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Retention of activity by the new generation platinum agent AMD0473 in four human tumour cell lines possessing acquired resistance to oxaliplatin

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Abstract

Four models of acquired resistance to the clinically-used platinum drug, oxaliplatin, have been established using human tumour cell lines *in vitro*; two colon (HCT116 and HT29) and two ovarian (A2780 and CH1). Levels of acquired resistance ranged from 3.0- to 15.8-fold with levels of resistance higher in the colon relative to the ovarian carcinoma cell lines. Notably, the platinum analogue, AMD0473, currently undergoing clinical evaluation, exhibited superior circumvention of acquired oxaliplatin resistance in comparison to either cisplatin or the trinuclear platinum BBR3464. Resistance in the two colon cell lines was unique to oxaliplatin itself among the platinum drugs studied. Acquired oxaliplatin resistance was not due to either reduced drug membrane transport or increased levels of glutathione in any of the four resistant lines. Following exposure to oxaliplatin, a lower level of platinum–DNA adducts was present in acquired oxaliplatin-resistant HT29 cells. In the remaining resistant lines, there was no change in the levels of platinum–DNA adducts relative to the parent lines. There was no change in *hMLH1* DNA mismatch repair gene status in any of the four cell line pairs. However, in an A2780 subline where loss of hMLH1 and a p53phe172 mutation occurred, 5-fold resistance to cisplatin was observed, but only 1.7-fold resistance to oxaliplatin and no resistance to AMD0473 were observed. Re-introduction of *hMLH1* into these cells caused no significant change in the sensitivity to cisplatin, oxaliplatin or AMD0473. These data show that acquired resistance to oxaliplatin may occur in cell lines (and therefore probably in the clinic) and in the four independent cell lines studied this was circumvented by AMD0473. Alongside previously described models of acquired resistance to cisplatin, these oxaliplatin-resistant cell line models may be useful in the evaluation of further novel platinum agents.

Keywords: ZD0473; Oxaliplatin; Resistance; Circumvention; Mismatch repair

1. Introduction

The platinum-containing drugs, cisplatin and carboplatin, play a significant role in the chemotherapeutic treatment of a number of human malignancies [1]. However, the two drugs are active against essentially the same tumour types and share cross-resistance with each other, and thus in recent years, several new platinum compounds have entered the clinic in attempts to broaden the clinical utility of platinum-based chemotherapy [2]. Foremost among these is the 1,2-diaminocyclohexane (DACH) platinum, oxaliplatin. This has shown utility, in combination with 5-fluorouracil, in the treatment of advanced colorectal cancer and is registered in France and some other European countries for this indication [3–5]. A phase II trial of oxaliplatin has also been completed in patients with advanced ovarian cancer previously pretreated with cisplatin or carboplatin [6]. Preclinical studies have also shown that oxaliplatin and cisplatin, when used in combination *in vitro*, may confer additive and possibly synergistic cell kill [7]. ZD0473 (cis-amminedichloro[2-methylpyridine]platinum(II); formerly known as AMD473 and JM473) is another 'new generation' platinum agent that is now undergoing worldwide phase II and III clinical trials [8]. AMD0473 is a sterically hindered agent that was developed on the basis of its ability

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to circumvent acquired cisplatin resistance in various preclinical *in vitro* cell line and *in vivo* tumours possessing acquired resistance to cisplatin [9,10]. Although the agent was designed primarily to be less susceptible than cisplatin to inactivation by thiol-containing species associated with tumour resistance (such as glutathione), AMD0473 also circumvented acquired cisplatin resistance due to impaired drug transport and enhanced DNA repair [10,11].

To date, little is known concerning mechanisms of acquired resistance to oxaliplatin and, moreover, whether newer generation platinum agents like AMD0473, may circumvent acquired oxaliplatin resistance. The first aim of this study was to establish representative laboratory models of acquired oxaliplatin resistance using two human colorectal carcinoma and two human ovarian carcinoma cell lines. Thereafter, the ability of AMD0473 (and cisplatin) to overcome resistance to oxaliplatin was determined. Another aim was to determine the role of some of the major biochemical features known to contribute to acquired cisplatin resistance in causing resistance to oxaliplatin in these cell line models. The factors studied were oxaliplatin transport, levels of glutathione and levels of platinum–DNA adducts. Finally, as differences in the recognition of DNA adducts formed by cisplatin and oxaliplatin have been shown [12], the role of the DNA mismatch repair protein, hMLH1 in determining sensitivity to AMD0473 and oxaliplatin has been studied using the four oxaliplatin resistant cell lines and in a human ovarian carcinoma isogenic pair of lines differing only in their hMLH1 status [13].

2. Materials and methods

2.1. Cell lines

Two human ovarian cancer cell lines (A2780 and CH1) and two colon tumour cells (HCT116 and HT29) were used. Acquired cisplatin and AMD0473-resistant sublines of the A2780 and CH1 cell lines have been previously described in Ref. [14]. Resistance to oxaliplatin was established by similar means by exposing cell lines to 0.5× concentration required for 50% inhibition (IC_{50}) repeatedly until the cells tolerated this concentration. Doses were then doubled and stable resistance established over a 6-month period of repeated exposure.

Additional A2780 sublines of defined DNA mismatch repair status were kindly provided by Dr P. Karran (Imperial Cancer Research Fund, UK). The parental A2780 SCA5 line (proficient in mismatch repair), a methylation tolerant variant which had lost mismatch repair function through loss of hMLH1 (A2780MNU1), and A2780MNU cells transfected with a vector containing *hMLH1* cDNA, named A2780pMLH1 have been previously described in Ref. [13].

All cell lines grew as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (Imperial Laboratories, Andover, UK), 2-mM L-glutamine and 0.5 μg/ml hydrocortisone in a 6% CO_2, 94% air atmosphere. Cells were free of *Mycoplasma* species.

2.2. Cell growth inhibition assay

A 96-h exposure, sulphorhodamine B (SRB) growth-inhibition assay was used as previously described by us for the study of platinum drugs [10,14]. Cisplatin was obtained from the Johnson Matthey Technology Centre, Sonning, UK and AMD0473 and oxaliplatin were the clinical formulations as used by Astrazeneca and Synofi-Synthelabo, respectively. BBR3464 was kindly provided by Dr N. Farrell, Virginia Commonwealth University, Richmond, USA. Drugs were dissolved in isotonic saline immediately prior to use. Briefly, cells (5×10^3) were seeded into 96-well plates and left at 37 °C overnight to allow attachment. A range of drug concentrations were added to quadruplicate wells for 96 h. Cells were then fixed with 10% trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. The IC_{50} values were derived from the drug concentrations that reduced the absorption by 50% of that in untreated control wells.

2.3. Measurement of glutathione (GSH) levels

Exponentially growing cells were harvested and total GSH was extracted using 0.6% sulphosalicylic acid and levels determined in an enzymatic assay using 5′5′-dithio-bis(2-nitrobenzoic acid) (DTNB) as previously described in Ref. [15]. GSH content was expressed as nmol GSH per mg of protein.

2.4. Measurement of platinum accumulation and DNA platination

The intracellular platinum accumulation after 2-h exposure to oxaliplatin was determined using flameless atomic absorption spectrometry (FAAS; Perkin-Elmer 1100B and HGA 700; limit of detection was 5 ng (1 nmol) platinum). This method has been previously described in Ref. [10]. Following drug exposure, cells were washed twice with ice-cold phosphate-buffered solution (PBS), the monolayer scraped into 2 ml ice-cold PBS and then sonicated on ice prior to FAAS. Cellular platinum levels were expressed as nmol platinum/mg protein.

For DNA platination studies, around 3×10^7 cells were exposed to oxaliplatin for 2 h, and the DNA extracted using phenol and ethanol precipitation following cell lysis [10]. DNA content was measured at 260 nm by a spectrometer and platinum levels were determined by FAAS.

2.5. Western blotting for hMLH1 DNA mismatch repair protein

Levels of hMLH1 protein were measured in the A2780 cell lines including those provided by Dr P. Karran, by western blotting. Total protein was extracted, 50 μg protein separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (8–16% Novex precast gels; 30 mA per gel for 1 h), transferred to nitrocellulose membranes and probed with an hMLH1 antibody (Pharmingen G168-15), and detected by enhanced chemiluminescence (ECL). The mismatch repair proficient parental A2780 cells were used as a positive control [12].

3. Results

Acquired resistance to oxaliplatin was successfully established in all four human tumour cell lines under study, within 6 months. There were no significant differences in the population doubling time between any of the resistant lines versus their respective parent line (data not shown).

3.1. Levels of resistance to oxaliplatin and cross-resistance to cisplatin and ZD0473

Using the 96-h SRB assay, the resistant sublines exhibited varying levels of acquired resistance to oxaliplatin (Fig. 1). Results are presented in the form of 'resistance factors' RF values (IC_{50} resistant/IC_{50} parent) as used previously by us (e.g. Ref. [10]). RF values were A2780 pair 3.7, CH1 pair 3.0, HCT116 pair 15.8 and HT29 pair 13.3. Hence within the same time-frame and using the same procedure, higher levels of resistance to oxaliplatin were obtainable in the two colon cancer cell lines in comparison to the two ovarian carcinoma lines. Individual IC_{50} values in μM (±standard deviation (S.D.), $n = 3$–4) for the lines to oxaliplatin, cisplatin, AMD0473 and BBR3464 are shown in Table 1. Levels of cross-resistance to either cisplatin or AMD0473 are shown in Fig. 1. The results show that AMD0473 partially or completely circumvented acquired oxaliplatin resistance in all four cell lines. Platinum drug resistance in the two colon cancer cell lines may be specific to oxaliplatin itself since non cross-resistance was also observed to cisplatin and the trinuclear platinum drug BBR3464 currently undergoing clinical study [16]. As expected, BBR3464 was markedly more potent than the other three platinum drugs [17]. In the CH1 pair of lines, where full cross-resistance was observed to cisplatin, only partial cross-resistance was observed to AMD0473. Cross-resistance in the two ovarian lines was also observed to BBR3464 (RF values of 3.0 in the A2780 pair and 15.0 in the CH1 pair; Table 1). As for cisplatin and AMD0473, no cross-resistance to BBR3464 was observed in either of the oxaliplatin-resistant colon cancer cell lines (RF values of < 1.5 in both HCT116 and HT29 pairs).

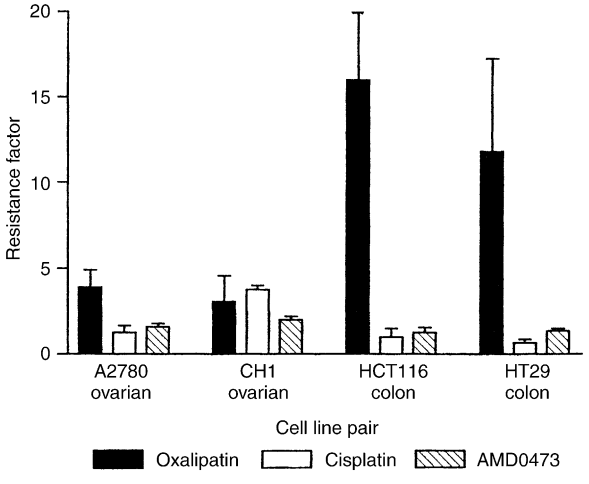

Fig. 1. Cross-resistance profile of the four acquired oxaliplatin resistant cell lines to cisplatin and AMD0473. Values (mean±standard deviation±S.D., $n = 3$–4) are shown as resistance factors, RF, IC_{50} resistant/IC_{50} parent line.

3.2. Mechanisms of resistance to oxaliplatin in the four acquired resistant cell lines

In view of published data for other platinum drugs (including oxaliplatin) (e.g. Refs. [14,15,18]), we determined whether higher levels of the thiol, glutathione, may be contributing to the acquired oxaliplatin resistance. Table 2 shows GSH levels in parent and oxaliplatin-resistant sublines. In each case, there was no significant difference in the levels between that of parent and acquired resistant subline.

Many acquired platinum-resistant sublines exhibit decreased platinum drug transport [14,19,20]. Accordingly, we measured platinum uptake in each of the four pairs of lines following exposure to oxaliplatin (Fig. 2a–d). Interestingly, results show that there were no marked differences in platinum uptake in any of the four oxaliplatin-resistant sublines. Over the concentration range

Table 1
Sensitivity of parent and acquired oxaliplatin resistant cell lines to oxaliplatin, cisplatin, AMD0473 and BBR3464

Cell line	Oxaliplatin	Cisplatin	AMD0473	BBR3464
A2780	0.15±0.04	0.52±0.20	1.15±0.4	0.01±0.007
A2780oxaliR	0.55±0.20	0.64±0.07	1.9±0.9	0.03±0.02
CH1	0.4±0.2	0.13±0.03	3±0.5	0.02±0.003
CH1oxaliR	1.2±0.5	0.51±0.1	5.4±1.9	0.3±0.1
HCT116	0.26±0.04	5.6±2.9	12.5±4.7	0.053±0.03
HCT116oxalR	4.1±1.6	4.4±1.6	17.7±10.4	0.076±0.03
HT29	0.66±0.3	3.4±0.6	17±1.0	0.074±0.04
HT29oxaliR	8.8±1.4	2.2±0.6	23.7±0.6	0.075±0.03

Values are IC_{50} in μM, 96-h drug exposure.

Table 2
Intracellular GSH levels in A2780, CH1, HCT116, HT29 and their acquired oxaliplatin-resistant cell lines

Cell line	nM GSH/μg protein Mean±S.D.
A2780	0.07±0.06
A2780oxaliR	0.09±0.05
CH1	0.25±0.1
CH1oxaliR	0.27±0.1
HCT116	0.19±0.02
HCT116oxaliR	0.19±0.08
HT29	0.3±0.1
HT29oxaliR	0.27±0.1

studied (10–50 μM), platinum uptake increased linearly with increasing drug concentration.

As with other platinum drugs, DNA is believed to represent the key intracellular target for oxaliplatin [21–23]. As an initial indication of the possible role of DNA binding in contributing to drug resistance, we measured global platinum–DNA levels in each of the pairs of lines following exposure to oxaliplatin (Fig. 3a–d). The only pair of lines to exhibit a significant change in platinum–DNA binding was the HT29 pair, where lower amounts of platinum was bound to the DNA of the acquired resistant cell line at each of the four concentrations examined (10, 25, 50, 100 μM). Levels of platinum–DNA were slightly higher in the A2780 oxaliplatin-resistant line while there was little difference in the CH1 or HCT116 pairs.

3.3. The role of the hMLH1 mismatch repair protein in determining sensitivity/resistance

Some reports have shown that, especially in the A2780 ovarian cell line, loss of DNA mismatch repair (especially hMLH1) may contribute to acquired cisplatin resistance [24]. We determined hMLH1 status by immunoblotting in the four paired lines and in additional A2780 lines of defined hMLH1 status (Fig. 4 for A2780 lines). There was no change in hMLH1 status in the CH1, HCT116 or HT29 pairs of lines. The A2780 parent cell line (including A2780 SCA5) and acquired oxaliplatin-resistant cell line all possess hMLH1. In contrast, and as previously shown in Ref. [13], A2780cisR and A2780MNU1 have lost hMLH1. As expected, the *hMLH1* transfected subline of A2780MNU1 (A2780p MLH1) showed expression of hMLH1.

Finally, we determined the sensitivity of the A2780 SCA5 (parent, mismatch repair proficient), A2780 MNU1 (loss of hMLH1) and A2780pMLH1 (*hMLH1*

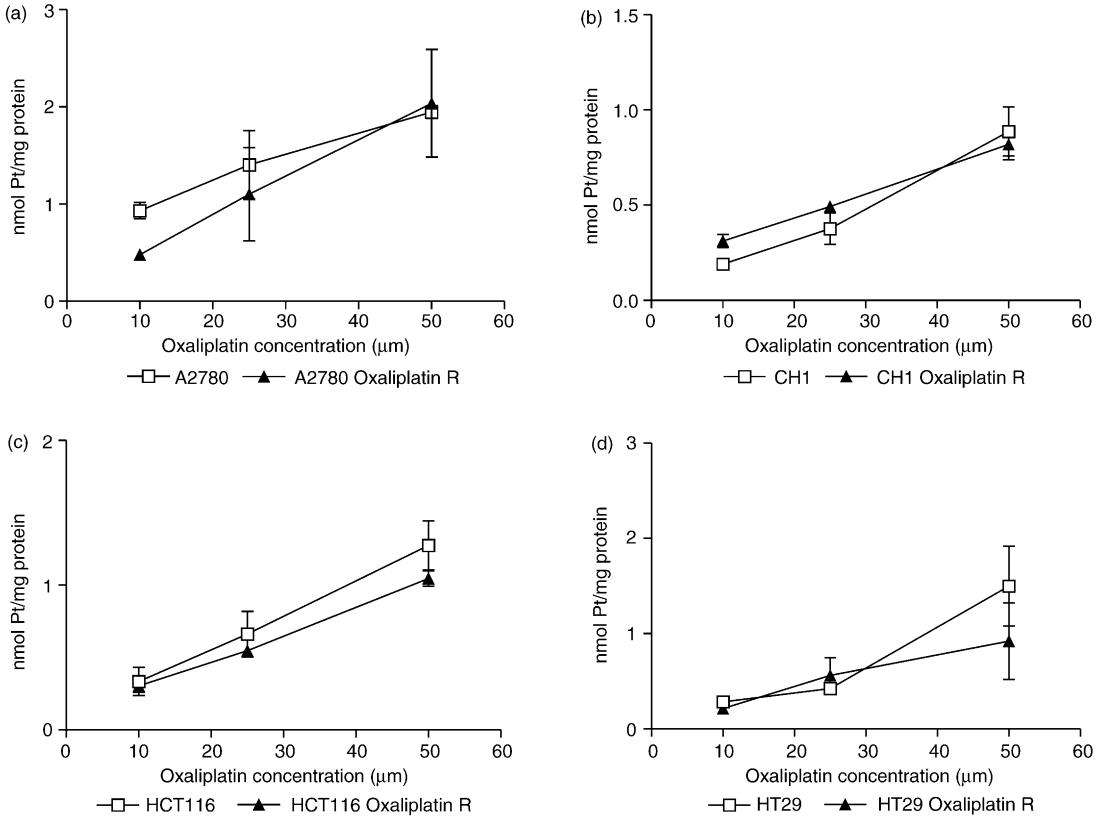

Fig. 2. Platinum drug uptake following exposure of (a) A2780 (□) and A2780oxaliplatinR (▲); (b) CH1 (□) and CH1oxaliplatinR (▲); (c) HCT116 (□) and HCT116oxaliplatinR (▲) and (d) HT29 (□) and HT29oxaliplatinR (▲). Values are means ±S.D. ($n=3$). Drug exposure was for 2 h and platinum levels determined by flameless atomic absorption spectrometry (FAAS).

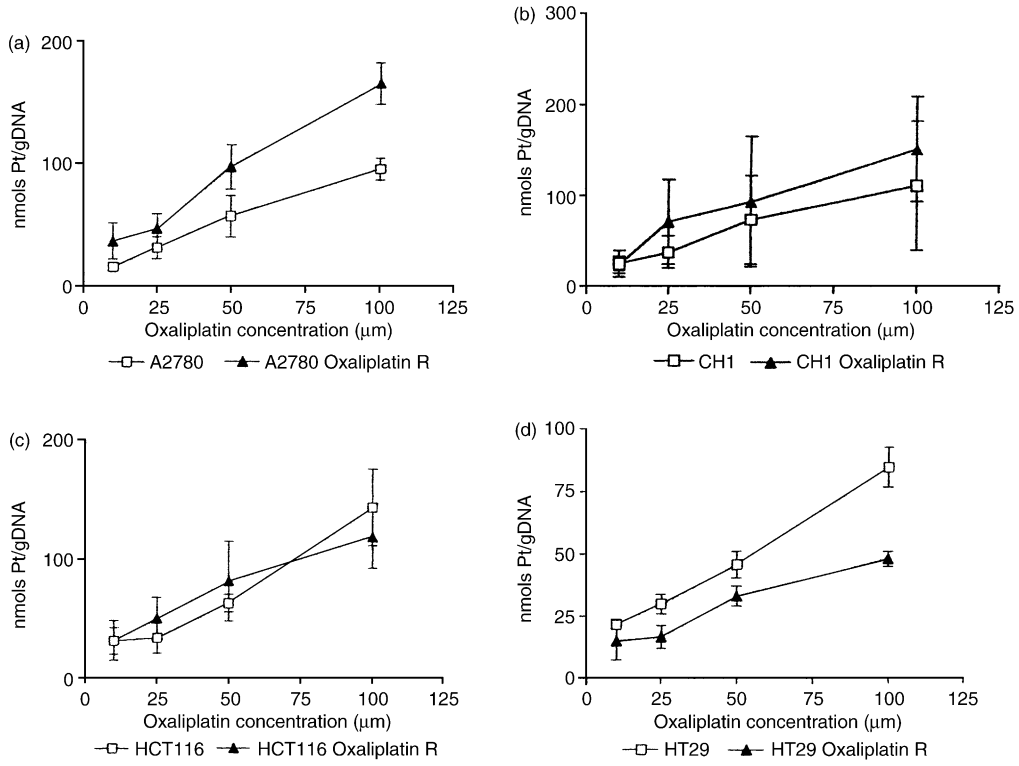

Fig. 3. Platinum–DNA adduct levels following exposure of (a) A2780 (□) and A2780oxaliplatinR (▲); (b) CH1 (□) and CH1oxaliplatinR (▲); (c) HCT116 (□) and HCT116oxaliplatinR (▲) and (d) HT29 (□) and HT29oxaliplatinR (▲). Values are means ±S.D. ($n=3$). Drug exposure was for 2 h and platinum levels on extracted DNA determined by FAAS.

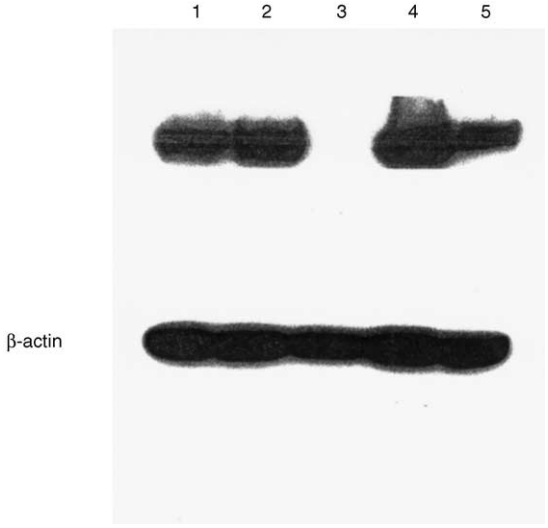

Fig. 4. Immunoblotting for hMLH1 mismatch repair protein. Lane 1, A2780; lane 2, A2780oxaliplatinR; lane 3, A2780MNU1; lane 4, A2780 SCA5; and lane 5, A2780pMLH1.

transfected back into A2780MNU1) to oxaliplatin, cisplatin and AMD0473 (Table 3). As previously shown in these lines for cisplatin [13], the A2780MNU1 was more resistant to cisplatin than the parent A2780 SCA5 line (5.2-fold). In contrast, A2780MNU1 was only 1.7-fold resistant to oxaliplatin under the same experimental conditions

Table 3
Sensitivity to cisplatin, oxaliplatin or AMD0473 in A2780 human ovarian carcinoma sublines of varying DNA mismatch repair status

Cell line	96-h IC_{50} (μM)		
	Cisplatin	Oxaliplatin	AMD0473
A2780 SCA5	0.52±0.06	3.0±0.4	10.8±0.8
A2780MNU1	2.7±0.4	5.0±1.0	10.3±6.7
A2780pMLH1	3.0±0.9	6.4±1.9	14.0±5.2

Values = mean±S.D., $n=3$.

and showed no resistance to AMD0473. Re-introduction of *hMLH1* into this cell line caused little change in the sensitivity to any of the three platinum drugs.

4. Discussion

Platinum-containing drugs play a significant role in the chemotherapeutic treatment of a variety of human cancers including ovarian cancer (with cisplatin and carboplatin) and colorectal cancer (with the recent introduction of oxaliplatin). However, tumour resistance to these agents represents a key determinant of long-term patient survival and much remains to be achieved to combat such resistance. One approach that has been extensively explored has been to design analogues of

cisplatin that address the major mechanisms of resistance shown to occur to cisplatin using cell lines. Hence, in the past 10 years, platinum complexes have been introduced into the clinic with increased lipophilicity to combat membrane transport mediated resistance (e.g. oxaliplatin, JM216), with decreased affinity for thiol-containing species like glutathione (e.g. AMD0473) and with markedly different DNA binding properties (e.g. BBR3464) [2].

This study has specifically addressed the issue of resistance mechanisms to oxaliplatin and their circumvention by AMD0473. Four human tumour cell lines (two colon where oxaliplatin has shown some clinical activity and two ovarian) have been made resistant to oxaliplatin by exposure *in vitro*. Resistance (ranging from 3.0- to 15.8-fold) was generated within 6 months exposure in all four cell lines, thus confirming as with other platinum drugs, that it is possible to generate acquired resistance to oxaliplatin *in vitro*. The most notable finding from this study was that AMD0473 was able to circumvent acquired oxaliplatin resistance in all four cell lines. Oxaliplatin resistance in the two colon cancer cell lines (where higher levels of resistance were observed relative to that seen in the two ovarian lines) appeared to be unique to oxaliplatin itself among the platinum drugs, as cisplatin, AMD0473 and BBR3464 all retained activity in these lines. By contrast, full cross-resistance was observed to both cisplatin and BBR3464 in acquired oxaliplatin-resistant CH1 ovarian carcinoma cells, whereas only partial cross-resistance was observed to AMD0473. These data add further support to the view that differences in the cellular pharmacology of platinum analogues can confer differences in the circumvention of resistance properties in cell lines *in vitro*.

Perhaps a surprising finding from our data was that two of the most commonly described mechanisms of resistance to cisplatin in cell lines, namely increased glutathione and reduced drug transport (see Ref. [25] for a review), did not appear to contribute to acquired oxaliplatin resistance in any of the four resistant cell lines. This is in contrast to recent reports of acquired oxaliplatin resistance by Pendyala and colleagues, including using A2780 cells [18,20]. They found that in A2780 oxaliplatin resistant sublines, elevated levels of glutathione (3.1- to 3.8-fold higher) contributed to resistance [18]. In addition, more recently, they have shown that the A2780 oxaliplatin resistant C25 subline also shows reduced drug uptake and reduced platinum–DNA adducts relative to the parent cell line [20]. This appears similar to A2780 cells possessing acquired resistance to cisplatin that have been described and exhibiting multiple mechanisms of resistance including reduced drug transport and enhanced GSH levels [19,26]. In contrast, in our hands, an A2780 oxaliplatin resistant cell line showed no difference from that of the parent line in terms of GSH levels, drug transport, overall platinum–DNA adducts or hMLH1 mismatch repair protein status. Thus it appears likely that in our oxaliplatin-resistant subline (as well as the CH1 and HCT116 sublines) resistance is likely due to alterations in DNA repair (possibly at the gene level as we have previously shown for cisplatin in acquired cisplatin-resistant CH1 cells [27]) and/or due to alterations in tolerance/apoptotic cell signalling [28,29]. Further experimentation is required to confirm this. Interestingly, some cellular signalling pathways (e.g. JNK and c-Abl) appear to differ in their response to DNA adducts formed by cisplatin versus oxaliplatin and be dependent upon the DNA mismatch repair status [30]. In HT29 oxaliplatin-resistant cells it appears that at least part of the observed resistance is due to less platinum–DNA adducts being formed for a given dose compared with the parental cells.

The DNA mismatch repair pathway has been the subject of considerable attention within the platinum resistance and analogue field for the past few years (e.g. Refs. [12,13,31]. Loss of hMLH1 has been described in at least two acquired cisplatin-resistant human ovarian carcinoma cell lines, 2008 [12] and A2780 [24]. However, we did not detect any loss of hMLH1 in our A2780 oxaliplatin-resistant cell line. While these studies suggest that loss of hMLH1 contributes to resistance to cisplatin, it has also been suggested that loss of mismatch repair does not affect sensitivity to oxaliplatin [12]. However, recent data suggest that in the A2780 model, defective p53 function is a major determinant of cisplatin resistance while defective mismatch repair is a minor, independent contributor [13]. Our data using the same cell lines show that A2780MNU1 cells are around 5-fold resistant to cisplatin. This is in broad agreement with previously reported data where a 4-fold resistance to cisplatin was reported in this line [13]. However, as well as loss of hMLH1, these cells also harbour a heterozygous p53phe172 mutation [13]. Interestingly, the A2780MNU1 cells were only 1.7-fold resistant to oxaliplatin and showed no resistance to AMD0473. Hence, both oxaliplatin and AMD0473 appear to be conferring growth inhibition in these cells independent of *TP53* mutation and loss of hMLH1. Furthermore, the difference in hMLH1 status in these lines appears to confer a minimal effect on platinum drug sensitivity since re-introduction of hMLH1 into A2780MNU1 did not result in any marked changes in platinum drug sensitivity. This is in agreement with previously reported studies with cisplatin in this pair of lines, where there was only a 1.3-fold change in sensitivity as measured by clonogenic assay [13].

In summary, these data in four human tumour cell lines, show that resistance to oxaliplatin, as with other platinum drugs, may occur and would be expected to occur clinically. Some mechanisms of resistance to oxaliplatin (e.g. in the two colon cell lines) may be unique

among the platinum drugs to oxaliplatin itself and did not involve changes in drug transport or levels of glutathione. In the CH1 ovarian cell line model, AMD0473 exhibited superior circumvention of oxaliplatin-resistance properties to that of either cisplatin or BBR3464. These cell line models may also be useful in the further *in vitro* evaluation of novel platinum agents where circumvention of clinical resistance to cisplatin (and oxaliplatin) remains a key goal.

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