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Different accumulation of cisplatin, oxaliplatin and JM216 in sensitive and cisplatin-resistant human cervical tumour cells

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ABSTRACT

The significance of reduced drug accumulation in resistance to cisplatin was investigated by using cisplatin, oxaliplatin and JM216 (hydrophobicity rank: JM216 > oxaliplatin > cisplatin) in human squamous cell carcinoma cell line A431 and its cisplatin-resistant counterpart A431/Pt. While cisplatin showed a resistance factor of 2.6, oxaliplatin and JM216 circumvented the resistance. Platinum accumulation after cisplatin exposure was lower (2.4-fold) in A431/Pt than in A431 cells, whereas a similar accumulation was found in the two cell lines when oxaliplatin or JM216 were used, thereby suggesting the capability of the latter drugs to bypass the accumulation defect. In the A431 cell line platinum accumulated to a similar extent after exposure to cisplatin, oxaliplatin or JM216, while in A431/Pt cells, Platinum accumulation depended on the hydrophobicity of the drug, and an increased hydrophobicity favours the uptake. No difference in efflux of cisplatin was found between the two cell lines. The values of platinum–DNA binding in A431 cells were similar for cisplatin and JM216 and higher than those of oxaliplatin. In A431/Pt cells: (i) Pt–DNA binding levels of JM216 remained as in sensitive ones; (ii) Pt–DNA levels of cisplatin and oxaliplatin were very similar and nearly two-fold lower than those of JM216. Such results, in this cell system characterized by a low level of cisplatin resistance, support a model whereby platinum uptake occurs by a mechanism of facilitated diffusion, perhaps involving a gated channel, which can be lost during the selection of the drug-resistant variant(s). The hydrophobicity of the drug can be the key to bypass resistance.

1. Introduction

Platinum (Pt) drugs represent an important class of anticancer agents. Cisplatin (CDDP, Fig. 1) is one of the most widely used anticancer drugs [1]. Despite its extensive use, several side effects [2] and the acquisition or presence of resistance to the drug undermines its clinical use [3]. These drawbacks have been the impetus for the development of new Pt compounds [3].

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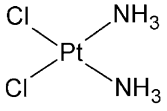
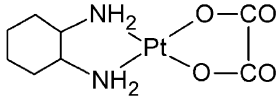
Among them, the interest of satraplatin [bis-(acetato)amminedichloro(cyclohexylamine) platinum(IV)] (JM216, Fig. 1) and of oxaliplatin {[(1R,2R)-1,2-cyclohexanediamine-N,N′][oxalato(2-)-O,O′] platinum(II)} (L-OHP, Fig. 1) has emerged, expanding the tumour types for which a Pt drug may be employed. Both drugs have shown potential use in CDDP/resistant tumours during preclinical evaluations [3]. JM216 has been chosen to enter clinical trials as orally active compound [4]. L-OHP has been proven to possess some activity in combination with thymidylate synthase inhibitors in patients with advanced colorectal cancer [5,6]. *In vivo*, resistance to CDDP can emerge after only a few courses of low-dosage chemotherapy [7] and, while the levels of resistance are in the order of two- to four-fold, the dominant mechanism accounting for it is unknown [8,9]. *In vitro*, resistance generated in cells exposed to increasing concentrations of CDDP generally reaches higher levels than *in vivo* and is multifactorial [9]. The factors accounting for cellular resistance include reduced drug accumulation, enhanced intracellular drug detoxification, increased repair and tolerance of Pt–DNA adducts and reduced apoptosis induction [10,11]. Reduced drug accumulation is a particularly common finding in CDDP-resistant cells, and the mechanism accounting for the decreased uptake remains uncertain [12,13]. As increased efflux usually does not occur in CDDP-resistant cells, it is important to gain insights into the parameters that govern the rate of CDDP influx in CDDP-sensitive and -resistant cells. The use of other Pt drugs characterized by different degree of hydrophobicity [14] could provide new understanding in defining mechanisms of Pt accumulation and possibly the basis of resistance overcoming, in particular when the acquisition of CDDP resistance is related to reduced drug accumulation.



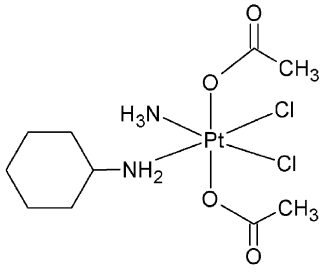
cis-diamminedichloroplatinum (II)

Cisplatin (CDDP)



[(*1R,2R*)-1,2-cyclohexanediamine-*N,N′*][oxalato(2-)-*O,O′*] Platinum (II)

Oxaliplatin (L-OHP)



bis-(acetato)amminedichloro(cyclohexylamine) Platinum (IV)

Satraplatin (JM216)

Fig. 1 – Platinum compounds.

Based on this background, in the present study we evaluated cellular accumulation and Pt–DNA adducts of Pt drugs exhibiting different hydrophobicity in a CDDP-sensitive (A431) and in its CDDP-resistant variant (A431/Pt) human squamous cervical carcinoma cell line [15]. When the major step for resistance to CDDP may be attributed to a defect of drug accumulation, our findings show that, in the sensitive cell line, the three drugs accumulate at the same level without the influence of their hydrophobicity. In the CDDP-resistant line, such a physico-chemical characteristic of the drug seems to be the key to facilitate the accumulation and to bypass resistance. The available results could support a mechanisms of facilitated diffusion involving a gated channel in the sensitive cell line. This facilitated diffusion seems to be lost during the selection of the drug-resistant variant(s).

2. Materials and methods

2.1. Cell lines and culture conditions

The human cervical squamous cell carcinoma cell line A431 and the CDDP-resistant A431/Pt subline were used in this study [15]. Establishment details and biological properties have been already described [15]. All the cell lines grew as monolayers at 37 °C in a 5% CO_2 atmosphere in RPMI-1640 medium (BioWhittaker Italia S.r.L., Milano, Italy) containing 10% heat-inactivated fetal calf serum (Sera-Lab, Crawley Down, Sussex, England) and 2 mM L-glutamine (Sigma–Aldrich S.r.L., Milano, Italy). Antibiotics were omitted from the medium to avoid interactions. The parental and the resistant lines were used from passage 2 to 20.

2.2. Platinum drugs

CDDP (Platamine®; Pharmacia & Upjohn S.p.A., Milano, Italy); L-OHP (Eloxatine®) was a gift from Sanofi-Synthelabo S.p.A., Milano, Italy; JM216 was provided by Dr. L.R. Kelland (Antisoma Research Laboratories, London, UK). Immediately before use, CDDP and JM216 were dissolved in 0.9% saline, while L-OHP was dissolved in water.

2.3. Assessment of cellular sensitivity to drugs

Cellular sensitivity to drugs was assessed by cell counting. Briefly, cells were seeded into three 6 well-plates (9.6 cm^2, Iwaki, Japan) at 6×10^4 cells/well and allowed to attach overnight. Drugs were then added, and exposure was for 1 h at 37 °C to a range of concentrations 35–500 μM. After drug exposure, the medium was removed and fresh drug-free medium was added. Plates were further incubated for 72 h. Cells were washed with prewarmed phosphate-buffered saline (PBS) and then harvested using a trypsin–EDTA solution (Sigma–Aldrich S.r.L., Milano, Italy). Cells were then counted using a microscope. All assays were performed in triplicate, and the values are the means ± standard deviation (S.D.) of three-independent experiments. IC_{50} is defined as the concentration resulting in 50% reduction in cell number as compared to untreated plates. The resistance factor (RF)

was calculated as:

$$RF = \frac{IC_{50}\ A431/Pt\ cells}{IC_{50}\ A431\ cells}$$

2.4. Determination of cellular Pt accumulation

For both cell lines, 1×10^6 cells were seeded in 100 mm tissue culture dishes (Iwaki) and, 24 h later, cells were exposed to freshly dissolved CDDP, L-OHP or JM216 at concentrations up to 500 μM. After 1 h of drug exposure, the medium was removed and the cells were washed three times with ice-cold PBS, scraped and harvested in double distilled water. Samples were freeze-dried overnight and Pt was solubilized in HNO_3 following standard procedures [16]. Total Pt content was determined by flameless absorption spectroscopy (FAAS) (Model 3300, Perkin-Elmer, Norwalk, CT). Under these conditions, the detection limit was 10 μg/l [16]. Pt levels were expressed as nmol Pt/10^6 cells, with cell number determined by counting parallel cultures. Experiments were performed in duplicate and the values are the means ± S.D. of three-independent experiments.

2.5. Drug efflux studies

Both cell lines were seeded as described above for cellular Pt accumulation studies. Twenty-four hours later, the A431 and A431/Pt cells were treated with 150 and 500 μM CDDP, respectively, to obtain, after 60 min, similar cellular Pt levels in the two cell lines (see Pt accumulation). At the end of the drug loading period, the medium was removed and the monolayers were washed three times with drug-free medium. The washing procedure time (90 s) was added on to the efflux time. Cells were then incubated further in fresh medium for various times up to 1 h. Efflux was stopped by removing the medium and washing three times the cells with ice-cold PBS. Cells were then processed, analysed for Pt content and counted as described above. Experiments were performed in duplicate and the values are the mean ± S.D. of three-independent experiments.

2.6. Quantification of Pt–DNA adducts

For both cell lines, 4.5×10^6 cells were seeded in 100 mm tissue culture dishes (Iwaki). After 24 h, the cells were exposed for 1 h to CDDP, L-OHP or JM216 (as described previously for Pt accumulation) at concentrations ranging up to 500 μM. After 1 h drug exposure period, the medium was removed and the cells were washed with ice-cold PBS (3× 10 ml), scraped and harvested in double distilled water (1.5 ml). DNA was extracted, using a combined phenol–Tris–HCl (Labtek S.r.L. Eurobio, Milano, Italy) and chloroform–ethanol–sodium acetate (Sigma) technique (modification of the method of Kirby and Cook [17]). Lysis of cells was carried out overnight at 37 °C, in the presence of a mixture of 10 mM Tris–HCl, 1 mM EDTA, 0.1 M NaCl, 1% SDS (all from Sigma), and 25 mg ml^{-1} proteinase K [32 UmAnson/mg] (Labtek). The amount and purity of DNA were determined by UV spectrometry (Model UV-1204, Shimadsu), measuring the absorption at 260 nm (A_{260}) and at 280 nm (A_{280}) [18]. The ratio A_{260}/A_{280} was in the range 1.7–1.9 [18]. Pt content of the samples was assessed by inductively coupled plasma mass spectroscopy (ICP-MS) (Elan 5000 Perkin-Elmer Sciex) [16]. DNA platination levels were expressed as pmol Pt/μg DNA. Experiments were made in duplicate and the values are the mean ± S.D. of three-independent experiments. The detection limit was 0.1 μg/l [16].

2.7. Statistical analysis

Data of Pt uptake, adducts and Pt efflux were plotted using a linear regression model. For all the plots a significant regression coefficient was found ($p < 0.01$ or less).

Statistical significance of the regression was evaluated by using unpaired two-tailed Student's *t*-test. Difference between linear regressions was obtained by comparing the slope with Student's *t*-test. A critical value of $p < 0.05$ was considered.

3. Results

3.1. Assessment of cellular sensitivity to Pt drugs

Cellular sensitivity to Pt drugs was determined by growth-inhibition assay after 1 h drug exposure. Cells were exposed to various concentrations of CDDP, L-OHP or JM216 (see Section 2). The IC_{50} values are shown in Table 1. Under our experimental conditions, the A431/Pt cell line was 2.6-fold more resistant to CDDP. No significant differences in IC_{50} values were observed when the two cell lines were exposed to L-OHP or JM216. The IC_{50} values of A431 cells were approximatively 5.3-fold and 1.8-fold higher for L-OHP and JM216, respectively, than CDDP.

3.2. Determination of cellular Pt accumulation

Total cellular Pt levels after 1 h drug exposure (range 35–500 μM) were measured by FAAS. Values are expressed in terms of nmol Pt/10^6 cells, and are shown in Fig. 2A–C. In both cell lines, exposure to CDDP resulted in a concentration-dependent linear increase in Pt levels. On the other hand, the slope was significantly steeper in A431 cells supporting that the uptake was higher in A431 cells than in the resistant variant (slope = 0.0071 ± 0.001, $r = 0.977$ in A431 and slope = 0.002 ± 0.0001, $r = 0.992$ in A431/Pt; $p < 0.001$). For L-OHP, Pt accumulation was similar in A431 and A431/Pt cells (slope = 0.006 ± 0.0001, $r = 0.988$ and slope = 0.006 ± 0.001, $r = 0.985$, respectively; p not significant). Similar levels of Pt were accumulated in A431 and A431/Pt cells after 1 h exposure to JM216 (slope = 0.008 ± 0.0001, $r = 0.991$ and 0.007 ± 0.0001, $r = 0.996$; p not significant). The levels of Pt accumulation in the same cell

Table 1 – Cell sensitivity to CDDP, L-OHP and JM216 in A431 and A431/Pt cells[a]

Drugs	A431 IC_{50} (μM)	A431/Pt IC_{50} (μM)	RF[b]
CDDP	35.55 ± 7.13	92.30 ± 20.5	2.6
L-OHP	187.60 ± 14.41	180.84 ± 4.11	0.96
JM216	65.40 ± 9.21	71.51 ± 5.4	1.09

[a] Cell sensitivity was assessed in cells exposed for 1 h to the drug and counted 72 h later.
[b] Resistance factor, calculated as: RF = (IC_{50} A431/Pt cells)/(IC_{50} A431 cells).

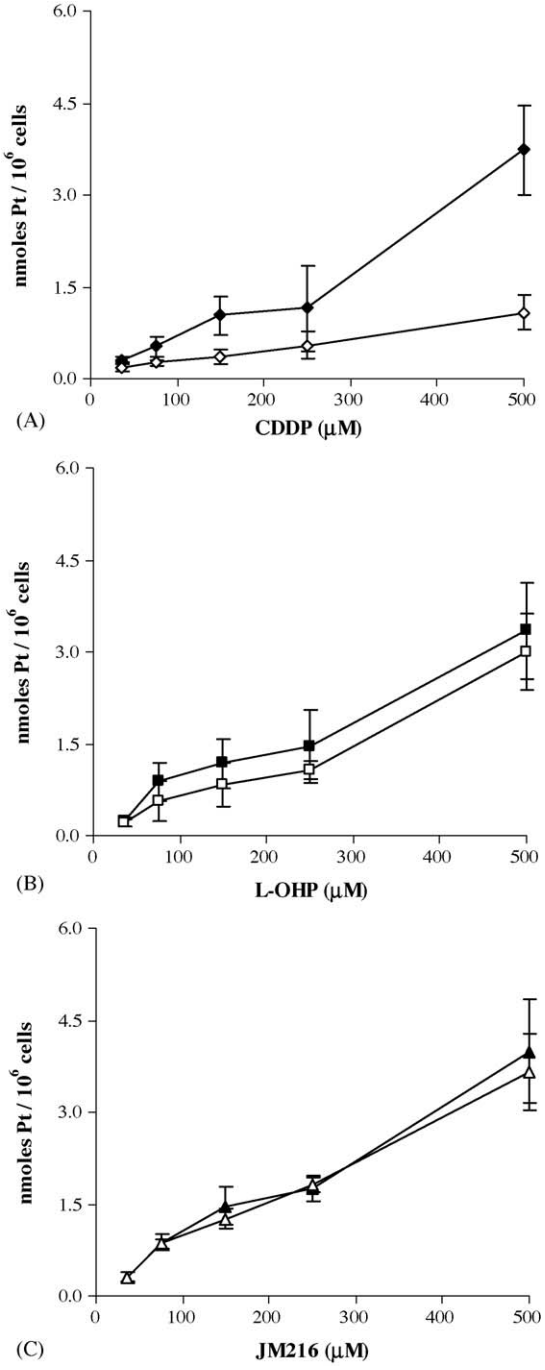


Fig. 2 – Pt accumulation after 1 h exposure to increasing concentration of: (A) CDDP in the A431 (◆) and in the A431/Pt (◇) cell lines; (B) L-OHP in the A431 (■) and the A431/Pt (□) cell lines; (C) JM216 in the A431 (▲) and the A431/Pt (△) cell lines. Data are means ± S.D. of three duplicate experiments.

line exposed to the three drugs is shown in Fig. 3A and B. In A431/Pt cells, cellular Pt accumulation was CDDP < L-OHP < JM216 (slope = 0.002 for CDDP, 0.006 for L-OHP and 0.007 for JM216) (Fig. 3B) whereas no difference between compounds was found in A431 cells (slope = 0.007 for CDDP, 0.006 for L-OHP and 0.008 for JM216) (Fig. 3A).

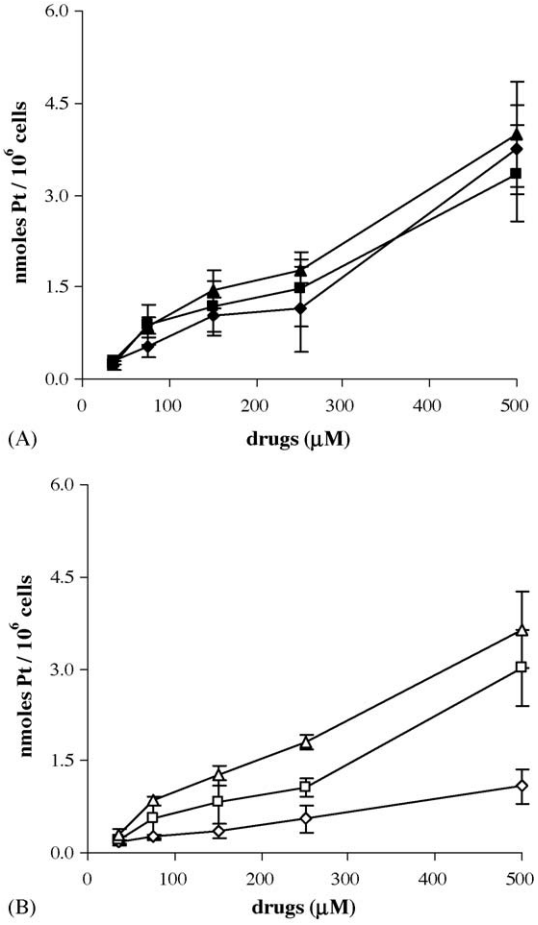


Fig. 3 – Comparison of the levels of Pt accumulation in: (A) A431 and (B) A431/Pt exposed to CDDP, L-OHP, JM216. Symbols are as reported in Fig. 2.

3.3. *Drug efflux studies*

Based on the Pt accumulation data, efflux studies were performed in A431 and A431/Pt cells exposed to CDDP. The time course of Pt efflux into drug-free medium was examined in A431 and A431/Pt cell lines, following a 60 min loading period, with 150 or 500 μM CDDP, respectively (Fig. 4). The

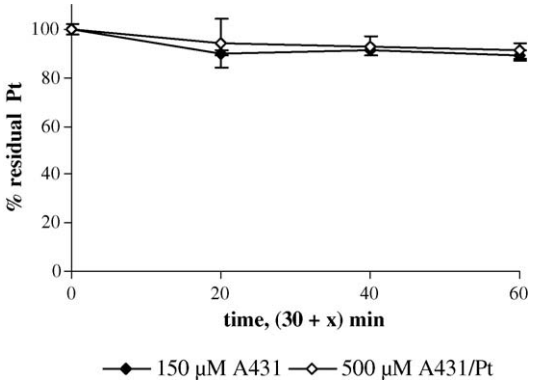


Fig. 4 – Efflux of CDDP over 60 min period from A431 (◆) and A431/Pt (◇) cells. Data are means ± S.D. of three duplicate experiments.

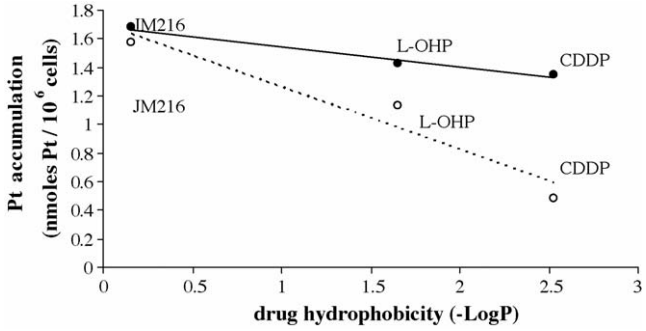


Fig. 5 – Relationship between drug accumulation and hydrophobicity (−log *p*) of the drugs in A431 ((●) and full line) [$y = -0.1429x + 1.6894$; $R^2 = 0.9754$] and A431/Pt ((○) and dotted line) [$y = -0.4399x + 1.6996$; $R^2 = 0.9317$].

retained intracellular Pt levels after incubation in drug-free medium for different times did not show significant differences in efflux (slope = −0.160 ± 0.080 and −0.140 ± 0.038 in A431 and A431/Pt, respectively; *p* not significant).

3.4. *Relationship between Pt accumulation and drug hydrophobicity*

To assess whether any relationship existed between the hydrophobicity of the drugs and cellular accumulation of Pt in A431 and A431/Pt cells, the mean values of the different Pt accumulations in the same range of drug concentrations (35–500 μM) were plotted as a function of ranking of the compounds in terms of hydrophobicity (JM216, log *p* = −0.16 ± 0.16; L-OHP, log *p* = −1.65 ± 0.21; CDDP, log *p* = −2.53 ± 0.28) [14]. We found a linear correlation between hydrophobicity and Pt accumulation (mean data) in both cell lines (Fig. 5). The slope for A431/Pt cells was steeper than that for A431 cell (slope = −0.440 and −0.143, respectively; see Fig. 5), although the statistical significance of the difference could not be determined because of the limited number of data.

3.5. *Analysis of Pt–DNA adducts*

The amount of Pt bound to DNA after 1 h exposure to various concentrations from 35 to 500 μM of CDDP, L-OHP or JM216 increased in both A431 and A431/Pt cells (Fig. 6A–C). With CDDP, DNA platination levels were significantly higher in A431 cells than in the resistant cell line throughout the entire range of tested concentrations (slope = 0.002 ± 0.0001 and 0.001 ± 0.0001, respectively; $p < 0.005$). No significant differences in DNA platination were observed between the two cell lines when JM216 was used (slope = 0.002 ± 0.0001 for both cell lines). As described for Pt accumulation, DNA platination after L-OHP exposure was slightly higher in A431 than in A431/Pt cells but the difference was not statistically significant. When comparing the Pt–DNA levels both in A431 and in A431/Pt cells after exposure to the three drugs (Fig. 7A and B) efficiency to form adducts was JM216 > CDDP > L-OHP in A431 and JM216 > CDDP ≅ L-OHP in A431/Pt cells.

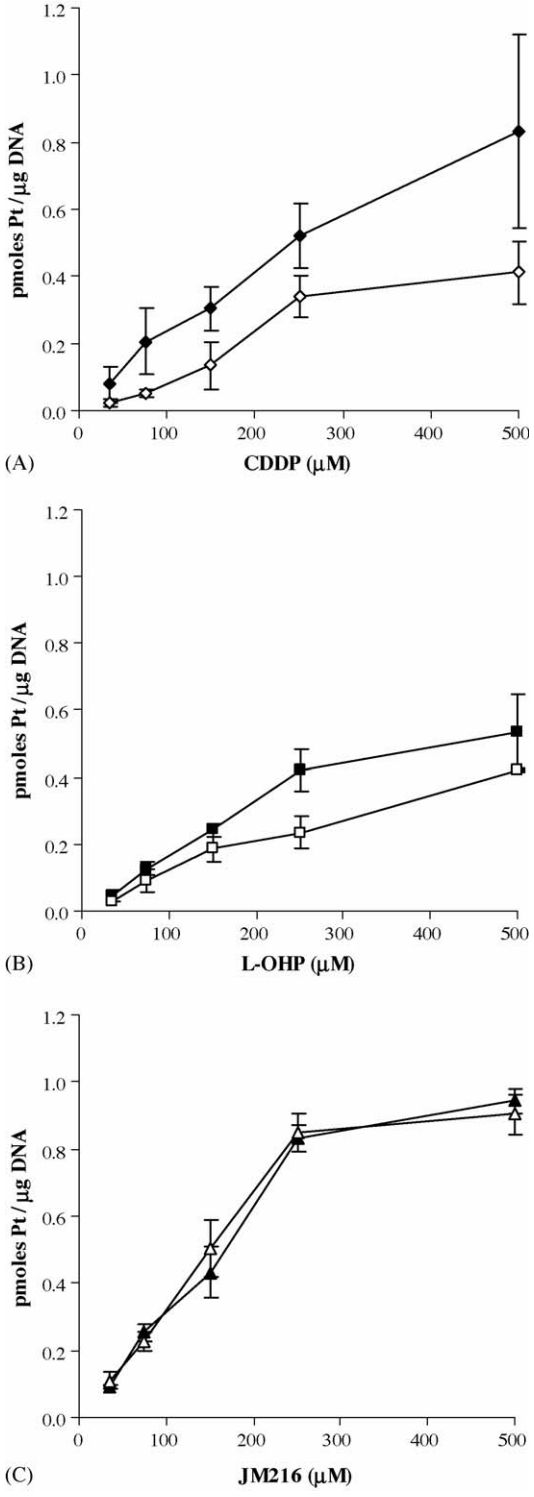


Fig. 6 – Pt–DNA binding after 1 h exposure to increasing concentrations of: (A) CDDP in the A431 (◆) and the A431/Pt (◇) cell lines; (B) L-OHP in the A431 (■) and the A431/Pt (□) cell lines; (C) JM216 in the A431 (▲) and the A431/Pt (△) cell lines. Data are means ± S.D. of three duplicate experiments.

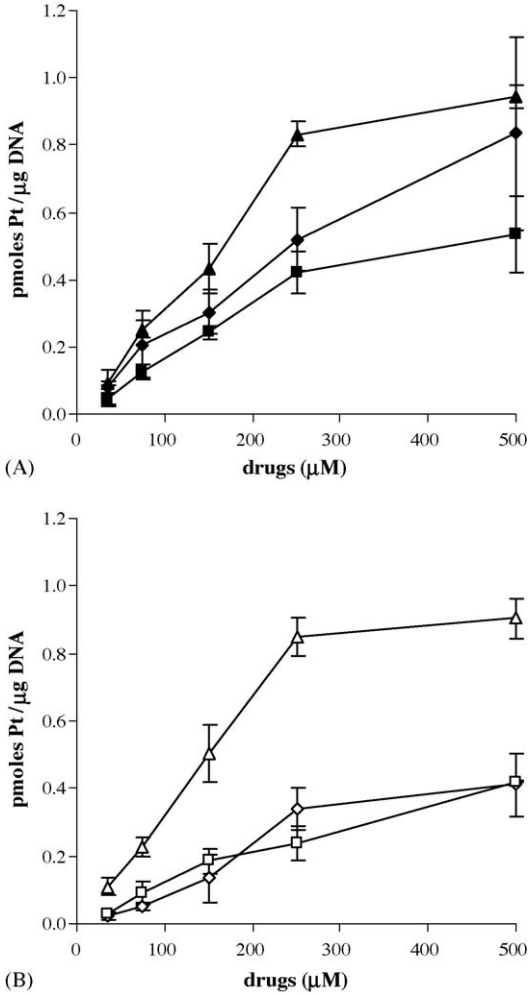


Fig. 7 – Comparison of the levels of Pt-DNA adducts in (A) A431 and (B) A431/Pt exposed to CDDP, L-OHP, JM216. Symbols are as reported in Fig. 6.

4. Discussion

In the present study, we investigated and compared the cellular effects of CDDP, L-OHP and JM216 on the human cervical squamous cell carcinoma cell line A431 and on its CDDP-resistant counterpart A431/Pt. This experimental model is characterized by a low degree of resistance (RF = 2.6) which provides a mimic of the clinical situation, where levels of resistance are of the order of two- to four-fold [9]. The parental and resistant cells share common properties in terms of morphology, growth, glutathione and metallothioneine content [15]. Among the proteins involved in the recognition of Pt-induced damage, the parental and resistant cells exhibit difference in levels of the DNA mismatch repair protein MSH2 (which is reduced in A431/Pt cells) but not in MLH1 content [15]. We now add that the two cell lines do not differ in terms of efflux for CDDP and the 2.4-fold reduction of drug accumulation in A431/Pt cells paralleled their resistance factor (RF = 2.6). These informations highlight the role of reduced drug accumulation in resistance and suggest that at least part of the mechanism(s) responsible for CDDP resistance is located in the plasma membrane. In A431 cells, our results indicate that, after 1 h incubation, CDDP, L-OHP and JM216 displayed similar levels of Pt accumulation that linearly increased with concentration. The situation was different when Pt–DNA binding was analysed. Firstly, Pt–DNA binding in parental cells exposed to equimolar concentration of the three compounds were different with the following order of efficiency: JM216 > CDDP > L-OHP. Secondly, in resistant cells DNA–Pt binding levels were similar for CDDP and L-OHP and lower than that of JM216. In spite of this pattern of DNA platination, A431/Pt cells were not resistant to L-OHP and JM216.

The hydrophobicity of the three Pt compounds used in this study increased in the following order: CDDP < L-OHP < JM216 [14]. In general, the non-specific diffusion through the double lipid layer of a cellular membrane is enhanced and/or faster when a drug is more liposoluble [14,19,20]. In the parental A431 cells, the slope of the line correlating hydrophobicity and Pt accumulation was almost flat and the most hydrophobic analogue, JM216, accumulated at levels comparable to those of the least hydrophobic CDDP. This strongly suggests that passive diffusion is not a limiting factor for Pt accumulation in A431 cells. The Gately–Howell's model of CDDP accumulation postulates that CDDP uptake could be due to both passive diffusion and facilitated diffusion through a gated channel (almost 50% each). Furthermore, this model predicts that analogues of CDDP that are more hydrophobic would not exhibit decreased uptake in resistant cells and would therefore have only limited or excluded cross-resistance [21]. Our results are in line with this model. However, the extent of Pt accumulation for the most hydrophobic compound, quite similar to that of the least hydrophobic one, suggests that a facilitated diffusion through a gated channel may be involved in Pt drug accumulation in A431 cells. This mechanism for facilitated diffusion appears to be lost during the selection of the drug-resistant variant, in which the defects in cellular accumulation of CDDP are not likely to involve the human copper transporter gene CTR1 [22], eventhough a major role in regulation of cellular influx of CDDP has been proposed for this transporter [23,24]. Although we can not exclude that differences in cellular accumulation may be due to other factors, including copper homeostasis proteins different from CTR1 and/or number of Pt binding sites in the cells and/or mechanisms for protein–Pt adduct formation/elimination, it appears that passive, hydrophobicity-dependent mechanisms for drug accumulation predominate in A431/Pt cells. The change between parental cell lines could be related, at least in part, to modification(s) of the chemical structure and/or composition or properties of the plasma membrane in the passage from sensitive to CDDP-resistant cell line. Recently, it has been reported that physical state changes of membrane lipids may be associated with the resistance of human lung adenocarcinoma A459 CDDP-resistant cells [25]. Furthermore, the application of surface enhanced infrared absorption spectroscopy (SEIRA) has shown a disordering in the lipid chain packing from resistant cells after application of CDDP [26]. Preliminary data (unpublished data from our laboratory) considering the analysis of the cell membrane lipids by proton magnetic resonance spectroscopy in A431 cell line and its resistant counterpart seem to point out alterations in the content of mobile lipids and phosphatidylcholine, according to

previously published results in other cell lines ([27,28] and references therein).

A further interesting result of our study is that in A431 cells, after 1 h of CDDP, L-OHP or JM216 application, in face of comparable levels of cellular Pt accumulation, DNA platination levels are in the order JM216 > CDDP > L-OHP. The DNA platination can be considered as a ligand substitution reaction in Pt complexes. It is well known that the rate of nucleophilic substitution of a ligand on square planar complexes, as in Pt(II), is very sensitive to steric hindrance, both in the substrate and in the nucleophile [29]. The presence of the chelated bulky ligand (DACH) [30,31] in the substrate may indeed interfere with the rate of DNA platination and consequently with the time of appearance of the drug cytotoxicity, at least if DNA is the major toxic target for these drugs. This implies that the differences in efficacy found between CDDP and L-OHP could be attenuated by using prolonged incubation time. Preliminary observations indicate that the antitumour activity of L-OHP is more dependent on duration of exposure to the drug than CDDP. Clinical implications can be derived from our findings: (i) DNA platination and possibly cytotoxicity following L-OHP exposure appear to be modulated by regimens of drug administration, (ii) the identification of the resistance profile for the predominant cell type in each tumour helps to adjust chemotherapy, avoiding aggressive therapy that could not be effective.

In conclusion, the present results, which suggest that a mechanism allowing facilitated diffusion of CDDP in drug-sensitive cells has been lost in CDDP-resistant cells, support the relevance of drug hydrophobicity in an attempt to bypass resistance. Such evidence could provide insights for the development of novel compounds/formulations and for optimization of therapeutic strategies.

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