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Cisplatin resistance in cell models: evaluation of metallomic and biological predictive biomarkers to address early therapy failure†

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Cisplatin, one of the most extensively used metallodrugs in cancer treatment, presents the important drawback of patient resistance. This resistance is the consequence of different processes including those preventing the formation of DNA adducts and/or their quick removal. Thus, a tool for the accurate detection and quantitation of cisplatin-induced adducts might be valuable for predicting patient resistance. To prove the validity of such an assumption, highly sensitive plasma mass spectrometry (ICP-MS) strategies were applied to determine DNA adduct levels and intracellular Pt concentrations. These two metal-relative parameters were combined with an evaluation of biological responses in terms of genomic stability (with the Comet assay) and cell cycle progression (by flow cytometry) in four human cell lines of different origins and cisplatin sensitivities (A549, GM04312, A2780 and A2780cis), treated with low cisplatin doses (5, 10 and 20 μM for 3 hours). Cell viability and apoptosis were determined as resistance indicators. Univariate linear regression analyses indicated that quantitation of cisplatin-induced G–G intra-strand adducts, measured 1 h after treatment, was the best predictor for viability and apoptosis in all of the cell lines. Multivariate linear regression analyses revealed that the prediction improved when the intracellular Pt content or the Comet data were included in the analysis, for all sensitive cell lines and for the A2780 and A2780cis cell lines, respectively. Thus, a reliable cisplatin resistance predictive model, which combines the quantitation of adducts by HPLC-ICP-MS, and their repair, with the intracellular Pt content and induced genomic instability, might be essential to identify early therapy failure.

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Significance to metallomics

Knowledge about cisplatin as a chemotherapy drug is extensive, including information about the several processes that contribute to its resistance, the main problem of using this chemical. However, this knowledge and information does not yet allow the early identification of resistant tumors/patients, one of the most desired aims of clinicians. In this work we have developed a model that might allow the early detection of chemical resistance and, more importantly, might do so mostly regardless of the resistance mechanism involved, because it determines the dynamics of adduct induction/repair, considering chemical influx/efflux and induced genomic instability.

1. Introduction

Among antitumor drugs, cisplatin (*cis*-diamminedichloroplatinum(II), cDDP) is one of the most extensively, and rather successfully, used in cancer treatment, either alone or in combination with other drugs.[1] However, intrinsic and/or acquired cellular resistance is one important drawback preventing its wider therapeutic use.[2,3]

The chemotherapeutic action of cisplatin is based on its capacity to damage DNA by covalently binding to N7-guanine residues, generating different types of adducts, such as monoadducts,

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and inter- and intra-strand crosslinks.[4–6] The most frequently generated cisplatin-induced adducts, intra-strand guanine–guanine (G–G) cross-links, can block DNA replication and transcription[6–8] and lead to cell cycle arrest and apoptosis.[6]

Tumors can become resistant to cisplatin by a variety of processes, such as decreased uptake/influx or increased efflux of the drug; low apoptosis capacity due to hypermethylation of gene promoters; increased DNA repair activity and/or increased DNA adduct bypass activity.[2,7,9,10] Since most of these processes are ultimately associated with the prevention of cisplatin–DNA adduct formation, or with their fast removal from the exposed DNA, the accurate detection and quantitation of DNA adducts may be a valuable tool in the early prediction of resistance.[11–15] However, there are some inconsistencies in the literature regarding the value of the cisplatin–DNA adduct level *per se* as a resistance predictive factor.[8,12]

It has been known for several years that cisplatin treatment is able to modify cell cycle progression,[16–18] and that this modification could be different for sensitive and resistant cells.[17] Furthermore, cisplatin can also induce genomic instability, which is generally measured as the presence of DNA strand breaks.[19–22] Nevertheless, studies combining these two parameters with cisplatin-induced adduct levels are scarce, and most of them make use of rather high and/or different cisplatin concentrations to evaluate each parameter,[13,21,23] thus preventing their comparison. Moreover, significant correlations among parameters have rarely been detected, probably due to difficulties in quantitating DNA adducts accurately.

In recent years, our group has developed an analytical methodology allowing the detection and accurate quantitation of cisplatin-induced DNA adducts.[24] This method, which is based on the specific detection of Pt by inductively coupled plasma mass spectrometry (ICP-MS), after separation of the adducts using high-performance liquid chromatography (HPLC-ICP-MS), has demonstrated very good performance for the detection and quantitation of cisplatin adducts *in vivo* both in rats[25] and in Drosophila.[26] Moreover, we have recently developed an approach to quantitatively compare intracellular platinum content and adducted DNA among cell lines.[27]

Considering all this, in this work we have tried to evaluate the cross-correlation of cisplatin-induced DNA adducts, intracellular Pt-content, cell cycle progression and drug-induced genomic instability (measured with the Comet assay), using four human cultured cell lines, A549, GM04312, A2780 and A2780cis, with different origins and cisplatin sensitivities.[23,28,29] Our final aim was to address the most accurate strategy to predict cisplatin sensitivity/resistance after short-time treatments with doses around the peak plasma concentration achieved in Pt-treated cancer patients.[30]

2. Material and methods

2.1. Chemical agent

Cisplatin or *cis*-diamminedichloroplatinum(II) (CAS No. 15663-27-1), from Sigma-Aldrich Spain®, was dissolved in sterile Milli-Q water, 18.2 Ω (Millipore, Bedford, MA, USA).

2.2. Cell lines and cultures

The A549 cell line is an adenocarcinoma human alveolar basal epithelial cell line and a model for squamous cell carcinoma,[28] which is usually treated with cisplatin; depending on the publications, this cell line is considered sensitive[28] or resistant[13] to this chemical.

The GM04312 SV40 transformed fibroblast cell line is a homozygous *XPA* mutant, which was derived from a Japanese patient of xeroderma pigmentosum.[31] It is cisplatin sensitive because of the lack of XPA activity,[29] which is a rate-limiting factor of the nucleotide excision repair (NER) system.[32]

The A2780 ovarian carcinoma cell line, a model in cisplatin treatment, is also sensitive to this chemical.[17,23]

The cisplatin-resistant ovarian carcinoma cell line A2780cis was derived from A2780 by continuous exposure to cisplatin.[33]

A549 and A2780 cell lines were kindly provided by Dr J. M. Pérez Freije (Department of Biochemistry and Molecular Biology, University of Oviedo), and they were authenticated at the Biotechnological and Biomedical Assay Unit from the Scientific and Technical Services (SCTs) at the University of Oviedo, using the AmpFLSTR®Identifier®Plus-PCR Amplification Kit (Thermo Scientific). The GM04312 cell line was obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. The A2780cis cell line was purchased from the European Collection of Cell Cultures through Sigma-Aldrich Spain®.

The GM04312 and A549 cell lines were grown in DMEM (LabClinics) and the A2780 and A2780cis cell lines in RPMI 1640 medium (Gibco); in both cases, the media were supplemented with 10% FBS (Gibco) and 5 μg mL^{-1} Plasmocin Prophylactic (InvivoGen). All of the cells were grown at 37 °C in a 5% CO_2 atmosphere. The A2780cis cells were incubated with 1 μM cisplatin every 3 passages to maintain their resistance. All cells were always used within no more than 30 passages.

2.3. Cell viability assay

An MTS CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was performed in 96 well plates. The GM04312, A549 and A2780 cells were seeded at 2×10^3 cells per well density, while the A2780cis cells were seeded at 12×10^3 cells per well. After 24 h for the GM04312, A549 and A2780 cells, and 48 h for the A2780cis cells, they were incubated for 3 h with nine different cisplatin concentrations (from 0 to 150 μM). Six wells were seeded for each concentration. Then, cisplatin was eliminated and fresh medium was added. After 24 h, MTS was added and the absorbance at 490 nm was measured one hour later with a Synergy™ HT Microplate Reader (BioTek instruments, Inc.; USA). At least two independent experiments were performed for each cell line.

2.4. Cell apoptosis and cell cycle assays

To adjust to growing characteristics, different numbers of cells were seeded in a six well culture plate for each cell line (0.75×10^6 cells per well for GM04312, 0.7×10^6 cells per well for A549, 0.5×10^6 cells per well for A2780 and 0.6×10^6 cells per well for A2780cis), to ensure that at least 10^6 cells per well attached after 24 h of

culture. The cells were treated with 0, 5, 10 and 20 μM cisplatin for 3 h; following cisplatin removal, the cells were washed with PBS, and cisplatin-free medium was added for an additional 24 h. Finally, the cells were removed and washed with cold PBS. The content of each well was divided into two tubes with 0.5×10^6 cells in each: one was used for apoptosis analysis and the other for cell cycle arrest analysis. Three independent experiments were performed for each cell line.

The Annexin V-Fluorescein IsoThioCyanate (FITC) apoptosis detection method (Inmunostep, Spain) was used for cell apoptosis analysis. Briefly, 0.5×10^6 cells from each cisplatin concentration were resuspended in 500 μL of 1X Annexin-binding buffer (10 mM Hepes/NaOH pH = 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$); 5 μL of Annexin V-FITC and 10 μL of 40 μg mL^{-1} propidium iodide were added and the cells were incubated for 15 min at RT in the dark. Then 200 μL of 1X Annexin-binding buffer was added and 10 000 events were analyzed in an FC-500 Series cytometer (Beckman Coulter) at the SCTs (University of Oviedo). The data were analyzed using RXP Analysis software (Beckman Coulter).

For cell cycle analysis, 500 μL of PBS was added to 0.5×10^6 cells from each concentration and then the cells were fixed overnight with 2 mL of cold 70% EtOH. The cells were centrifuged (5 min, 1200*g*) to eliminate EtOH and washed with PBS. Afterwards, 400 μL of Vindelov's reagent (RNAse 10 μg mL^{-1}, propidium iodide 75 μg mL^{-1} and Nonidet P-40 0.001% in Tris-buffered saline pH = 7.6) was added and 10 000 events were analyzed in the same cytometer at the SCTs (University of Oviedo). Data were analyzed using "ModFit LT" (Verity Software House).

2.5. Cell treatments for Pt quantitation (intracellular and DNA-adducts) and the Comet assay

Confluent cells (90%) from each cell line, in T-75 flasks, were treated for 3 h with 15 mL of 5, 10 and 20 μM cisplatin. After treatment (AT), cisplatin removal and three thorough cell washes with PBS, cells were harvested at two times: (1) AT-0, the culture was immediately treated with 1 mL of 10× trypsin (LabClinics) to recover the cells; and (2) AT-1, 15 mL of new cisplatin-free medium was added, and the cells were recovered 1 h later as already described. In all of the experiments, negative controls were treated with culture medium.

2.6. Total intracellular Pt quantitation

After treatment, both at AT-0 and AT-1 times, all the cells in one T-75 flask (approximately 9×10^6 cells) were recovered and freeze-dried. The cell pellets were weighed and were digested with 500 μL of 65% HNO_3 (Suprapur, Merck) for 1 hour at 70 °C, and then with 500 μL of 30% H_2O_2 for 3 h at 70 °C, to produce transparent solutions suitable for Pt determination by ICP-MS.[27] The Pt concentration obtained thereafter was corrected by the dry cell mass. Two independent experiments were performed for each cell line.

2.7. Cisplatin-induced DNA adduct quantitation

Genomic DNA was extracted and purified from approximately 2/3 of the recovered cells from a T-75 flask, using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA), at a ratio of $3–4 \times 10^6$ cells per extraction. The kit was used according to the manufacturer's instructions.

DNA concentration was checked first using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific Inc.), and then by ICP-MS monitoring P after the samples, which had been digested with 100 μL of HNO_3 (65%) and 60 μL of H_2O_2 (30%), were introduced into the system by a flow injection setup. The ^{31}P signal was calibrated using digested calf thymus DNA standards, as previously described.[27] DNA adduct identification and quantitation were performed by HPLC-ICP-MS.[24] Briefly, DNA from each extraction was incubated with 5600U of S1-Nuclease overnight. The digestion products were passed through a 10 kDa pore-size ultrafiltration membrane (Amicon Ultra-Millipore) at 14 000*g* for 15 min to remove the enzyme. 20 μL of the purified solution was injected into the chromatographic system,[24] which included a Narrow Bore Zorbax Eclipse XDB-C18 column (Agilent). 20 mM ammonium acetate buffer (pH 6.5) was used as phase A and 90% MeOH with 20 mM ammonium acetate buffer (pH 6.5) was used as phase B. The gradient was set as follows: 0–5 min 3% B, 5–35 min 15% B, and 35–40 min 15% B. Quantitation was performed by ICP-MS detection (Pt monitoring).[27] At least three independent experiments were performed for each AT time and cell line.

2.8. Alkaline Comet assay

The alkaline Comet assay was performed as previously described.[34] Briefly, 15×10^4 cells, from the same T-75 flask used for the quantitation of adducts, were analyzed per slide in 0.5% low melting point (LMP) agarose (Invitrogen). These slides were subjected to 1 h lysis, 20 min denaturing at pH > 13, and 20 min electrophoresis at 0.81 V cm^{-1} and 300 mA, at 4 °C in the dark. After neutralization and fixation, each slide, coded for blind analysis, was stained with 40 μL of ethidium bromide (0.5 μg mL^{-1}) and 1 μL of fluorescence protector Vectashield® (VECTOR laboratories, Inc. Burlingame). Nucleoids were visualized at 400× magnification with an OlympusBX61 fluorescence microscope, equipped with fluorescence filters and an Olympus DP70 CCD-coupled camera (at SCTs, University of Oviedo). Nucleoids from 50 cells per slide were scored and photographs were analyzed with Komet 5 (Kinetic, UK). For each cell line, three slides were analyzed per cisplatin concentration, and at least three independent experiments were carried out for each AT time and cell line. 0.3 mM of methyl methanesulfonate (MMS) was used as the positive control.

2.9. Statistical analyses

The effects of cisplatin treatments on the analyzed variables, within each cell line, were determined using paired Student's *t* tests to compare treatments with negative controls, and linear regression analyses to study whether the response increased with concentration.

Half-maximal inhibitory concentration (IC_{50}) values were estimated with Probit analysis to obtain linear dose–response relationships, using the SPSS program (version 21.0.0.0).

Comparisons among cell lines for each variable, and among AT times within each cell line, were performed by comparing the respective linear regression slopes.

To check for statistically significant relationships among the analyzed variables, univariate linear regressions were used. In addition, multivariate regression analyses, including a stepwise method, were used to analyze the most useful variables for the prediction of viability and apoptosis, as indicators of resistance. Pearson correlation coefficients are reported as quality measurements. To study the reliability of these analyses, three different datasets were considered: (1) all the cell lines together; (2) only the sensitive cell lines A2780, GM04312 and A549; and (3) only the ovarian cell lines A2780 and A2780cis.

3. Results

3.1. Cell line sensitivity: viability

Cisplatin sensitivity was evaluated in four human cell lines by addressing viability after 3 h of treatment. The results showed clear differences between the resistant A2780cis cell line and all of the sensitive cell lines, and among the sensitive cell lines, the A549 cell line seemed to be less sensitive than the other two at low cDDP doses (Fig. 1A).

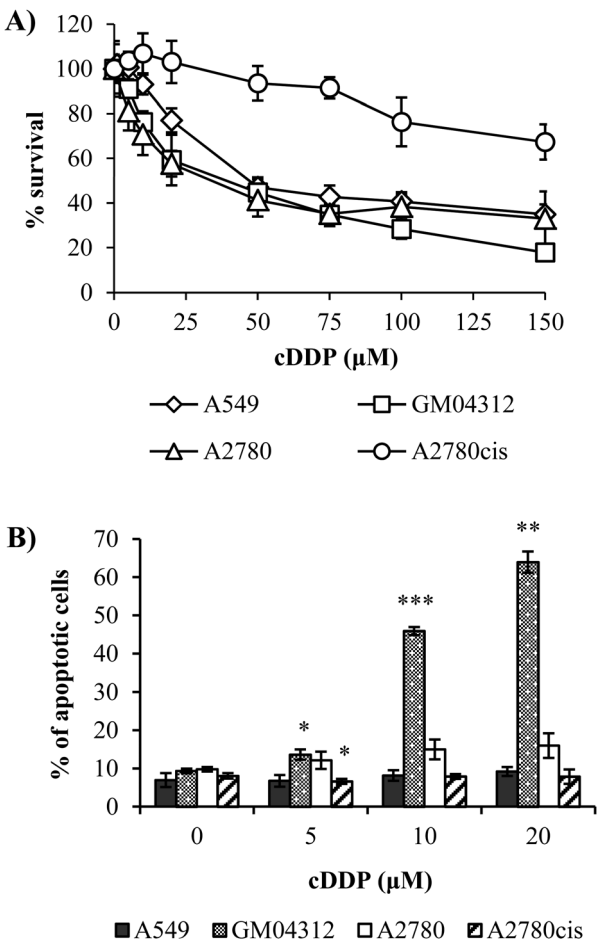

Fig. 1 Cell sensitivity measured 24 h after the end of the 3 h treatment: (A) viability analysis: MTS determined cell survival. (B) Apoptosis analysis: pooled early and late apoptotic cells estimated by flow cytometry. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared with the corresponding negative control. All values are arithmetic means ± standard errors.

Table 1 Cisplatin IC_{50} values. The IC_{50} values and their respective confidence intervals were calculated with Probit analyses, from the MTS viability data

Cell line	IC_{50} (μM)	Confidence intervals	
A549	65.4	50.1	89.1
GM04312	35.5	30.3	41.3
A2780	38.9	30.1	49.6
A2780cis	2123.9	804.5	39433.2

From these results, two different sets of data were extracted. Firstly, the IC_{50} values were estimated for all the cell lines (Table 1). GM04312 and A2780 were the most sensitive cell lines, A549 presented more resistance, and A2780cis was clearly the most resistant one.

Secondly, comparisons among cell lines were performed considering the results at cisplatin concentrations of 5, 10 and 20 μM and the respective negative controls using linear regression analyses. The results show that for the sensitive cell lines (GM04312, A2780 and A549) the regression slopes of dose–viability relationships were negative, with rather good fits and statistically different from zero, indicating that survival decreased with increased drug concentration (Table 2). For the resistant cell line (A2780cis) the regression slope was not different from zero (Table 2), since survival was similar at all tested concentrations. Differences among cell lines were only statistically significant between the resistant A2780cis cell line

Table 2 Linear regression analyses to study treatment responses. Regression slopes, their standard errors and significance, and the regression fit (R^2), for viability and apoptosis, estimated 24 h after treatment, and for the intracellular Pt content and cisplatin-induced G–G adducts, estimated at AT-0 and AT-1 times

Variable	AT time	Cell line	Regression slope ± S.E.	R^2	p value
Viability		A549	−1.24 ± 0.27	0.91	0.042
		GM04312	−2.09 ± 0.17	0.99	0.004
		A2780	−2.00 ± 0.39	0.93	0.032
		A2780cis	0.13 ± 0.22	0.14	0.627
Apoptosis		A549	0.13 ± 0.03	0.89	0.056
		GM04312	2.94 ± 0.61	0.92	0.037
		A2780	0.31 ± 0.081	0.88	0.062
		A2780cis	0.01 ± 0.05	0.03	0.803
Intracellular Pt content	AT-0	A549	0.69 ± 0.01	0.99	0.000
		GM04312	0.70 ± 0.02	0.99	0.000
		A2780	1.97 ± 0.13	0.99	0.003
		A2780cis	0.17 ± 0.00	0.99	0.000
	AT-1	A549	0.29 ± 0.05	0.95	0.023
		GM04312	0.25 ± 0.02	0.98	0.007
		A2780	0.66 ± 0.02	0.99	0.000
		A2780cis	0.24 ± 0.01	0.99	0.000
Cisplatin adducts	AT-0	A549	2.30 ± 0.32	0.96	0.015
		GM04312	2.73 ± 0.12	0.99	0.001
		A2780	1.75 ± 0.03	0.99	0.000
		A2780cis	0.45 ± 0.06	0.96	0.016
	AT-1	A549	2.17 ± 0.30	0.96	0.015
		GM04312	4.26 ± 0.36	0.99	0.005
		A2780	1.67 ± 0.31	0.93	0.030
		A2780cis	0.54 ± 0.16	0.85	0.076

and the most sensitive cell lines A2780 and GM04312 ($p = 0.04$ and $p = 0.009$, respectively).

3.2. Cell line sensitivity: apoptosis

Cell apoptosis was determined as another cisplatin sensitivity marker. The percentage of apoptotic cells (early and late combined) increased with increased cisplatin concentration in most of the cell lines studied, except in the resistant one (A2780cis) (Fig. 1B). However, the increase was statistically significant only for the *XPA* mutant cell line (GM04312), as revealed by Student's *t* tests and linear dose–apoptosis regression slopes (Table 2).

3.3. Cell cycle analysis

The percentage of G1 cells decreased as the cisplatin concentration increased in all of the cell lines, except GM04312 (Fig. 2). Such descents were linked to increases in G2 cell percentages for A549 and A2780cis at all concentrations, and for A2780 at the lower tested concentrations.

Increases in S cell percentages were detected for the A2780 cells at 20 μM, and for the *XPA* mutant cells (GM04312) at 5 μM cisplatin concentration. In these last cells, increases in G1 cell percentages were linked to decreases in G2 and S cell percentages, at 10 and 20 μM cisplatin concentrations, respectively.

3.4. Total intracellular Pt quantitation

The results show that intracellular cisplatin increased with increasing drug concentrations at AT-0 time (Fig. 3A). The highest intracellular Pt content corresponded to the sensitive A2780 cell line, as indicated by its regression slope (Table 2). Since the Pt content was quantitated and referenced to cell mass, direct comparisons among cell lines, and experimental conditions for each cell line, could be performed. Statistically significant differences were found between A2780 and the rest, at both AT-0 and AT-1 conditions, and between A2780cis and A549 and GM04312 at AT-0.

Comparisons between AT-0 and AT-1 showed that at AT-1 time the levels of intracellular Pt still present in the sensitive strains were between 32 and 68% of those at AT-0 time, depending on the cell line and concentration (Fig. 3B). This finding revealed that a statistically significant cisplatin efflux from these cells had occurred. However, no relevant differences were found for A2780cis between AT-0 and AT-1 times.

3.5. Cisplatin-induced DNA adduct quantitation

Our methodology, with an overall recovery of about 50% (considering hydrolysis, ultrafiltration and column recovery),[35] enabled the detection of only one Pt species that corresponded to G–G intra-strand crosslinks (Fig. 4). The concentration of this species after 3 h of treatment at AT-0 and AT-1 times, for all of the cell lines, is shown in Fig. 3C and D, respectively. Except for the resistant A2780cis cells at AT-1 time, the slopes of dose–response regression analyses were statistically higher than zero (Table 2). Comparisons among cell lines at AT-0 time revealed clear differences between the three sensitive and the resistant cell lines, as well as between the GM04312 and A2780 cell lines. Differences in adduct levels were also detected between GM04312 and the other cell lines, and between A549 and A2780cis, at AT-1 time, as indicated by their respective

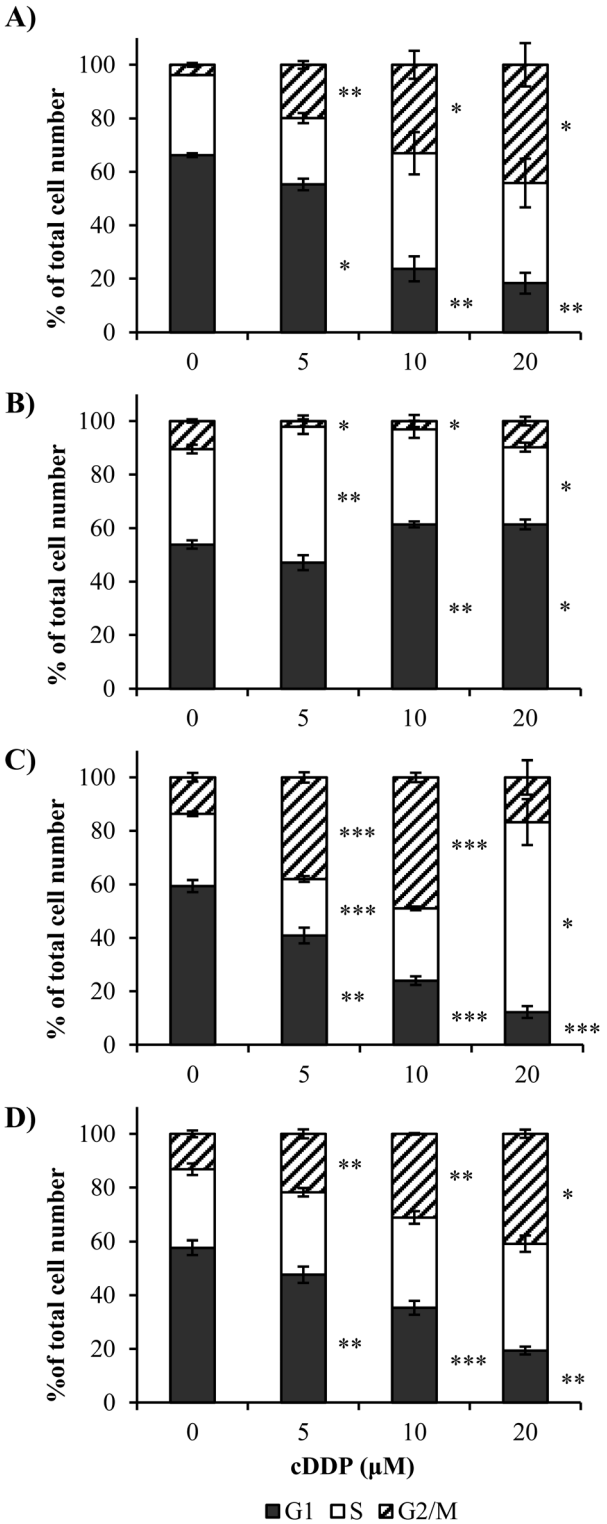

Fig. 2 Cell cycle progression estimated 24 h after the end of the 3 h treatment. Percentage of cells in G1, S and G2/M phases, determined by flow cytometry: (A) A549, (B) GM04312 (C) A2780 and (D) A2780cis. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared with the corresponding negative control. All values are arithmetic means ± standard errors.

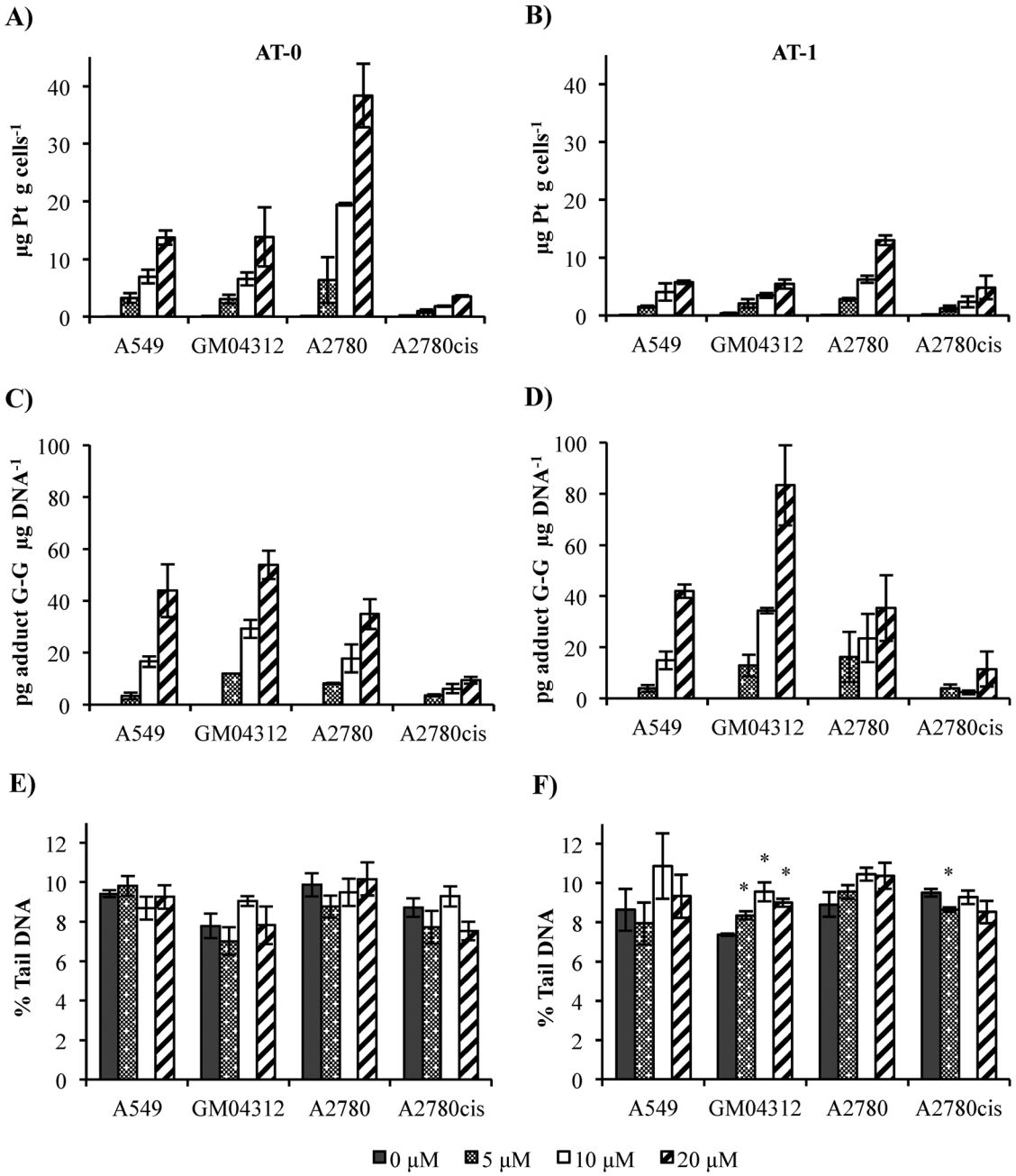

Fig. 3 Quantitation of cisplatin effects at two times after treatment (AT-0 and AT-1): (A and B) the intracellular Pt content, (C and D) cisplatin-induced adduct levels, and (E and F) induced genetic instability, estimated as %Tail DNA. *$p < 0.05$ when compared with the corresponding negative control. Treatment effects on A–D were estimated with linear regression analysis (see Section 2.9). All values are arithmetic means ± standard errors.

regression slopes (Table 2). In addition, although some minor differences could be detected between AT-0 and AT-1 times for each cell line, statistically significant differences in regression slopes were only found for the *XPA* mutant GM04312 cell line.

3.6. Genomic instability: Comet assay results

No effects of cisplatin treatment were detected for any cell line at AT-0, whereas the positive control MMS induced 32.02, 35.10, 45.04 and 54.28%Tail DNA for A549, GM04312, A2780cis and A2780 cells, respectively, all statistically significant ($p < 0.001$). No differences in %Tail DNA were found among cell lines at any AT time, nor between AT-0 and AT-1 times for any of the studied cell lines (Fig. 3E and F).

Analysis within each cell line showed increases in %Tail DNA for GM04312 and A2780, but statistically significant only for GM04312, and a decrease for A2780cis, all of them at AT-1 time.

3.7. Predictive variables for viability and apoptosis

The predictive ability of all the analyzed parameters for cell cisplatin sensitivity/resistance was studied by uni- and

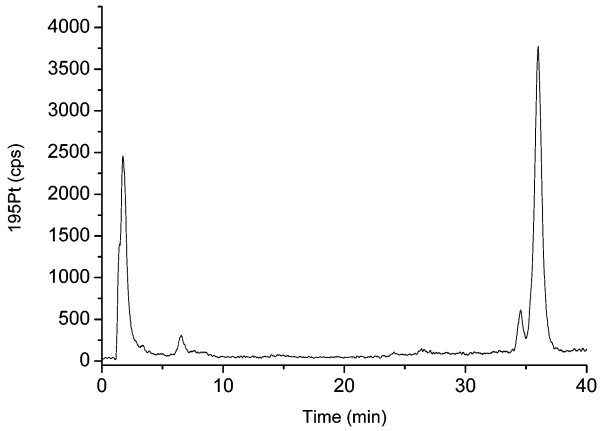

Fig. 4 Example of a chromatogram for a DNA sample obtained from treated cells (A549 cells treated with 20 μM cisplatin). The trace shows the signal for ^{195}Pt in ICP-MS. The peak at 2.5 min corresponded to free Pt. The double peak at 35 min was assigned to the cisplatin–GG adducts, as described before.[27,54]

multivariate regression analysis, together with correlation studies. The AT-1 adduct levels presented statistically significant regression slopes and rather good R^2 fits (Table 3). In fact, they were the best predictors (or equally good) for cell viability and apoptosis in the three analyzed datasets (the raw data are provided in Table S1, ESI†).

When datasets corresponding to all of the cell lines and the sensitive cell lines were considered, the predictive capability of AT-1 adducts increased when combined with AT-0 intracellular Pt (Table 3). However, when considering sensitive and resistant cells of the same origin, like A2780 and A2780cis cells, the predictive ability of AT-1 adducts was considerably improved when combined with AT-1 DNA strand break results (Table 3) (all data are provided in Table S2, ESI†).

Finally, the combination of AT-1 adduct levels, AT-0 intracellular Pt content, AT-1 DNA strand breaks and percentage of G1 cells provided rather good predictive value for cell viability and apoptosis, with R^2 fits between 0.89 and 0.997 and all statistically significant (ESI†, Table S3).

4. Discussion

The aim of the present work was to obtain the best model to predict cisplatin sensitivity/resistance; for this aim, cisplatin-induced DNA adducts were evaluated alone and/or in combination with total intracellular Pt content and cell biological response parameters, like induced cell cycle changes and genomic instability.

Table 3 Predictive analysis with viability and apoptosis as dependent variables. Univariate regression data (in bold) with adducts AT-1 as an independent variable. Multivariate regression data with adducts AT-1 as the first independent variable and intracellular Pt AT-0 as the second variable, for all three datasets, and also with %Tail DNA AT-1 as the second variable, for A2780 and A2780cis cell lines (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Model dependent *vs.* independent variables						
Dependent variable	Independent variables	B slope	Confidence intervals 95%		R^2	F Anova
(A) All cell lines						
Viability	**Adducts-AT-1**	**−0.624**	**−0.840**	**−0.408**	**0.733**	**38.356*****
	Adducts-AT-1	−0.405	−0.570	−0.239	0.906	62.456***
	Intracellular-Pt-AT-0	−0.826	−1.191	−0.461		
Apoptosis	**Adducts-AT-1**	**0.600**	**0.373**	**0.827**	**0.697**	**32.139*****
	Adducts-AT-1	0.745	0.492	0.998	0.774	22.252***
	Intracellular-Pt-AT-0	−0.545	−1.103	0.013		
(B) Sensitive cell lines						
Viability	**Adducts-AT-1**	**−0.561**	**−0.802**	**−0.320**	**0.729**	**26.900*****
	Adducts-AT-1	−0.376	−0.525	−0.228	0.935	64.840***
	Intracellular-Pt-AT-0	−0.768	−1.092	−0.443		
Apoptosis	**Adducts-AT-1**	**0.609**	**0.312**	**0.905**	**0.676**	**20.902****
	Adducts-AT-1	0.743	0.424	1.063	0.763	14.485**
	Intracellular-Pt-AT-0	−0.560	−1.258	0.139		
(C) Ovarian cancer cell lines						
Viability	**Adducts-AT-1**	**−1.340**	**−1.837**	**−0.844**	**0.879**	**43.662****
	Adducts-AT-1	−0.931	−1.481	−0.382	0.949	46.213**
	%Tail-DNA-AT-1	−9.753	−19.388	−0.118		
	Adducts-AT-1	−0.836	−2.502	0.831	0.894	20.990**
	Intracellular-Pt-AT-0	−0.501	−2.069	1.066		
Apoptosis	**Adducts-AT-1**	**0.247**	**0.119**	**0.375**	**0.788**	**22.323****
	Adducts-AT-1	0.144	−0.002	0.290	0.905	23.813**
	%Tail-DNA-AT-1	2.463	0.090	5.017		
	Adducts-AT-1	0.149	−0.293	0.591	0.802	10.156*
	Intracellular-Pt-AT-0	0.097	−0.319	0.513		

Using cell viability and apoptosis as resistance indicators, we have analyzed the response of four human cell lines to low doses of cisplatin treatment.

In agreement with previous results,[7,8,36] no changes in cell viability, apoptosis or cell cycle progression were detected immediately after cisplatin treatment, and therefore they were measured 24 h after the end of the treatment. Intracellular Pt content, cisplatin G–G intra-strand crosslinks, and DNA strand breaks were determined immediately after treatment (AT-0 time) and also one hour later (AT-1 time). In this way, it was possible to estimate not only the Pt influx and efflux[37] but also adduct dynamics (formation *versus* repair/removal).[38] As described for other human cells,[35] only G–G intra-strand crosslinking adducts were detected in the analyzed cells.

The GM04312 cells were the most cisplatin sensitive, as indicated by the IC_{50} values and dose–viability and dose–apoptosis regression analyses. They were followed by the A2780 cells, with very similar viability but no significant apoptosis, and by the A549 cells, which showed higher viability and no induced apoptosis. This rank was closed by the A2780cis cells, which did not show growth inhibition nor induced apoptosis at any assayed dose.

This cell sensitivity rank was corroborated by the cell cycle data, since G1-phase arrests (observed before for important DNA damage in XPA-deficient cells[39]) were only detected in the GM04312 cells at the highest drug concentrations. Moreover, S-phase arrests (indicative of high DNA damage[16]) were already detected at cisplatin doses of 5 μM for the GM04312 cells and 20 μM for the A2780 cells. For the A2780 cells at lower doses, and for the A549 and A2780cis cells at all cisplatin concentrations, the results revealed cell cycle arrests at the G2/M-phase, as described before for cisplatin treatments,[18] even after low dose exposure.[8]

Considering that low levels of XPA protein were specifically related to cisplatin sensitivity[40] and to increased cell apoptosis,[41] the highest sensitivity of the GM04312 cells was most likely due to the lack of XPA protein.[7] This possibility was supported by the increases in adduct levels detected in these cells at AT-1 time, compared to AT-0 time, demonstrating the absence of DNA adduct repair. Furthermore, DNA repair capacity would also explain differences between GM04312 and A549 cells in adduct levels at AT-0 time, since their cisplatin influx and efflux were almost identical. Nevertheless, the fact that cell viability was similar for GM04312 and A2780 cells following cisplatin treatment suggests that other repair pathways (different from NER) could be contributing to the cisplatin-induced DNA damage response, at least at low doses.[10,22]

Despite similarities in cell viability with the GM04312 cells, the sensitivity of the A2780 cells to cisplatin cannot be linked to a deficiency of DNA repair. However, it might be related to their higher Pt influx, as described,[27,37,42,43] even for other platinum chemicals,[44] which could be related to high levels of the copper transport protein CTR1,[23] although recently the influence of this type of protein was considered controversial.[45] Nevertheless, since high Pt influx was not accompanied by high levels of induced DNA adducts, the drug effects on cell viability in these A2780 cells must be related to cisplatin activity in the cell cytoplasm, and not specifically related to apoptosis, because the apoptotic cell number did not significantly change at different Pt concentrations. In this regard, although the lack of induced apoptosis in the A2780 cell line after treatment with cisplatin doses as high as IC_{90} had been previously reported,[17] controversial data may be found in the literature.[46,47]

On the other hand, the lack of differences in cisplatin adducts between AT-0 and AT-1 times not only for the A2780 cells but also for the A549 and A2780cis cells (all repair active cells), demonstrated that there is an equilibrium between adducts being repaired/removed and new adducts being formed by the remaining intracellular cisplatin, at least in the 1 h time lapse studied, as suggested before.[48] Besides, the repair/removal of cisplatin-induced DNA adducts in these cells seemed to be a relatively slow process, as described for A2780[23] and the other cells;[8,23,49] however, it is important to address that no adducts were detected 24 h after the end of treatment (data not shown).

The intermediate viability of the A549 cells against cisplatin treatment agrees with their classification as cisplatin sensitive[28] or resistant.[13] Our results are also in agreement with the previously described lack of cisplatin-induced apoptosis in this cell line.[15,50] Moreover, comparisons to the GM04312 and A2780 cells suggest that some factor other than adduct levels and cisplatin influx/efflux must be influencing A549 sensitivity/resistance to this drug.

In contrast, the cisplatin resistance detected in the A2780cis cells might be clearly related to the low DNA-adduct levels which, in turn, could also be ascribed to the low intracellular Pt content, previously claimed as the cause of their *cis*-resistance.[37,43,48] Furthermore, as expected, the A2780cis cells did not show cisplatin-induced apoptosis.[17,46]

According to all these results, cisplatin-induced adduct levels depended on the intracellular Pt content within each cell line, but not necessarily when all of the cell lines were considered together. Furthermore, differences in intracellular cisplatin at AT-0 and AT-1 times revealed a considerable cisplatin efflux after one hour for all the cell lines except A2780cis, which was much higher and faster than those reported before.[14,23,37,48]

In the analysis of genomic stability, the Comet assay is the most widely used tool.[34] However, detection of DNA strand breaks after cisplatin treatment is not a straightforward result; some authors reported them in different cells and organisms,[20,26] and others reported their absence.[20,51,52] Although some authors have used the Comet assay to detect and quantitate cisplatin-induced DNA adducts,[15,22,51] we have used it to study which DNA adduct levels would induce detectable genomic instability. Our results show that cisplatin induced DNA strand breaks only in GM04312 cells at AT-1 time. This indicates that DNA strand breaks, and therefore genomic instability, were detected when adducts were accumulating because the cells were not repairing them, and that DNA damage was linked to large cell cycle changes and to apoptosis.

Although we have worked with only four human cell lines, and indeed further experiments in different cancer cell models, and in a larger number of cell lines, are required as confirmation, our highly promising findings allowed us to develop a model in which cisplatin-induced adducts seem to be fundamental in the prediction of resistance. Considering the data together, although cisplatin sensitivity/resistance could be related to induced-DNA

adducts in the case of GM04312 and A2780cis cells, but not in A549 and A2780 cells, the univariate regression analyses revealed that G–G intra-strand adduct levels were good predictors of viability and apoptosis, as reported for squamous carcinoma cells of the head and neck.[14] Specifically, the AT-1 adduct levels were better predictors than the AT-0 ones, suggesting the relevance of the ratio adduct formation/adduct repair, and therefore the importance of time when analyzing the effects of cisplatin treatments in cell stability.[7,8,36] Time importance was stressed by the fact that only the %Tail DNA at AT-1 time correlated with the other analyzed variables (Table S4, ESI†). In addition, the combination of AT-1 adduct levels with intracellular Pt content at AT-0 time, in multivariate regression analyses, improved the predictive value when using cell lines of different origins. However, when comparing matched sensitive and resistant ovarian cancer cells, the predictive value of AT-1 adduct levels was considerably increased when the genomic instability data (as AT-1 %Tail DNA) was introduced into the equation. This result supported the potential of the Comet assay in the analysis of the chemotherapy response.[53]

5. Conclusions

We have developed an experimental model that might be a useful tool to predict cisplatin resistance. It has allowed us first to confirm that in our analyzed cell lines of different origins, cisplatin sensitivity/resistance was dependent on DNA adduct formation, and second to demonstrate the relevance of their repair/removal. Likewise, our model demonstrates that the relationship between adduct formation and cisplatin influx is not always direct; it depends on the analyzed cell type, suggesting the existence of a relevant cytoplasmic mechanism for cisplatin sensitivity. Moreover, our work demonstrates that the detection of DNA strand breaks with the Comet assay, after cisplatin treatment, is possible when the levels of induced adducts are capable of altering cell cycle progression and the percentage of apoptotic cells. Data on genomic instability could also be a valuable tool in this type of analysis, at least when comparing sensitive and resistant cells with the same genetic background.

Nevertheless, the model is based on the data obtained from only four cell lines, and although their consistency and cross fit, as well as the fact that the analyzed cell lines were derived from different tissues and tumor cells with different cisplatin sensitivities, make them reliable enough, surely the model would benefit from the analysis of a broader range of cell lines.

Author contributions

Conception and design: L. M. Sierra, E. Blanco-González, M. Montes-Bayón, M. Espina, M. Corte-Rodríguez, and M. I. Sierra. Development of methodology: M. Espina, M. Corte-Rodríguez, L. M. Sierra, E. Blanco-González, and M. Montes-Bayón. Acquisition of data: M. Espina, M. Corte-Rodríguez, and L. Aguado. Analysis and interpretation of data: L. M. Sierra, M. Espina, and P. Martínez-Camblor. Writing, review and/or revision of the manuscript: L. M. Sierra, E. Blanco-González, M. Montes-Bayón, M. Espina, M. Corte-Rodríguez, and M. I. Sierra.

Conflict of interest

The authors declare that there are no conflicts to disclose.

Abbreviations

AT	After treatment
AT-0	Immediately after treatment
AT-1	One hour after treatment
cDDP	Cisplatin (*cis*-diamminedichloroplatinum(II))
CTR1	CTR1 human protein
FITC	Fluorescein IsoThioCyanate
G–G	Guanine–guanine intrastrand adduct
Hepes	2-[4-(2-Hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid
HPLC-ICP-MS	High performance liquid chromatography–inductively coupled plasma–mass spectrometry
IC_{50}	Half-maximal inhibitory concentration
ICP-MS	Inductively coupled plasma–mass spectrometry
LMP	Low melting point
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt
NER	Nucleotide excision repair
PBS	Phosphate buffered saline
SCTs	Scientific and technical services at the University of Oviedo (Spain)
SV40	Simian virus 40
XPA	*XPA* human gene
XPA	XPA human protein

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