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Mechanisms of drug resistance to the platinum complex ZD0473 in ovarian cancer cell lines

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Abstract

Acquired drug resistance to the sterically hindered platinum drug ZD0473 (formerly known as JM473 and AMD473) and currently being tested in phase I clinical trials, has been studied in two human ovarian carcinoma cell lines (CH1 and A2780) where previously, acquired cisplatin resistance has been described. Common mechanisms of resistance were observed in A2780 acquired cisplatin and ZD0473R (resistant) lines (including reduced drug transport and DNA platination, increased glutathione (GSH) and loss of the *MLH1* DNA mismatch repair gene). However, contrasting mechanisms were observed in the CH1 sublines. While ZD0473 retained activity against the acquired cisplatin resistant sublines, cisplatin did not circumvent acquired ZD0473 resistance. The *trans* platinum complex JM335 circumvented resistance in CH1cisR and A2780ZD0473R lines, but not in A2780cisR or CH1ZD0473R cells. Overexpression of metallothionein (MT) in A2780 cells by stable gene transfection resulted in protection from the growth-inhibitory effects of cadmium chloride (3.8-fold) and a range in protection with platinum drugs (from 7-fold with cisplatin, but only 1.3-fold with ZD0473). Overall, the results show that some mechanisms of resistance to ZD0473 are shared with those previously described in the same parental lines for cisplatin (e.g. in A2780), but in the CH1 lines, differing mechanisms were apparent. Moreover, ZD0473 possesses distinct cellular pharmacological properties in comparison with cisplatin with respect to reduced interactions with MTs, a thiol-containing species associated with tumour resistance to cisplatin.

Keywords: Platinum; ZD0473; Resistance; Thiols

1. Introduction

While cisplatin is a widely used and active chemotherapeutic agent, there has been an extensive search over 20-plus years to discover other platinum compounds possessing either less toxicity (e.g. carboplatin), oral bioavailability (e.g. JM216) or able to circumvent intrinsic and/or acquired tumour resistance [1]. Laboratory and clinical studies have shown that the mechanisms underlying tumour resistance to cisplatin are multifactorial. These include decreased drug transport, increased cellular detoxification due to increased glutathione (GSH) and metallothionein (MT), changes in DNA repair involving increased nucleotide excision repair (NER) and/or loss of mismatch repair (MMR), increased tolerance of DNA adducts and alterations in the apoptotic cell death pathway [2]. Currently, there are at least three platinum complexes in clinical trial which have shown some evidence of circumvention of cisplatin resistance in preclinical *in vitro* and *in vivo* tumour models: oxaliplatin, BBR3464 and ZD0473 [3].

Cis-[amminedichloro(2-methylpyridine)] platinum (II) (ZD0473, formerly AMD473, JM473) was synthesised to possess a reduced susceptibility to binding to thiols compared with cisplatin. Many studies, including our own using a panel of human ovarian carcinoma cell lines, have shown that inactivation of cisplatin by GSH contributes to resistance [4–7]. Mechanistic studies showed that ZD0473 was indeed less susceptible than cisplatin to inactivation by thiols, both in cell-free conditions using thiourea, methionine or GSH or within human ovarian tumour cells where GSH levels were artificially raised [8–9]. ZD0473 also showed a promising level of activity in preclinical cell lines and xenograft models of acquired cisplatin resistance including against cell lines where decreased platinum transport or enhanced DNA repair contributed to resistance [8–11]. Toxicological studies in rodents showed myelosuppression

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to be dose-limiting with no nephro- or neurotoxicity [10]. Based on these promising preclinical properties, ZD0473 entered phase I trials at the Royal Marsden NHS Trust hospital in November 1997 under the auspices of the UK Cancer Research Campaign.

The aim of the present studies was 2-fold: (1) as with other anticancer 'cytotoxics', drug resistance is likely to represent a limiting factor to the clinical utility of ZD0473. Therefore, in order to obtain an early-stage understanding of the mechanisms of resistance to ZD0473, and whether such mechanisms are similar or dissimilar to those previously described for cisplatin, acquired resistance to ZD0473 has been deliberately established and studied using two human ovarian carcinoma cell lines where acquired cisplatin resistant models have already been described [2]. In addition to GSH, increasing MT levels have also been shown to contribute to acquired cisplatin-resistance [12–14]. The effects of high MT levels on growth inhibition of ZD0473 was also investigated by stable transfection of *MTIIA* cDNA into an ovarian cancer cell line.

2. Materials and methods

2.1. Cell lines

Three parental human ovarian carcinoma cell lines were used in this study — A2780 [15] and CH1 [16]. A2780 and CH1 cell lines were selected and made resistant to ZD0473 since these lines are relatively sensitive to cisplatin. Acquired cisplatin-resistant sublines have been described previously and the lines and xenograft counterparts were used in the preclinical development studies of the oral platinum drugs JM216 and ZD0473 [8,17]. Lines were made resistant to ZD0473 by *in vitro* exposure over a 7-month period to increasing concentrations of drug from 0.5 to 12.5 μM. Cells were exposed continuously to ZD0473 for 4 days after which medium was removed and replaced with drug-free medium for 2 weeks prior to the next dose escalation. The resulting acquired resistant lines were not cloned, although some positive selection, i.e. of cells with a growth advantage, for example, may have occurred in culture.

All cell lines were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Imperial Laboratories, Andover, UK), 2 mM L-glutamine and 0.5 μg ml hydrocortisone in a humidified 6% carbon dioxide, 94% air atmosphere. Lines were confirmed to be free of microbial or *Mycoplasma* contamination throughout the course of study.

2.2. Chemotherapy agents and other chemicals

Cisplatin, carboplatin, JM216, AMD473 (ZD0473) and JM335 were synthesised by Johnson Matthey Technology Centre (Reading, Berkshire, UK) or AnorMED (Langley, BC, Canada). Structures of the platinum agents have been published previously [8,9,16–18]. Unless otherwise stated, all other chemicals were obtained from Sigma Chemicals (Poole, UK).

2.3. Growth inhibition assay

Cytotoxicity (2 and 96 h) was measured by the sulphorhodamine B (SRB) growth inhibition assay as previously described [17–19]. The mean absorbance of each drug was expressed as a percentage of the control untreated cells and plotted versus drug concentration from which the IC_{50} concentration was derived.

2.4. Platinum uptake and DNA platination

The effect of concentration on ZD0473 uptake (2 h) in the cell lines was determined as previously described [8], using flameless atomic absorption spectrometry (FAAS) (Perkin Elmer 1100B and HGA 700; detection limit was approximately 5 ng (1 nmol) platinum). Cellular platinum levels were expressed as nmol platinum per mg of protein.

For DNA platination experiments, cells (3×10^7) were exposed for 2 h to different concentrations of ZD0473. Cells were processed and DNA extracted as previously described [8]. DNA content was measured by spectrophotometry (260 nm) and platinum levels determined by FAAS. A_{260}/A_{280} ratios were between 1.75 and 1.8 for all samples.

2.5. Measurement of GSH

Total GSH content was measured as previously described using an enzymatic assay involving extraction in 0.6% sulphosalicylic acid and glutathione reductase [4,20]. GSH content was expressed as nmol GSH per mg of protein.

2.6. Protein estimation and Western blotting

This was performed as previously described using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation and transfer to nitrocellulose membranes and visualisation by enhanced chemiluminescence (ECL) [20]. Equal protein (50 μg) was loaded and the antibody used for detection was hMLH1 (Pharmingen). Blots were repeated three times.

2.7. Transfection of the MTIIA gene into A2780 cells

A2780 cells were selected since this was the cell line used for previous studies of increasing GSH [8] and the line possesses relatively low MT levels. The *MTIIA* gene (the isoform most frequently overexpressed in cisplatin

resistant cell lines; [21]) was kindly provided by Dr Horst Lohrer (Gray Laboratory, Mount Vernon, UK) within an autonomously replicating bovine papillomavirus (BPV) vector. The gene encoding *MTIIA* was excised from the BPV vector as a Nco*I*–Bsr*BI* 707 bp fragment containing all three exons. This was cloned, using *Xho1* and an additional Kozak sequence, into the multiple cloning site of vector pCIpuro (F179) where stable expression is driven by the cytomegalovirus (CMV) promotor. *MTIIA* integrity and alignment was checked by restriction digest patterns and polymerase chain reaction (PCR)-based sequencing. Transfections of vector alone (F179) and sense *MTIIA* (F215) were performed using lipofectamine (Life Technologies) and clones selected for resistance to the selectable marker puromycin (at predetermined concentrations toxic to all non-transfected cells).

2.8. Immunohistochemistry for MT

Although immunoblotting for MTIIA was also used for the detection of MT in the puromycin-resistant transfects, the performance of the antibody (MT-E9 DAKO) was erratic resulting in immunohistochemistry (IHC) also being used. IHC was performed on paraffin-embedded sections using the DAKO catalysed signal amplication system. The primary antibody was mouse anti-MT IgG diluted 1:50 in phosphate-buffered saline (PBS).

2.9. Statistical analyses

When appropriate, statistical significance was tested using a two-tailed Student's *t*-test; a P value of $\leqslant 0.05$ was considered as significant. Values are means ±standard error of the mean (SEM).

3. Results

3.1. Characterisation of ZD0473 acquired resistant cell lines

Acquired resistant ZD0473 cell lines were established using the A2780 and CH1 ovarian carcinoma cell lines (Table 1). Doubling times were similar between the parental and resistant lines and resistance factors were approximately 2.7 to 3.3-fold for A2780ZD0473R and approximately 3.3 to 4.2-fold for CH1ZD0473R. Studies to determine the mechanisms of resistance in these lines initially concentrated upon commonly described features of acquired cisplatin resistance, namely reduced drug accumulation and decreased DNA platination [2]. Results of transport and DNA platination experiments following a 2 h exposure to ZD0473 in the A2780 and CH1 parental and resistant cell lines are shown in Fig. 1(a–d).

Table 1
Doubling times and sensitivity to ZD0473 of CH1 and A2780-acquired ZD0473 resistant cell lines[a]

Cell line	Doubling time (h)	IC_{50} 2 h exposure (μM)	IC_{50} 96 h exposure (μM)
A2780	12.7±0.5	32.4±2.7	1.32±0.23
A2780ZD0473R	12.7±0.6	108±21	3.6±0.3
(RF)	–	3.3	2.7
CH1	23.7±2.1	26.9±6	1.26±0.16
CH1ZD0473R	23.7±0.7	112.7±4.2	4.1±0.2
(RF)	–	4.2	3.3

RF, Resistance Factor = IC_{50} resistant/parent line.
[a] Values = mean±standard error of the mean (SEM), $n \geqslant 3$.

In both acquired resistant cell lines there was a significant reduction in platinum accumulation when compared with their respective parental line; mean reduction of 1.7±0.1 fold ($P < 0.05$) in A2780ZD0473R (Fig. 1a) and 1.5±0.2-fold, $P < 0.05$, in CH1ZD0473R (Fig. 1c). Accumulation was generally linear with increasing concentration over the 25–100 μM range studied. In terms of DNA platination, the A2780ZD0473R line showed consistently lower levels than A2780 (Fig. 1b) but this difference (1.5±fold across all data points) only reached statistical significance at the 100 μM dose level. Similarly, DNA within CH1ZD0473R cells showed less platination than in CH1 (mean difference of 1.3±fold) although none of the differences were significant (Fig. 1d). Platinum adduct removal experiments showed no significant differences in the rates of removal between resistant and sensitive lines (data not shown; over 72 h postexposure to obtain equal DNA platination).

The levels of the two major sulphur-containing species previously shown to contribute to acquired cisplatin resistance, GSH and MT, were also measured. Both resistant lines contained significantly higher GSH levels than their respective parental lines: A2780ZD0473R was 1.5-fold higher ($P < 0.05$) and CH1ZD0473R was 2.7-fold higher ($P < 0.05$). Levels (in nmol GSH/mg protein) were 11.3, 17.5, 7.3 and 19.5 in A2780, A2780ZD0473R, CH1 and CH1ZD0473R, respectively. By immunohistochemistry, A2780ZD0473R generally showed higher levels of MT than A2780, whereas staining was higher and similar in the CH1 pair of cell lines (data not shown).

As a further means to assess the possible contribution of increased MT levels in the acquired resistant lines, sensitivity to cadmium chloride was determined in the two pairs of cell lines, using 96 h of continuous exposure. In addition, cross-resistance profiles to other platinum drugs, cisplatin, carboplatin, the oral drug JM216 and the *trans* platinum JM335, were obtained (Fig. 2). In contrast to the immunohistochemistry data, the A2780ZD0473R line showed no cross-resistance to cadmium chloride whereas 4.2-fold resistance was observed with CH1ZD0473R. Similar levels

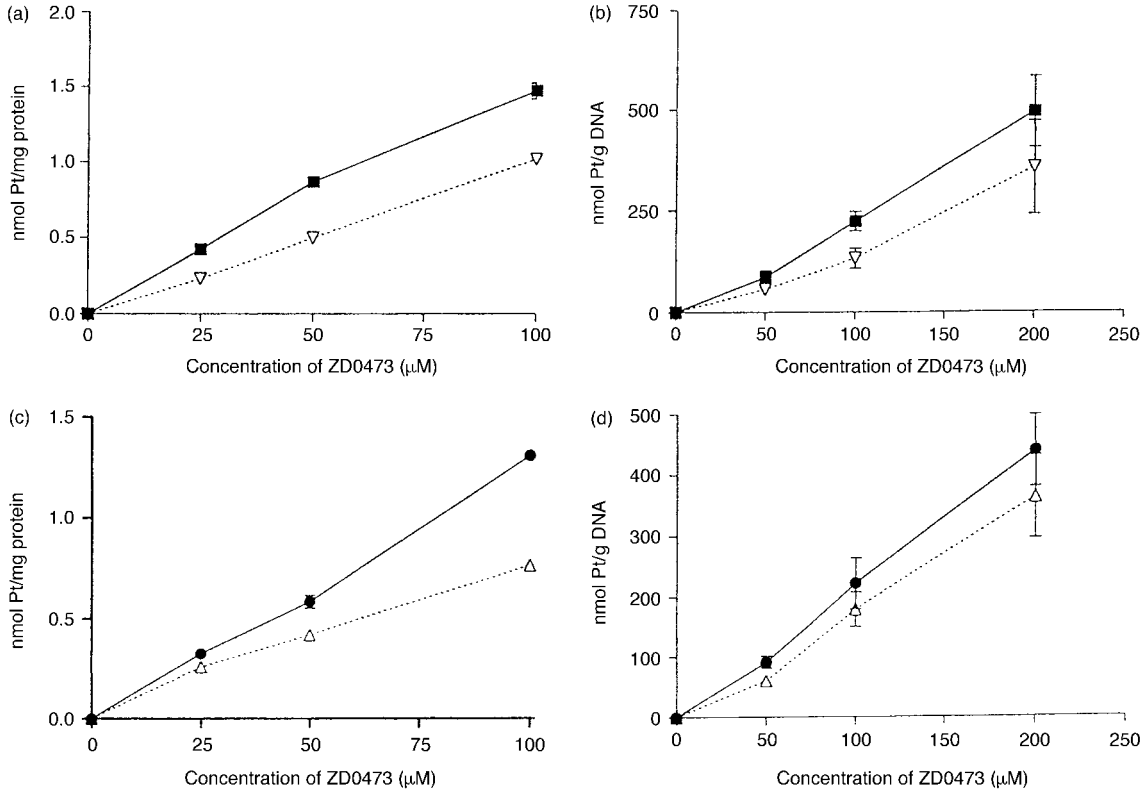

Fig. 1. Platinum (Pt) uptake (a) and (c) and DNA platination (b) and (d) immediately following a 2 h exposure of A2780 (■) versus A2780ZD0473R (▽) (a) and (b) or CH1 (●) versus CH1ZD0473R (△) (c) and (d) to ZD0473. Values are means±standard error of the mean (SEM), $n \geqslant 4$.

of cross-resistance to that obtained with ZD0473 itself were observed to cisplatin, carboplatin and JM216 in both resistant lines. In contrast, a differential response was observed to the *trans* platinum complex JM335; acquired ZD0473R resistance was completely circumvented by JM335 in the A2780 resistant-line (RF of 0.9) but not in the CH1 resistant-line.

In the A2780 acquired cisplatin-resistant cell line, loss of the DNA mismatch repair protein hMLH1 has been reported [22]. We measured levels of hMLH1 (and other mismatch repair proteins) by immunoblotting in the two pairs of resistant lines (as well as in a further series of acquired platinum-resistant sublines). There was no loss of hMLH1 in any of the CH1-derived lines, although

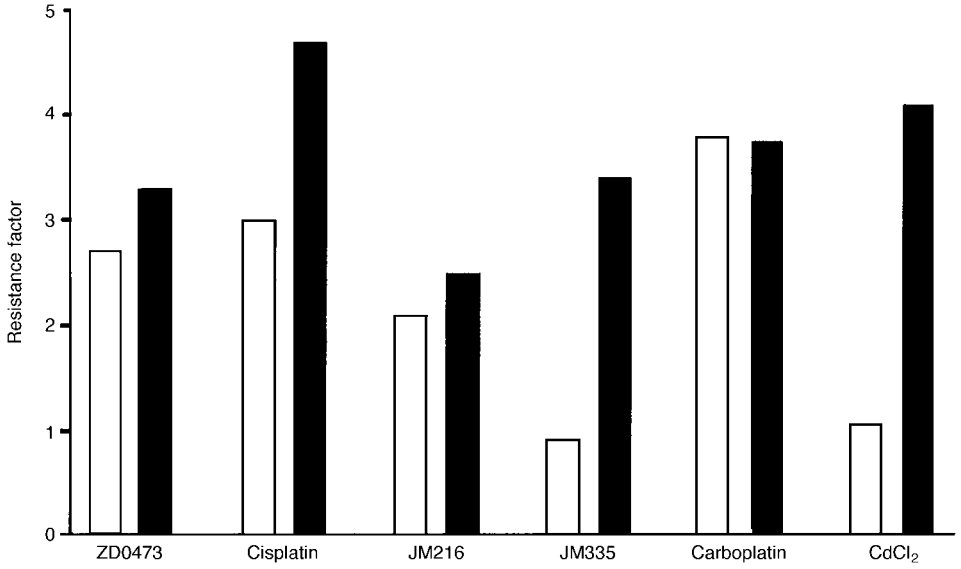

Fig. 2. Cross-resistance profile of A2780ZD0473R (open bars) or CH1ZD0473R (solid bars) to platinum drugs and cadmium chloride. Resistance factor = IC_{50} resistant/parent line following 96 h of drug exposure; values are the means from five independent experiments.

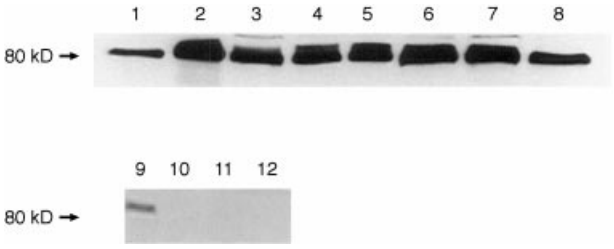

Fig. 3. Immunoblot for hMLH1 in CH1 and CH1ZD0473R and A2780 and A2780ZD0473 cell lines and additional acquired platinum-resistant sublines. Lane 1, positive control; lane 2, CH1JM149R; lane 3, CH1JM335R; lane 4, CH1JM118R; lane 5, CH1ZD0473R; lane 6, CH1JM216R; lane 7, CH1CisR; lane 8, CH1 parental cell line; lane 9, A2780 parental cell line; lane 10, A2780JM149R; lane 11, A2780-JM335R; lane 12, A2780ZD0473R.

there was a slight variance in levels. In contrast, there was a loss of hMLH1 (but not other mismatch repair proteins; data not shown) in A2780ZD0473R (and A2780JM149R and A2780JM335R) in comparison with the parental A2780 cell line (Fig. 3).

3.2. *Effect of transfection of* MT *into A2780 cells*

Clones representing vector control (F179) and *MT* transfect (F215) were used. Doubling times were similar at 13.1±0.5 h for the A2780 vector (F179) and 11.7±2.6 h for the A2780-*MT* (F215). Expression of *MT* was detected and confirmed in A2780-*MT* relative to vector control by immunohistochemistry and by PCR for the presence of full genomic sequence of *MTIIA* (data not shown).

The growth-inhibitory properties of cadmium chloride and a series of platinum complexes (cisplatin, carboplatin, JM216, the *trans* platinum complex JM335 and ZD0473 itself) in the vector control versus A2780-*MT* were determined (Fig. 4). There was no difference in sensitivity to any of the agents between the vector control line and the parental A2780 line. Overexpression of MT in A2780 cells resulted in 3.8-fold resistance to cadmium chloride and varying levels of resistance to each of the platinum complexes from 7-fold for cisplatin, 2.8-fold for carboplatin and 3.1-fold resistance to the oral drug JM216. Interestingly, the degree of resistance was lowest for ZD0473 (only 1.3-fold).

4. Discussion

ZD0473 is a new sterically hindered platinum-based compound currently undergoing clinical evaluation. This study describes the first investigation of the potential mechanisms of acquired resistance to ZD0473 and also investigates the effects of increasing intracellular thiol levels (in this case MTs) on the drug's growth-inhibitory properties.

Two acquired ZD0473-resistant human ovarian carcinoma cell lines have been established from CH1 and A2780 cells and the mechanisms of resistance compared with those previously described for acquired cisplatin resistance in these lines [15,23]. In contrast to resistance mechanisms observed in acquired cisplatin-resistant CH1 cells (no differences in uptake, DNA platination, GSH levels or sensitivity to cadmium chloride [23] —

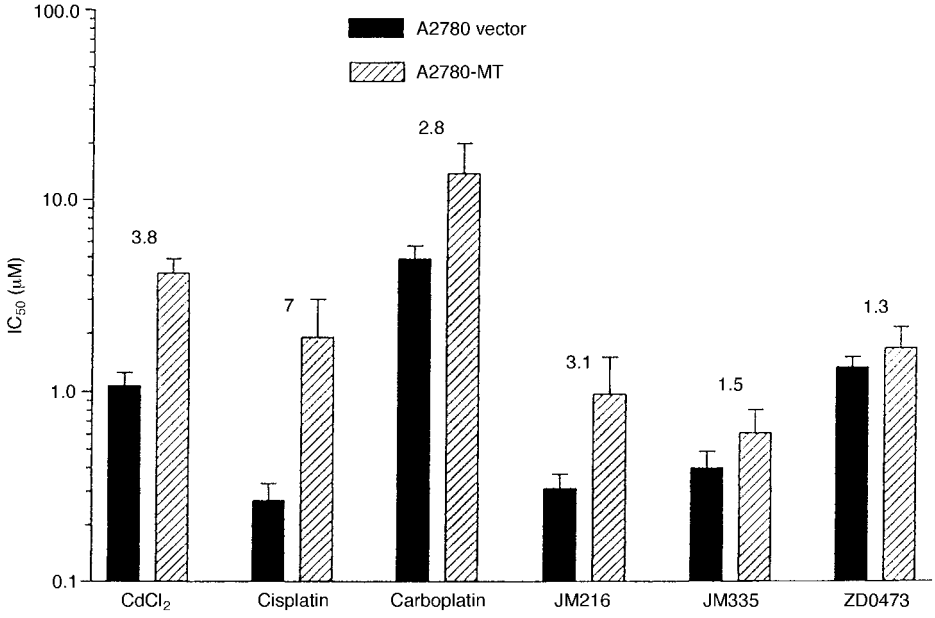

Fig. 4. The effect of overexpression of MT in A2780 cells on sensitivity to cadmium chloride and platinum drugs (after 96 h of drug exposure). IC_{50} values for A2780 vector control (solid bars) or A2780-MT (hatched bars) are means±standard error of the mean (SEM) from $n \geqslant 4$. Values on figure = fold-difference in IC_{50} A2780-MT/A2780 vector.

the CH1ZD0473R cell line exhibited multiple mechanisms of resistance with decreased uptake and platination and increased GSH levels and resistance to cadmium chloride. However, in common with acquired cisplatin-resistant A2780 cells [8,15,18], the A2780ZD0473R cell line also exhibited reduced uptake and DNA platination and increased GSH levels. In addition, as observed previously for A2780cisR cells [22] and another acquired cisplatin-resistant human ovarian carcinoma cell line [24], A2780ZD0473R cells had lost the DNA mismatch repair gene h*MLH1*. We have also noted an increased expression of the anti-apoptotic protein Bcl2 in A2780ZD0473R cells compared with the parental cell line [25].

Cross-resistance patterns with the acquired cisplatin-resistant [8] and acquired ZD0473-resistant lines were generally similar in that partial to full cross-resistance was observed to most of the other platinum-containing drugs studied (e.g. carboplatin and JM216). However, there were some intriguing findings, notably with the *trans* platinum complex JM335 where a lack of cross-resistance (resistance factor <2) was seen in CH1cisR and A2780ZD0473R lines but not in the A2780cisR and CH1ZD0473R lines. This circumvention of cisplatin or ZD0473-acquired drug resistance by JM335 does not appear to correlate with a particular biochemical mechanism(s) of resistance. While there was a lack of cross-resistance seen with ZD0473 in the two acquired cisplatin-resistant lines [8], cisplatin did not retain activity in either of the two acquired ZD0473R lines, suggesting distinct mechanisms of action for the two drugs as previously described [8,9].

The effect of modulating intracellular thiols (MT) resulted in some interesting effects on the growth-inhibitory properties of ZD0473. Increased levels of MT are often (but not universally) observed in cisplatin-resistant cancer cells [12,13,21]. Overexpression of MT by stable gene transfection in A2780 cells resulted in protection from the growth-inhibitory effects of cadmium chloride (3.8-fold) and all five platinum drugs studied when compared with the isogenic vector control. However, the degree of protection differed markedly across the platinum drugs with most protection seen with cisplatin (7-fold) and least (only 1.3-fold) with ZD0473. These cellular data are supportive of previous observations showing the relative lack of affinity for thiol-containing species both *in vitro* and within cells for the sterically hindered ZD0473 versus cisplatin [8,9]. Hence, as with tumours possessing increased levels of GSH [4,7], ZD0473 when compared with cisplatin, may also be useful in the treatment against tumours possessing relatively high MT levels.

In summary, these data indicate that some mechanisms of acquired resistance to the novel sterically hindered platinum ZD0473 are shared with those previously observed in the same parental cell lines with cisplatin (e.g. reduced uptake, increased GSH, loss of *hMLH1* in A2780 sublines). However, in the CH1 sublines, differing mechanisms were observed. Moreover, these results add support to previous findings that ZD0473 possesses unique cellular pharmacological properties with respect to interactions with thiol-containing species associated with tumour resistance to cisplatin.

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