47.

Furthermore, we examined the effects of an increasing dose of LA-12. As shown in Fig. 2, exposure to low concentrations of the drug (1–100 nmol/l) for 12 h resulted in modest accumulation of endogenous, full-length (FL) p53 and p53/47 in tested cell lines, which was retained over the full period of the experiment. In contrast, treatment with higher concentrations (> 100 nmol/l) led to a striking loss of both p53 and p53/47 proteins in the three cell types. As exposure of cells to a low concentration results in a sustained increase in p53 levels, a further longer time course was performed. As shown in Fig. 3a, use of the drug at 1–10 nmol/l for either 24 or 48 h resulted in a strong increase in the levels of endogenous and exogenous p53 and p53/47 proteins, however, only FL p53 was detectable in the BT549 cells. Consistent with the previous dose experiments, ramping LA-12 to a higher concentration (500 nmol/l) led to a reduction of p53 and p53/47.

Taken together, these results indicate that LA-12 at low concentrations induces accumulation of p53 and p53/47, whereas use of the drug at high concentrations for longer

Fig. 1

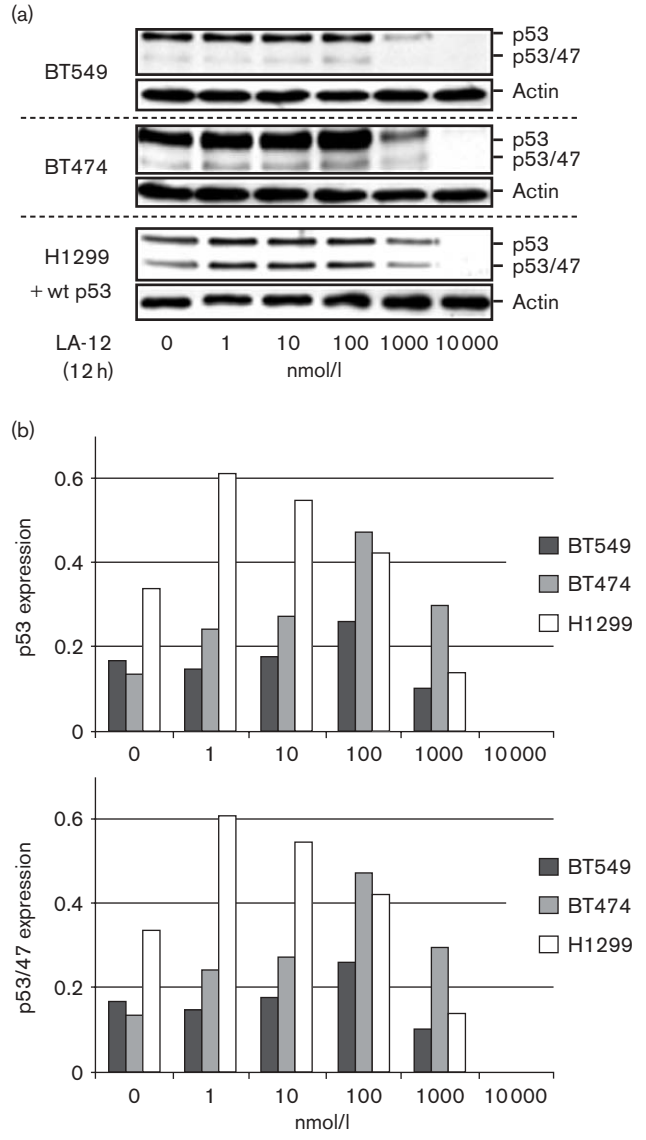


The effect of LA-12 on the expression of endogenous and exogenous p53 and p53/47 protein. (a) Comparison of both p53 and p53/47 protein expression in response to 1 μmol/l LA-12, 3.3 μmol/l cisplatin and 0.1 μmol/l doxorubicin. (b) Analysis of MDM2, p53 and p53/47 protein expression in the cell lines as indicated. H1299 cells were transiently transfected with wt p53 cDNA or mock transfected with empty pcDNA3 vector. (c) Densitometry of protein levels shown in (b) normalized to actin.

periods results in their loss. One plausible explanation is that a high-level of the drug may dysregulate the balance between the expression and degradation of the p53 proteins. To examine this, we determined the relative influence of proteasome-dependent degradation on the observed changes in the levels of p53 isoform expression. As shown in Fig. 3b, treating cells with the proteasomal inhibitor MG132 blocks the proteasomal degradation of p53, thus resulting in an increase of p53 (compare lanes 1 and 3, Fig. 3b). Pretreating cells with MG132 before exposure to a high concentration of LA-12 (10 μmol/l) partially prevents the loss of endogenous p53 in BT549 and BT474 cells (compare lanes 2 and 4, Fig. 3b). In contrast, combined MG132/LA-12 treatment of transiently transfected H1299 cells does not influence the exogenous p53 and p53/47 protein expression in comparison with LA-12 treatment alone (see lanes 2 and 4, Fig. 3b). These data suggest that using a high concentration of LA-12 results in enhanced degradation of p53 and this effect is more apparent in the BT549 and BT474 cells harbouring endogenous p53, thus explaining the faster disappearance of mutant p53 in the BT549 and BT474 cells.

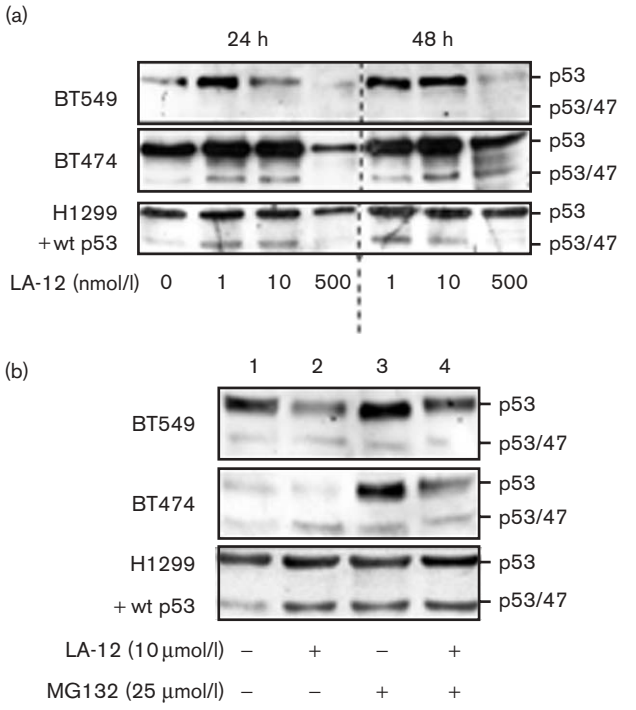
The ability of LA-12 to induce loss of p53 is highly unusual, because the canonical response of cells to cytotoxic agents is the robust stabilization and accumulation of p53 [22]. Posttranslational activation of p53 is achieved through covalent modifications particularly phosphorylation and acetylation, which increases protein stability and induces DNA binding [10]. To assess the effects of LA-12 on the covalent modifications of p53 and p53/47, we used 2-D gel electrophoresis. Probing untreated cell lysates from wt p53-transfected H1299 cells with monoclonal antibody specific for FL p53 protein (DO-1) reveals a long horizontal streak of spots adjacent to the 50.2-kDa protein marker (Fig. 4a). Exposing cells to 10 μmol/l LA-12 for 2 h induced a significant change in the FL p53 profile coupled with loss of the clustering of spots in a lower pH that could be

Fig. 2



Dose-dependent expression of p53 and p53/47 proteins. (a) The effect of LA-12 doses on endogenous and exogenous p53 and p53/47 proteins expression. (b) Densitometry of protein levels normalized to actin.

Fig. 3



The 24 and 48-h effects of LA-12 treatment and determination of the role of proteasome in degradation of p53 and p53/47 proteins. (a) Cells BT549, BT474 and H1299 transiently transfected with wt p53 cDNA were exposed to LA-12 for the time and dose as indicated in figure. (b) To assess the role of proteasomal degradation, the cells were pretreated with MG132 (25 μmol/l, 30 min) before LA-12 (10 μmol/l, 4 h) or DMSO vehicle treatment. Cells were harvested and subsequently immunoblotted for p53 proteins using the CM1 antibody.

associated with alterations in the number of charged phosphate moieties after phosphorylation. p53/47 was examined using a polyclonal sera (ACMDD) targeted to the N-terminal residues of the protein, which correspond to amino acids 40–49 in FL p53. This serum recognizes both FL p53 and N-terminally truncated p53/47, unlike DO-1 that recognizes only FL p53 protein. Probing untreated lysates with ACMDD yields a small horizontal clustering of p53/47 proteins (Fig. 4b). Partial loss of signal could be explained by LA-12-induced phosphorylation of this region, as many of the serine residues within ACMDD docking site are known to be phosphorylated during p53 activation resulting in epitope masking.

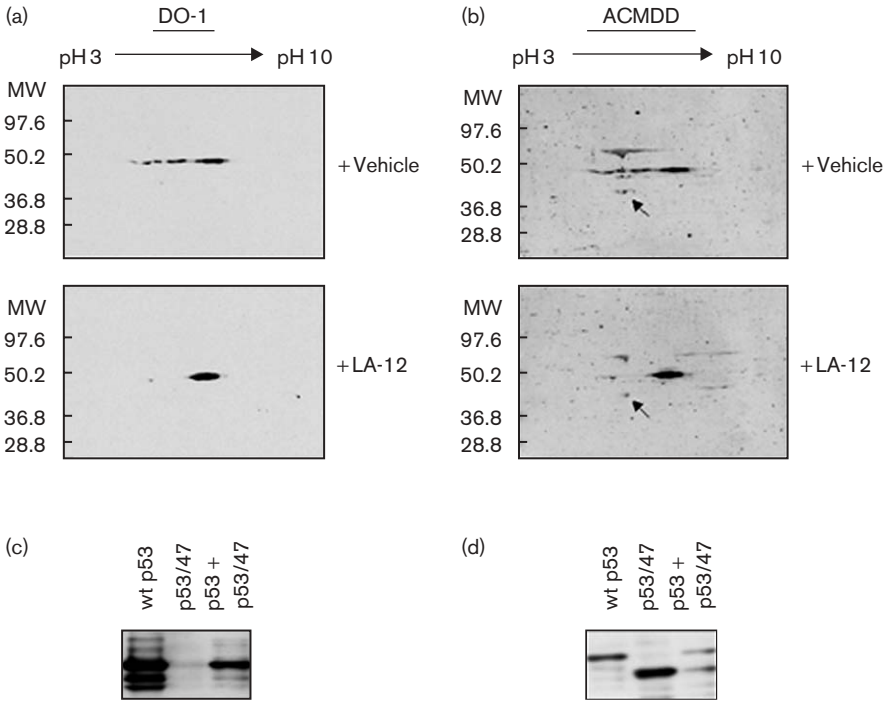
Cell cycle determination

To correlate the drug-induced changes in p53 and p53/47 expression with physiological changes in cell cycle and apoptosis, a fluorescence-activated cell sorting (FACS) analysis was performed. In asynchronized, p53-null H1299 cells, 48-h LA-12 treatment induced a modest G_2/M arrest and apoptosis in a dose-dependent manner (Fig. 5). These effects, however, were significantly augmented by p53 resulting in a large proportion of the cells arrested in the G_2/M phase. In comparison, cisplatin and doxorubicin exhibited smaller changes in cell cycle profile in p53-null cells than LA-12; however, in cells harbouring wt p53, the two agents induce a strong arrest in the S-phase in contrast to the predominant G_2/M phase arrest induced by LA-12.

Analysis of gene expression associated with p53-mediated signal transduction

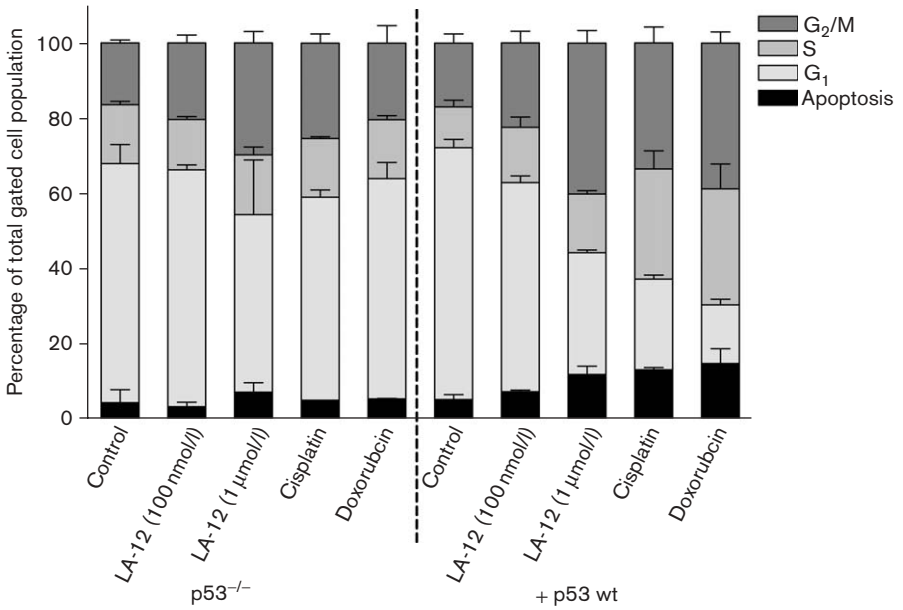
To understand how LA-12 induces different responses, we determined the effect of LA-12 on p53-directed transcription using a gene macroarray designed to profile the mRNA expression of a panel of 96 key genes involved in p53 signalling. Table 1 details the relative changes in

Fig. 4



Analysis of p53 and p53/47 posttranslational modifications in response to LA-12 treatment. H1299 cells were transfected with wt p53 cDNA or mock transfected with empty pcDNA3 vector before treatment with LA-12 (10 μmol/l) or vehicle (DMSO) for 2 h. Arrows indicate p53/47-positive signals. FL p53 protein was detected with DO-1 antibody (a) and the alternatively initiated p53/47 protein was detected with ACMDD rabbit polyclonal sera (b). The specificity of DO-1 antibody to FL p53 protein only (c) and specificity of ACMDD rabbit polyclonal sera to both FL p53 and p53/47 proteins (d) is shown by one-dimensional gel analysis of H1299 cells transfected with wt p53, p53/47 and a mixture of wt p53 and p53/47. FL, full-length.

Fig. 5



Cell cycle analysis. H1299 cells transiently transfected with empty vector or vector expressing wt p53 were treated with either LA-12 (as indicated, 48 h) cisplatin (3.3 μmol/l, 48 h) or doxorubicin (0.1 μmol/l, 24 h). Both floating and adherent cells were subsequently harvested and analyzed by fluorescence-activated cell sorting to determine the proportion of the total gated cell population in each phase of the cell cycle.

Table 1 The effect of acute 2-h LA-12 treatment

Activity category	Gene	Mock	Mock + LA-12 (120 min)	wt p53	wt p53 + LA-12 (120 min)
Apoptosis					
1,2	DAXX	1.00	2.31	2.41	4.31
1,2	PUMA	1.00	1.25	1.51	2.18
Cell-cycle control					
2,3	AD022	1.00	0.84	1.30	2.37
2,3	Cdk1	1.00	0.58	0.95	1.56
2	$p21^{Waf1}$	1.00	0.97	7.21	10.88
1,2	B99	1.00	1.99	0.95	2.88
1,2	Chk1	1.00	2.02	1.58	1.83
2	HIPK2	1.00	0.88	1.75	2.81
2	MAP4	1.00	0.87	0.94	1.91
1,2	p14(ARF)	1.00	1.79	1.26	2.60
2	CAK	1.00	0.93	1.14	2.52
2	HIF1A	1.00	0.83	0.74	1.54
	Hsp70	1.00	0.32	0.56	0.80
DNA repair					
3	APEX	1.00	0.78	0.52	1.42
2,3	GADD45	1.00	0.42	1.61	3.27
p53 modification and interactions					
2	E1B-AP5	1.00	0.94	0.98	1.25
2,3	MDM2	1.00	0.63	6.44	8.20
	CSNK1A1	1.00	0.50	0.87	1.04
1	JNKK2	1.00	2.63	0.50	2.88
	PML	1.00	0.37	0.55	0.84
	DNA-PK	1.00	0.54	0.66	0.80
Housekeeping					
	GAPDH	1.00	1.00	1.00	1.00
	RPL13A	1.00	1.15	1.00	1.05
	β-actin	1.00	0.99	1.01	0.92

Activity categories. 1, Upregulated by LA-12 independent of p53; 2, genes dependent on p53 expression for induction by LA-12; 3, genes repressed by LA-12 in mock cells, but upregulated by LA-12 in p53-positive cells.

response to acute LA-12 (10 μmol/l) treatment in H1299 cells with or without wt p53.

Acute 2-h LA-12 treatment of p53-null H1299 cells results in modulation of 21 of the 96 genes measured by the array. Genes including those linked to the DNA damage response (e.g. ATR, Chk1) are elevated, several of which are known p53 kinases [23], whose activation could account for the drug-induced changes in p53 modification observed in the 2-D analysis (Fig. 4). Effectors of cell cycle arrest and apoptosis (DAXX, B99) are enhanced, whereas other genes required for proliferation and survival are repressed [heat shock protein 70 (Hsp70), Cdk1] indicating that LA-12 is capable of activating the DNA damage response and in the absence of p53 can exert a limited but significant cytotoxic effect on cellular processes. Transfection of H1299 cells with wt p53 resulted in upregulation of 46 p53 transcription targets, including the classical p53-responsive genes $p21^{WAF1}$, MDM2, PUMA, GADD45. Expression of these genes was further enhanced after treatment with acute LA-12 in p53-transfected H1299 cells. The specific increase in MDM2 mRNA confirms the enhanced expression of MDM2 protein, whose p53-dependent induction by the drug was shown by Western blotting (Fig. 1b).

Of the different groups of p53 response genes analyzed, it is mainly the cell cycle regulatory genes that are modulated after the acute treatment, with few changes noted in genes linked to the induction of apoptosis. This, however, may merely reflect the short time course utilized, and is in line with the report that in response to cell stress p53 will preferentially promote cell cycle arrest to allow recovery before triggering apoptosis [24]. With this in mind a second series of macroarrays were performed using a lower dose of LA-12 (1 μmol/l) for 48 h alongside cisplatin and doxorubicin. Both cisplatin and doxorubicin were used at concentration and time known to induce comparable levels of p53 and cell death [25]. Gene expression profiles were determined in H1299 cells as well as in A2780 cells with the aim to get, as much as possible, true view of molecular signalling pathways linked with wt p53 in tumour cells. Only genes showing similar expression patterns in both cell lines were included in Table 2 as candidate genes that execute real biological functions in the cancer cell.

A total of 70 genes were modulated in the mock-transfected H1299 cells after 48 h incubation including genes of all categories, and in particular, increased expression of many apoptotic genes compared with the earlier acute treatment was evident. In the course of comparing the profile of gene expression in the H1299 and A2780 cells, a total of 26 genes showing similar expression pattern in response to drug treatment in both cell lines were detected (Table 2). The extended 48-h LA-12 incubation period resulted in greater increases in the expression of DNA response and repair genes, such as ATR, Chk2/Rad53, as well as heightened expression of genes commonly associated with p53 activation, such as the cell cycle control and apoptotic genes. Additionally, we observed strong modulation of many other genes pertinent to cellular maintenance, such as NFκB, Sp1 and Rb, underlining that over a longer time period LA-12 can exert powerful effects on the cell independently of p53 status.

Discussion

Cisplatin-based chemotherapy is a standard treatment for different cancers. Resistance to chemotherapy, however, is still a complex problem. Although approximately 50% of patients are already resistant to chemotherapy, a substantial number of those who were originally responsive develop resistance to platinum-based chemotherapy during the course of their treatment [26]. Earlier works demonstrated that the efficacy of various chemotherapeutic agents, including cisplatin, requires a functional p53 protein [27,28], and p53 mutations are in correlation with resistance to platinum-based chemotherapy and shortened survival [29].

During the analysis of mRNA expression of key genes involved in p53 signalling, we compared the effect of acute 2-h and extended 48-h LA-12 treatment. Use of LA-12 for 48 h resulted in enhanced transcription of many

Table 2 The effect of extended LA-12 treatment in comparison with cisplatin and doxorubicin

		H1299		H1299				A2780		
Activity category	Gene	Mock	Mock + LA-12	wt p53	wt p53 + LA-12	wt p53 + cisplatin	wt p53 + doxorubicin	Control	LA-12	Cisplatin
	p53	–	–	1.00	2.38	2.89	1.18	1.00	1.60	1.27
Cell-cycle control										
1,2,5	$p21^{Waf1}$	1.00	1.63	1.00	3.09	3.85	3.51	1.00	1.20	4.65
1,4,5	Rel A	1.00	2.37	1.00	0.75	14.02	5.64	1.00	0.86	1.50
1,2	14-3-3	1.00	1.85	1.00	2.24	3.38	1.58	1.00	1.74	1.81
2,3	Cdk1	1.00	0.77	1.00	1.68	1.39	2.34	1.00	1.13	1.30
2	Chk1	1.00	1.06	1.00	1.85	2.26	2.90	1.00	3.99	3.49
1,5	Chk2	1.00	4.23	1.00	1.53	0.77	1.72	1.00	1.29	1.08
1	WIG1	1.00	2.91	1.00	2.22	2.90	1.17	1.00	1.75	2.13
1,4,5	c-myc	1.00	2.53	1.00	0.04	2.24	4.87	1.00	0.88	12.51
1,5	Rb	1.00	2.32	1.00	2.19	8.46	5.24	1.00	0.89	2.38
2,6	$p14^{Arf}$	1.00	0.97	1.00	2.09	1.90	9.31	1.00	1.10	1.13
Apoptosis										
2,6	PUMA	1.00	1.13	1.00	2.13	1.83	9.97	1.00	1.20	1.13
1,2	DAXX	1.00	1.56	1.00	2.52	3.57	1.79	1.00	1.48	1.52
1,2	APR-3	1.00	1.70	1.00	2.99	2.74	1.39	1.00	1.56	1.49
1,2,5	DR5	1.00	2.07	1.00	2.77	6.53	2.69	1.00	2.84	3.95
p53 modification and interactions										
1,2	ATM	1.00	1.49	1.00	2.29	3.46	1.82	1.00	1.28	1.18
1,2,6	ATR	1.00	1.35	1.00	2.02	2.00	4.34	1.00	1.61	1.07
1,2	CSNK2A2	1.00	1.45	1.00	2.26	2.47	1.21	1.00	1.41	1.56
2	p300	1.00	0.83	1.00	1.24	1.71	1.80	1.00	1.25	2.02
2,5	MDM2	1.00	0.88	1.00	0.98	1.47	0.71	1.00	1.08	3.24
4	JIP2	1.00	1.07	1.00	0.61	0.95	5.54	1.00	0.60	0.91
Other genes										
1,2,6	NFκB	1.00	1.84	1.00	2.68	2.59	6.53	1.00	1.61	2.33
1,2	TRAF5	1.00	1.95	1.00	2.15	2.43	4.52	1.00	1.98	2.49
2,3	TRAF4	1.00	0.65	1.00	4.57	5.11	1.94	1.00	3.18	3.61
1,5,6	GADD45	1.00	5.66	1.00	2.14	0.90	3.43	1.00	2.66	0.57
2	Sp1	1.00	0.81	1.00	1.33	1.79	1.57	1.00	1.49	1.49
Housekeeping										
	GAPDH	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	RPL13A	1.00	1.27	1.00	1.00	1.02	1.42	1.00	1.21	1.28
	Cycl. A	1.00	1.00	1.00	0.50	0.77	1.34	1.00	1.07	1.17

Activity Categories. 1, Upregulated by LA-12 independent of p53; 2, genes dependent on p53 expression for induction by LA-12; 3, genes repressed by LA-12 in mock cells, but upregulated by LA-12 in p53-positive cells; 4, activated by LA-12 mock and repressed by LA12 plus wt; 5, large differences between LA-12 and cisplatin in p53 wt cells; 6, Differences between doxorubicin, LA-12 and cisplatin.

p53 transcription targets at a greater magnitude than that resulted from the acute time course, thus indicating that the 2-h time point is not sufficient to allow a maximal response to the drug.

As LA-12 and cisplatin are platinum-based drugs, it would be expected that both compounds would exert their cytotoxic effects via the same mechanism (DNA damage) resulting in comparable gene expression patterns. Indeed, earlier studies have shown that platinum(IV) metal centres are readily reduced by cellular components such as small molecules involved in redox balance in the body [30] to form the platinum(II) analogues that bind more rapidly to DNA. This fact is in agreement with findings that the treatment of tumour cells with adamplatin(IV) (LA-12) does not result in DNA modifications that would be markedly different from those produced by cisplatin [31]. Therefore, it is not surprising to see relatively similar changes in patterns between LA-12 and cisplatin (Table 2, columns 4 + 5 and 8 + 9) indicating that DNA is a major target of LA-12 as well. There are, however, noticeable exceptions with cisplatin eliciting larger augmentations especially in cell cycle regulatory genes, such as $p21^{WAF1}$, Rel A, c-myc, Rb and the apoptotic gene DR5. Conversely, a greater induction of the DNA repair-related gene GADD45 is seen with LA-12. It is also important to note that in addition to full length p53 we observe that p53/47 is clearly upregulated in response to DNA damage induced by LA-12 as well as by cisplatin and doxorubicin (Fig. 1). This is intriguing, as it has been shown that p53/47 can promote apoptosis independent of p53 [13,14], and that the differential expression of p53 isoforms has been shown to regulate p53 transcription activity [32], and thus p53/47 may play a key role in maintenance of cellular integrity. The FACS and macro-array techniques used here, however, cannot differentiate between p53-directed and p53/47-directed signalling, and future studies will seek to determine the contribution of p53/47 to the p53 stress response.

Doxorubicin (adriamycin), another commonly used therapeutic anticancer drug, was also included in the study to compare the p53 stress response activated by different types of DNA lesions. In contrast to cisplatin, which

binds to DNA guanine residues and forms cross-linkages inside or among the DNA chains [33,34], doxorubicin interferes with the topoisomerase II–DNA complex, leading to the formation of double-stranded breaks of DNA [35], thereby activating a different subset of damage response pathways. This is reflected in differential upregulation of DNA damage response genes, including ATR, GADD45 and also p53R2 by doxorubicin compared with cisplatin and LA-12. Likewise, the alterations in gene expression induced by doxorubicin compared with those by cisplatin and LA-12 are different for many p53 proapoptotic transcription targets, such as Reprimo, PUMA, Noxa and others including p73 and Apaf-1.

The partially different gene expression profile induced by LA-12 compared with that by cisplatin indicates that they have distinct modes of action accounting for the differential activation of both p53-dependent and p53-independent gene targets. These apparent differences may be because of the effects of other pharmacological factors associated with antitumour effects of LA-12, such as enhanced accumulation of the drug in cells, strong inhibition of DNA polymerization by the platinum adducts, enhanced persistence of the adducts owing to their low repair efficiency and DNA–protein cross-linking, which are different from the effects of these factors in the mechanism underlying the activity of cisplatin [31]. It is likely that these differences explain the ability of LA-12 to induce cell death in cisplatin-resistant cells [4,18] or cells lacking a functional p53. The ability to use lower dosage in combination with differential activation suggests that LA-12 may also be beneficial in reducing the harmful side effects associated with other platinum-based therapies.

Results derived from cell cycle determination are consistent with previous reports [18,19] that in certain cells LA-12 and cisplatin induce different dynamics of cell cycle arrest, thus reflecting the different patterns of gene expression induced by the two drugs. Additionally, the observation that LA-12 induces cell cycle arrest in the null transfected cells (albeit at a lower efficacy than in cells harbouring p53) shows that LA-12 activates both p53-dependent and p53-independent apoptotic and cell cycle arrest pathways and may therefore be an appropriate agent for use against cells lacking p53 or harbouring a nonfunctional or mutant form of the tumour suppressor.

Western blot analyses indicate that LA-12 promotes posttranslational modifications of both p53 and p53/47. It is in coincidence with recent biochemical and genetic studies indicating that several genotoxic treatments stabilize p53 through posttranslational modifications. Especially N-terminal phosphorylations are important for stabilizing p53 and are crucial for acetylation of C-terminal sites, which in combination lead to the full p53-mediated response to genotoxic stresses. Posttranslational modifications also promote different interactions between p53 and other proteins and with different target gene regulatory elements to facilitate cell cycle arrest, apoptosis or DNA repair [36]. Several p53 posttranslational modifications seem to modulate its transcriptional activity in a promoter-specific and cell-type-specific manner. It is supported by studies indicating that activation of specific promoters depends on specific p53 phosphorylations [37,38]. It remains to be determined how these changes in protein phosphorylation and/or acetylation in response to specific kinases/phosphatases or acetyltransferases/deacetylases, as well as changes in protein stability, play a role in promoter selectivity and affect the duration and magnitude of the transcriptional output after exposure of cancer cells to LA-12.

The partially different expression profile induced by LA-12 compared with cisplatin could be explained by the unique ability of LA-12 to induce loss of p53 at higher drug concentrations. Both A2780 cells with intrinsic wt p53 and H1299 cells transiently transfected with wt p53 showed loss of p53 in response to LA-12 treatment, however, the rate of degradation was significantly lower in comparison with cells harbouring a mutant p53 (Fig. 1b). In case of both wt p53 H1299 and A2780 cells, MDM2 was elevated in a pattern that mirrored the transient accumulation of p53/47 and loss of FL p53 (Fig. 1b), thus indicating a MDM2-dependent mechanism of p53 degradation. Although MDM2 can drive the degradation of both mutant and wt p53, several reports show that the ability of MDM2 to function as an ubiquitin ligase is less important in the degradation of mutant p53, which is heavily ubiquitinated in an MDM2-independent manner [39,40].

Although the cytotoxic effect of cisplatin is believed to result mainly from its interaction with DNA, it is known that cisplatin may also disrupt the function of some proteins, including Hsp90 [41]. For example, treatment of neuroblastoma cells with cisplatin led to an immediate inhibition of the hormone binding of the glucocorticoid receptor, followed by proteasome-dependent degradation of the receptor [41]. Some authors suppose that LA-12 serves as a prodrug for the chemotherapeutically active platinum(II) analogue *cis*-[$PtCl_2(NH_3)$(1-adamantylamine)] [adamplatin(II)], which is presumably the complex that binds to DNA [31]. It is possible to suppose that this platinum(II) analogue can interact with proteins analogous to cisplatin as well, resulting in stronger effect. Inhibition of Hsp90 induces the degradation of mutant p53 by the dissociation of p53 and MDM2 from Hsp90 [40], thus interpreting more efficient degradation of p53 in cell lines expressing mutant p53. In the absence of Hsp90 activity, the less stable unfolded p53 mutants

preferentially associate in a complex with Hsp70 and C-terminus of Hsp70-interacting protein (CHIP), where CHIP is responsible for ubiquitination and degradation of these mutants [42].

In conclusion, we demonstrate that the novel platinum(IV) compound LA-12 is a highly potent cytotoxic agent that promotes the rapid induction of DNA stress pathways leading to a blockade in the cell cycle and death. Although LA-12 is capable of disrupting cellular proliferation regardless of the p53 status of the cell, the potency of the drug is greatly enhanced by the presence of a functional p53. In this scenario, LA-12 induces the posttranslational modification and accumulation of p53 resulting in strong activation of many p53 response genes and heightened induction of cell cycle arrest and apoptosis. Importantly, we showed that LA-12 induces a profile of transcriptional changes that is different from the related drug, cisplatin, thus providing the molecular basis for the ability of LA-12 to effectively kill cisplatin-resistant cells.

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