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The cellular pharmacology of oxaliplatin resistance

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Abstract

Oxaliplatin is a third generation platinum compound that differs from cisplatin and carboplatin in having a broader spectrum of antitumour activity. Molecular studies suggest that oxaliplatin adducts are recognised and processed differently than those produced by the earlier generation Pt-containing drugs. We report here studies on the kinetics of the development of oxaliplatin resistance, and the changes in the cellular pharmacology of oxaliplatin that accompany the emergence of the resistant phenotype in five parental human tumour cell lines and their sub-lines selected for acquired oxaliplatin resistance *in vitro*. During selection, resistance did not substantially increase until after at least six cycles of oxaliplatin treatment. Oxaliplatin demonstrated schedule-dependency with a 1-h exposure being substantially less cytotoxic than a continuous exposure. Whole cell uptake was linear with concentration, but uptake in the resistant cells averaged only 27 ± 10 S.D.% of that in the sensitive cells. Pt accumulation in DNA was markedly reduced in four of the five resistant cell lines, but this did not correlate with either IC_{50} or total cellular accumulation. Four of the five resistant sub-lines also demonstrated increased tolerance to adducts in DNA that ranged from 3.1 to 7.6-fold. We conclude that development of acquired resistance to oxaliplatin is accompanied by independent defects in both whole cell uptake and in adduct formation.

Keywords: Oxaliplatin; Resistance; Ovarian cancer

1. Introduction

Oxaliplatin is a third generation platinum compound that differs from cisplatin and carboplatin in several important ways. First, in some model systems it has activity against cisplatin- and carboplatin-resistant tumour cells [1–3]. Second, it has a different pattern of toxicity being neither predominantly myelosuppressive or nephrotoxic [2]. Rather, its major dose-limiting toxicity is a somewhat unusual form of neurotoxicity [4]. Third, it has activity in at least one major tumour type, colon carcinoma, against which cisplatin and carboplatin have essentially no activity [5].

When tested against a panel of human tumour cell lines, oxaliplatin was found to be more potent than cisplatin in most model systems when cytotoxicity was measured by a growth rate assay [1]. The pattern of activity of cisplatin and oxaliplatin in the NCI 60 cell line panel suggests significant differences in their mechanisms of action, and diversity in the biochemical pathways involved in protecting cells against these agents [3]. Indeed, some differences between the molecular pharmacology of cisplatin and oxaliplatin have already been identified. Like the first- and second-generation platinum drugs, oxaliplatin is believed to cause cytotoxicity through its ability to form adducts in DNA [6,7]. However, some differences between the molecular pharmacology of the adducts produced by cisplatin and oxaliplatin are already known. For example, loss of DNA mismatch repair results in cisplatin resistance consistent with the concept that this repair system functions as a detector for cisplatin adducts [8]. However, loss of mismatch repair does not cause oxaliplatin resistance [9]. Whereas exposure to cisplatin strongly activates the c-Jun NH2-terminal and c-Abl kinases, treatment with oxaliplatin does not [10]. Structural modelling suggests that the bulky diaminocyclohexane

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ring of oxaliplatin protrudes into the major grove in such a way as to cause steric hindrance to whatever DNA binding proteins trigger this event [11].

Biochemical pharmacology studies have demonstrated that cells can become resistant to cisplatin by virtue of a reduction in the uptake of the drug, increased detoxification through interaction of cisplatin with intracellular thiols, and alterations in the ability of the cell to recognise and process cisplatin–DNA adducts. The current paradigm is that the DNA adducts produced by the platinum drugs are recognised by a detector, the detector activates a series of damage-responsive signal transduction pathways, and this in turn triggers apoptosis [8]. Failure of any one component in this sequential process would be expected to result in drug resistance.

The mechanisms underlying resistance to oxaliplatin have not been as well defined. We have prepared oxaliplatin-resistant variants of five human tumour cell lines and used these to investigate the biochemical and molecular mechanisms by which the cells have acquired resistance. We report here studies on the changes in the cellular pharmacology of this drug that accompany the emergence of resistance.

2. Materials and methods

2.1. Reagents

Cisplatin was gift from Bristol-Myers Squibb (Princeton, NJ, USA), and oxaliplatin was a gift from Sanofi Research, Inc (Malvern, PA, USA).

2.2. Cell lines and selection strategy

Four human ovarian carcinoma cell lines (2008, A2780, IA-9 and IGROV-1) and one squamous cell carcinoma of the head and neck (UMSCC10b) were grown under standard tissue culture conditions. Cell lines were grown at 37 °C in an environment of 5% CO_2 in Roswell Park Memorial Institute (RPMI)1640 and 10% fetal calf serum (FCS) with the exception of the 2008 line, which was grown in RPMI1640 and 5% heat-inactivated FCS. Before starting selection, the sensitivity of all five parental cells lines was determined by clonogenic assay performed as previously reported from this laboratory in Ref. [9]. A full concentration-survival curve over the first two logs of tumour cell kill was determined, and the IC_{50} and IC_{90} concentrations estimated by interpolation. A standard protocol for selection for oxaliplatin resistance was applied to the 2008, A2780, IGROV-1 and UMSCC10b cell lines (the oxaliplatin-resistant IA-9/OX15 cells were derived using a similar selection scheme to that used in the laboratory of Dr Tito Fojo). Oxaliplatin was added to a culture of 80% confluent cells to a final concentration equal to the IC_{90}; the drug was left in the culture. At the point that the surviving population again resumed logarithmic growth, the cells were sub-cultured into new flasks, and the process was repeated again. In most cases, each cell line was exposed three times to the same concentration of oxaliplatin before the concentration was incrementally increased by 1.2- to 2.0-fold. As the cells became more resistant, it was often possible to increase the selecting concentration at a more rapid pace. The selection process continued until the cells were at least 3-fold resistant. The level of resistance was measured after the cells had been removed from oxaliplatin exposure for a minimum of 2 weeks.

2.3. Colony assay

Colony assays were performed as previously reported in Ref. [12] using triplicate cultures of 200 cells per 35 mm plate grown in 5 ml of medium. Following colony formation (10–14 days), the dishes were rinsed twice with phosphate buffered solution (PBS), fixed with 100% methanol and stained with a 1% crystal violet solution. Groups of >50 cells were scored as colonies using a ChemiImager™ 400 (Alpha Inotech, San Leandro, CA, USA). The data reported are the mean $\pm$standard error of the mean (S.E.M.) of a minimum of three independent experiments each performed with triplicate cultures at each drug concentration tested.

2.4. Oxaliplatin uptake and adduct formation

Cells growing at 80% confluence in T-150 flasks were incubated in fresh medium containing oxaliplatin at concentrations up to 200 uM for 1 h. The cells were then washed twice with 5 ml ice-cold PBS, and then the cells were scraped free in 1 ml of PBS and transferred to a centrifuge tube. The flask was rinsed a second time with an additional 1 ml PBS that was transferred to the same centrifuge tube. The cells were pelleted by centrifugation, the supernatant removed, and the pellets stored at -20 °C until they were ready for analysis. For isolation of DNA, the washed cells were incubated overnight at room temperature in a lysis buffer containing 0.67% Triton X-100, NaCl 2.6 M, 133 mM ethylene diamine tetra acetic acid (EDTA), and 2.6 M Tris–HCl (pH 8.0). DNA was isolated by phenol-chloroform extraction and dissolved in Tris-EDTA (TE) buffer (pH 8.0). DNA concentration was determined by absorbance at 260 nm. Aliquots of the DNA were digested in 1 M HCl at 75 °C for 2 h and the hydrolysate was used for the quantitation of Pt by flameless atomic absorption spectrophotometry (Perkin-Elmer Model 2380). All measurements of uptake and adduct formation were performed with duplicate cultures, and all experiments were repeated twice. To correct for

variability in the baseline of the atomic absorption spectrometer between runs, accumulation of Pt in DNA was normalised by setting the intercept of the regression line for the parental cells to zero.

2.5. Statistics

All the pharmacologic data were analysed by use of a two-sided paired Student's *t* test with the assumption of unequal variance.

3. Results

3.1. Oxaliplatin sensitivity

All five oxaliplatin-resistant cell lines (2008R7, A2780R4, IA9OX15, IGROV-1R6, and UMSCC10bR5) were derived by repeated *in vitro* treatment with progressively higher concentrations of oxaliplatin starting with cells that had never been exposed to this drug before. Four of these lines (2008, A2780, IGROV-1 and UMSCC10b) were selected for resistance in this laboratory, and one (IA-9) was selected in the laboratory of Dr Tito Fojo at the National Cancer Institute. For the four lines prepared in this laboratory, the degree of resistance relative to the parental cells was measured by determining the IC_{50} repeatedly during the selection process. Fig. 1 shows that there was little increase in resistance during at least the first six cycles of drug exposure. However, following 6–16 cycles of oxaliplatin treatment, resistance increased more rapidly, depending on the cell line. Of note is the fact that resistance emerged much more rapidly in the A2780 cell line than in the 2008, IGROV-1 or UMSCC10b lines.

Table 1 presents the IC_{50} value for each cell line determined using either a 1-h exposure to oxaliplatin or a continuous exposure produced by leaving the oxaliplatin in the culture during the full period of colony formation (10–14 days). The oxaliplatin-selected sublines were from 6.7 to 31.1-fold resistant when exposed to oxaliplatin for 1 h. As measured by IC_{50} values, the potency of oxaliplatin averaged 30 ± 16 (standard deviation (S.D.))-fold less against the parental cells when exposure was limited to just 1 h than when the cells were exposed continuously. For the resistant cells, oxaliplatin was 50 ± 15 (S.D.)-fold less potent on a 1-h exposure schedule than on the continuous exposure schedule.

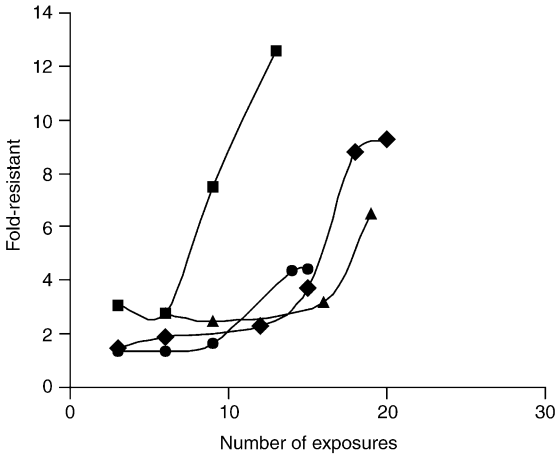

Fig. 1. Kinetics of the appearance of oxaliplatin resistance during *in vitro* selection. ◆, 2008; ■, A2780; ▲, IGROV-1; ◆, UMSCC10b.

The measured degree of resistance for each pair of sensitive and resistant cells also varied with exposure schedule. The magnitude of resistance was greater when measured using a 1 h exposure for three of the five sublines; in the remaining two the level of resistance was similar with both exposures. There was not a very good correlation between the degree of resistance measured with the 1-h exposure and that measured with the continuous exposure ($r = 0.46$, $P > 0.05$) suggesting that different defence mechanisms may predominant with different exposure schedules.

3.2. Oxaliplatin uptake

Fig. 2 presents plots of total cellular accumulation of Pt/g cellular protein as a function of oxaliplatin concentration at the end of a 1-h exposure. To correct for variability in the baseline of the atomic absorption spectrometer between runs, accumulation of Pt in DNA

Table 1
Oxaliplatin IC_{50} values as determined by clonogenic assay

	IC_{50} for 1-h exposure (μM)[a]			IC_{50} for continuous exposure (μM)[a]		
Parental cell type	Parental cells	Resistant sub-line[b]	Fold-resistant	Parental cells	Resistant sub-line[b]	Fold-resistant
2008	9.5	295.8	31.1	0.51	4.7	9.2
A2780	6.0	82.7	13.8	0.11	1.4	12.7
IA-9	1.8	50.0	27.8	0.11	1.0	9.1
IGROV-1	4.5	30.0	6.7	0.19	1.2	6.3
UMSCC10b	6.4	44.6	7.0	0.18	0.8	4.4

[a] Mean of a minimum of three independent experiments each performed with triplicate cultures at each drug concentration tested.
[b] Names of resistant sub-lines: 2008R7, A2780R4, IAOX15, IGROV-1R6 and UMSCC10bR5.

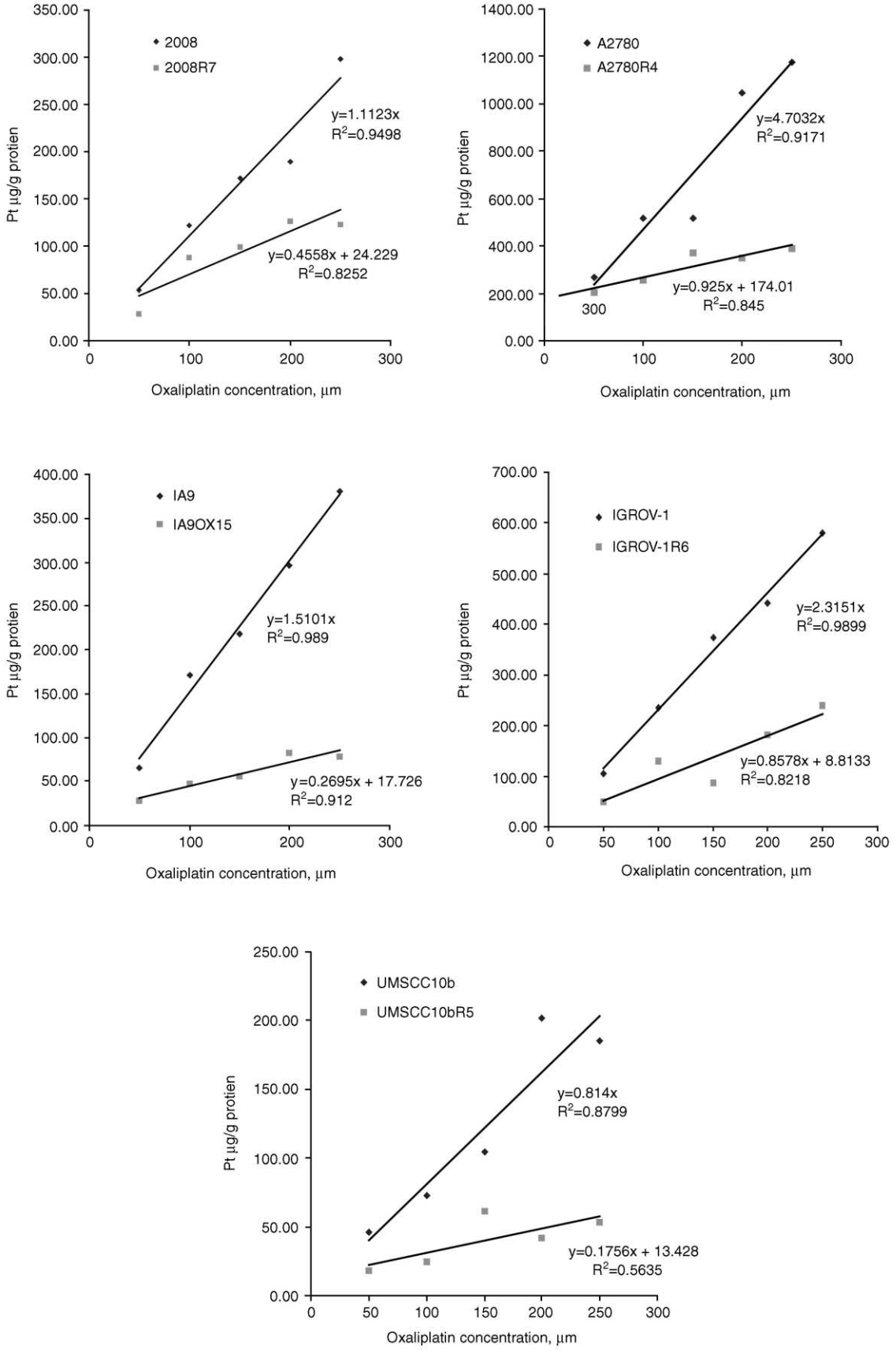

Fig. 2. Pt in DNA following a 1-h exposure to oxaliplatin as a function of drug concentration.

was normalised by setting the intercept of the regression line for the parental cells of each pair to zero. Pt accumulation was linear with time over the first hour of uptake (data not shown). As shown in Fig. 2, Pt accumulation at the end of 1 h was linear with oxaliplatin concentration in both the sensitive and resistant members of all five pairs of cell lines. Even at an external concentration of 250 uM there was no suggestion that the uptake mechanism was saturated. In all five resistant sub-lines there was a substantial impairment of Pt accumulation. Mean uptake in the resistant cells was only 27 ± 10 (S.D.)% of that in the sensitive cells.

One might expect to find a correlation between IC_{50} and uptake; however, this was not the case. The correlation coefficients were <0.10 for both the sensitive parental cells and the resistant sub-lines. Likewise, one might expect that the cell pairs demonstrating the largest difference in sensitivity would be those with the largest difference in uptake, but this was also not true ($r^2 = 0.15$, $P > 0.05$). These results indicate that the amount of oxaliplatin becoming associated with the cell is not the sole or even predominant determinant of its potency. In this regard, although cells were washed three times after exposure to oxaliplatin, the assay used detected drug truly inside the cells, as well as drug possibly tightly associated with the external surfaces.

3.3. Pt accumulation in DNA

The total amount of Pt per μg DNA after a 1-h exposure to increasing concentrations of oxaliplatin up to 250 uM was measured for each cell line. This measurement reflects the net rate of adduct formation and its repair over the first hour of drug exposure rather than the absolute value of either process. Pt accumulation in DNA was linear with oxaliplatin concentration for all the lines in a pattern similar to that for whole cell uptake presented in Fig. 2. Table 2 presents the slope of the plot of ng pt/μg DNA as a function of oxaliplatin concentration for each cell line. Several points are noteworthy. First, DNA accumulation in the five oxaliplatin-sensitive parental cell lines varied over a 7-fold range; in the five resistant lines it varied over an 18-fold range. Second, in four of the five resistant sub-lines there was a substantial reduction in accumulation relative to the parental line. Accumulation in these four sub-lines averaged only 20 ± 16% (S.D.) of that in the sensitive cells. The one outlier was the IGROV-1R6 sub-line which demonstrated no defect in DNA accumulation relative to the parental IGROV-1 line.

Table 2
Pt accumulation in DNA as a function of oxaliplatin concentration

Parental cell type	Slope of regression line[a]		
	Sensitive parent	Resistant sub-line	Ratio of slopes
2008	56.5	5.1	11.1
A2780	259.0	93.2	2.8
IA-9	141.0	45.1	3.1
IGROV-1	50.0	58.4	0.9
UMSCC10b	376.0	16.7	22.5

[a] Mean of two independent experiments each performed with duplicate cultures.

Correlations were sought between IC_{50} and Pt accumulation in DNA. One might expect to find a correlation between Pt accumulation in DNA and sensitivity to the cytotoxic effect of oxaliplatin; however, no significant association was identifiable. One might also expect that better uptake into the whole cell would correlate with better uptake into DNA. This was true for the five resistant cell lines ($r = 0.77$, $P < 0.05$), but not for the sensitive sub-lines or all the lines considered together ($r = 0.43$, $P > 0.05$). Finally, one might have expected that the greater the degree of resistance, the greater the reduction in accumulation of Pt in DNA. However, no association between these parameters was demonstrable for either the sensitive or resistant cells. These results suggest that there are substantial differences between the cell lines in how efficiently oxaliplatin that has gained access to the interior of the cell is delivered to the DNA, and in how efficiently apoptosis is triggered by a given load of DNA adducts.

3.4. Adduct tolerance

Adduct tolerance can be estimated by measuring the amount of Pt in the DNA at a concentration that kills a fixed fraction of the population. Because the amount of Pt that accumulates in the DNA is so small when the cells are exposed to an IC_{50} concentration, it is difficult to measure accurately. However, it can be adequately quantified when the cells are exposed to a concentration that is 10 times the IC_{50}. This approach has been used by other investigators [13] and is a reasonable way of dealing with the problem of limited instrument sensitivity because there is a log-linear relationship between the fraction of surviving clonogenic cells and oxaliplatin concentration over this range (data not shown).

Whereas one might have expected that the amount of Pt in DNA required to kill the cell would be a constant, this was clearly not true. The data presented in Table 3 show that all 4 of the resistant ovarian carcinoma sub-lines were substantially more tolerant of oxaliplatin adducts in their DNA than the parent from which they were derived. The degree of tolerance ranged from 3.1-fold for the IA9OX15 sub-line to 7.6-fold for the A2780R4 sub-line. In contrast, the one head and neck carcinoma oxaliplatin-resistant sub-line demonstrated a reduction in tolerance such that there was greater cell kill per ng Pt in the DNA than in the parental cells. Consistent with the concept that the efficiency with which cells detect oxaliplatin adducts in DNA, or

Table 3
Adduct tolerance as measured by the amount of Pt in DNA 1 h following exposure to oxaliplatin at a concentration equivalent to 10 times IC_{50}[a]

Parental cell type	pg Pt/μg DNA		
	Sensitive parent	Resistant sub-line	Ratio of resistant to sensitive
2008	4.8	16.5	3.4
A2780	12.3	93.9	7.6
IA-9	7.9	24.2	3.1
IGROV-1	3.4	20.0	5.9
UMSCC10b	26.7	10.1	0.38

[a] Mean of two independent experiments each performed with duplicate cultures.

propagate an injury signal to the apoptotic machinery, differs between cell lines, there was no discernable correlation between the IC_{50} and adduct tolerance as measured in this way ($r < 0.07$ for both sensitive and resistant cells). Likewise the resistant sub-lines showing the greatest difference in sensitivity to the cytotoxicity of oxaliplatin were not those demonstrating the greatest adduct tolerance.

The ratios for each of the cellular pharmacological parameters analysed are presented together in Table 4 for ease of comparison.

4. Discussion

Although oxaliplatin has a different spectrum of activity against human tumours than cisplatin and carboplatin, the studies reported here document that resistance emerges with kinetics that resemble those for the first and second generation platinum-containing chemotherapeutic agents. In addition, some features of the cellular pharmacology of these drugs are similar, and the oxaliplatin-resistant cells share some of the major characteristics often found in cisplatin-resistant cells (reviewed in Ref. [14]).

As shown in Fig. 1, there was heterogeneity among ovarian carcinoma cell lines with respect to the rate at which acquired resistant emerged during repeated selection *in vitro*, and there was some suggestion that the degree of resistance increased more rapidly once a threshold degree of low level resistance had been attained. No direct comparison of the rate of emergence of resistance during selection with either oxaliplatin or cisplatin has been reported. The only comparable data available on the rate of emergence of resistance to cisplatin in an ovarian carcinoma cell line [15] suggest that cisplatin resistance may develop somewhat more rapidly than oxaliplatin resistance. However, caution is required since these studies were performed with different cell lines and heterogeneity between cell lines clearly influences this process.

The results of the current studies indicate that the potency of oxaliplatin increases with the duration of exposure. Raymond and colleagues [2] compared the potency of oxaliplatin on 1 h and continuous 14-day exposure schedules against a large number of fresh human tumours using a clonogenic *in vitro* assay system. As measured by the fraction of tumours in which the drug produced $>50\%$ reduction in colony formation, oxaliplatin was approximately 2-fold more potent on a continuous than on a 1-h exposure schedule. The potency difference for the 10 cell lines included in the current study varied from 15- to as high as 63-fold. Both cisplatin and carboplatin demonstrate similar changes in potency as a function of exposure schedule (S.B. Howell, University of California, La Jolla, CA, USA). It is important to note that oxaliplatin is unstable in medium containing physiological concentrations of chloride; the oxalato group is displaced by chloride with a half-life of approximately 11 h. Thus, the apparent schedule-dependency may be due, in part, to greater conversion of the drug to an alternative form with greater potency.

In order to kill, it is currently believed that oxaliplatin must get into the cell, it must be delivered to the nucleus and form adducts with DNA, and the adducts must be detected by a system capable of generating a signal that activates an intact apoptotic pathway. The results presented here demonstrate that there is heterogeneity between cell lines for many of these steps. However, decreased drug uptake in all five pairs, decreased platinum accumulation in DNA in four out of five pairs (all except IGROV-1) and increased tolerance for adducts in four out of five pairs (all except UMSCC10b)

Table 4
Ratios of cellular pharmacological parameters for oxaliplatin-resistant relative to oxaliplatin-sensitive cell lines

Cell line pairs	Ratio of platinum			
	Fold-resistance	Uptake ratio	Accumulation in DNA	Ratio of adduct tolerance
2008R7/2008	9.2	0.4	0.09	3.4
A2780R4/A2780	12.7	0.2	0.36	7.6
IA9OX15/IA-9	9.1	0.18	0.32	3.1
IGROV-1R6/IGROV-1	6.3	0.37	1.3	5.9
UMSCC10bR5/UMSCC10b	4.4	0.2	0.04	0.38

strongly indicate that there are common features that underlie the oxaliplatin-resistant phenotype. Little is known about how the platinum-containing chemotherapeutic agents enter the cell [16], but it is of interest that both cisplatin and oxaliplatin uptake, measured over minutes to hours, increases linearly with concentration up to very high external drug concentrations [17]. As was observed for oxaliplatin in our oxaliplatin-resistant sub-lines, impaired uptake of cisplatin is commonly found in cell lines that are selected for resistance to cisplatin (reviewed in Ref. [16]). Rixe and colleagues [3] reported that oxaliplatin uptake was reduced as well in KB and A2780 cell lines selected for resistance to cisplatin.

As shown by the measurements made in this study, the vast majority of the oxaliplatin that enters the cell never becomes associated with the DNA. It is reasonable to expect that the efficiency of mechanisms that mediate delivery of oxaliplatin from the cytoplasm to the DNA might differ from tumour to tumour. This provides at least a conceptual basis upon which to explain the relatively large differences observed in the accumulation of Pt in DNA. The intracellular level of glutathione and other thiols has long been thought to be a determinant of the cytotoxicity of cisplatin [18,19], and El-akawi and colleagues [20] reported that glutathione levels were increased 2.5- and 8-fold in A2780 cells that were selected for 8- and 12-fold oxaliplatin resistance, respectively. The cellular metallothionein level has been studied extensively as a potential determinant of acquired resistance to cisplatin. Metallothioneins are thiol-rich intracellular proteins which play a role in the metabolism and storage of many heavy metals, including zinc, cadmium and copper. It is thought that an increased metallothionein level can contribute to the detoxification of cisplatin due to binding to the abundant sulphhydryl groups. Increased levels of metallothioneins have been identified in some cisplatin-resistant cell lines [21]. In addition, some cell lines which have been exposed to heavy metals express higher levels of metallothionein and display cross-resistance to cisplatin [22]. To our knowledge, there have been no studies on the interaction between metallothionein and oxaliplatin specifically.

The concept that resistance may be mediated by enhanced tolerance to adducts in DNA is rapidly gaining support. Several mammalian DNA polymerases capable of bypassing adducts that normally arrest replication have recently been identified [23–25]. Enhanced activity of such enzymes would be expected to permit survival under conditions of adduct load that would normally be lethal. Vaisman and colleagues [26] have recently reported that human DNA polymerases β, γ, and η can bypass oxaliplatin, as well as cisplatin, adducts in DNA. Both p53 and DNA mismatch repair proteins appear to play a role in modulating the extent to which bypass replication occurs [27,28]. The pharmacological results reported here suggest that enhanced adduct tolerance is commonly found in cells with acquired oxaliplatin resistance, and it will be of substantial interest in future studies to determine the extent to which this can be attributed to enhanced bypass replication.

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