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ORIGINAL ARTICLE

The relative activity of cisplatin, oxaliplatin and satraplatin in testicular germ cell tumour sensitive and resistant cell lines

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Abstract
Background Germ cell tumours (GCT) can become resistant to cisplatin, which is associated with a relatively poor prognosis. Oxaliplatin and satraplatin have been developed to overcome cisplatin resistance in other cancers, but their effect in cisplatin resistant (cisR) GCTs is unclear. In this work we address this issue by comparing their efficacy in three paired sensitive and cisR GCT cell lines.
Methods Three established cisplatin sensitive (cisS) and resistant cell line pairs were used (GCT27, GCT27r: SUSA, SUSAr: 833k, 833kr). Viability was assessed using a luciferase based ATP assay and EC_{50} and EC_{80} concentrations were calculated. Western blot analysis and flow cytometry was used for further assessment.
Results Sensitivity to the three platinum compounds was broadly similar in the three cisS lines GCT cell lines (EC_{50} = 0.27–0.51 μM for cisplatin, 0.52–0.79 μM for oxaliplatin, 0.31–1.26 μM for satraplatin). EC_{50} values for cisplatin in the three cisR sub lines were 1.8- to 3.8-fold higher than in the sensitive parental lines. Cross resistance to satraplatin and oxaliplatin occurred in all three cisR cell lines (resistance factor 1.9–4.4), with the exception of oxaliplatin in the 833Kr (resistance factor 0.9). Differences in the effect of specific drugs on cell cycle distribution, p53, p21 and MDM2 were observed.
Conclusions These data suggest that satraplatin and oxaliplatin could theoretically be used in chemo-naive GCTs and support the further clinical evaluation of these agents in this setting. The mechanism of cross resistance to these drugs appears multifactorial.

Keywords Oxaliplatin · Cisplatin · Satraplatin · Germ cell tumour · Cell line

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Introduction

The introduction of cisplatin chemotherapy to the treatment of testicular germ cell tumours (TGCTs) was a major clinical breakthrough [6] which has resulted in cure for the majority of patients when combined with etoposide [5]. GCTs are more sensitive to cisplatin than other common tumours, although the reasons for this remain unclear [2, 19].

Despite this increased sensitivity, a small proportion of GCT patients relapse with cisplatin resistant (cisR) disease, although the drug is still often used as second line treatment in such patients [5, 17]. Cisplatin resistance can be induced in vitro by exposing GCT cell lines to increasing concentrations of the drug [12, 22, 28]. Such cell lines provide a useful model system for investigating potential mechanisms of cisplatin resistance. Identifying these mechanisms has been elusive, although differences in cisplatin uptake and DNA repair mechanisms have been implicated [12, 24] and p53 functional status may also be involved [21].

A number of cancer types are resistant to cisplatin, with resistance in most being multifactorial. For this reason oxaliplatin and satraplatin have been developed to overcome cisplatin resistance, with promising results [11, 20]. There has been a reluctance to move away from cisplatin in the treatment of GCTs due to its high cure rate as first line therapy.

However, the outcome of patients with relapsed GCT is less good and it appears that other drugs such as oxaliplatin, which has been shown to overcome cisplatin resistance in other tumour types, may be useful. In this study we have therefore investigated the efficacy of two new platinum agents, oxaliplatin and satraplatin, in GCT cell line pairs comprising the cisplatin sensitive (cisS) parental line and a cisR sub line. We have also investigated the effect of these drugs on the expression of p53 and the p53 target proteins p21 and MDM2.

Methods

Drugs

Stock solutions of cisplatin (CDDP), oxaliplatin and satraplatin (JM-216) were prepared at 20, 5 and 5 mg/mL, respectively. These solutions were aliquoted into Eppendorf tubes and stored at −40°C until use (each tube used only once).

Cell lines

Three established cisS and cisR cell line pairs were used (GCT27, GCT27r: SUSA, SUSAr: 833k, 833kr) [12, 22, 28]. These resistant lines were all developed by growing the sensitive cell lines in increasing concentrations of cisplatin over a long period of time. The GCT27 and 833K cell line pairs were obtained from the Institute of Cancer Research Laboratories (Dr Shipley, Sutton, Surrey, UK), and the Susa cell line pair from University College Hospital (Prof Masters, London, UK). Cells were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum and 1% Penicillin/Streptomycin. All cell lines were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Determination of drug activity

Exponentially growing cells were set at a density of 5×10^4 cells per well in 96-well plates. After leaving cells to adhere overnight, study drugs were added at zero (vehicle only) and five non-zero concentrations. Cells were then incubated for a further 72 h in the presence of drugs, prior to measuring viability using a luciferase based ATP assay (Vialight, Cambrex, Cambridge, UK). As cells start to undergo apoptosis they lose the ability to re-generate ATP, which becomes depleted.

Drug activity data was summarised as % viable cells (based on ATP concentration) relative to control cells. EC_{50} concentrations for each cell line and drug were derived using a modified E_{MAX} equation in GraphPad Prism (San Diego, CA, USA). The EC_{80} concentration was derived from the fitted EC_{50} concentration and Hill slope for each compound in each cell line. This was then used to determine the relative sensitivity of cell line pairs to the platinum compounds studied at the EC_{80} concentration.

All three drugs were evaluated in each experiment and the summary activity results (EC_{50} and EC_{80} concentrations) were derived from a minimum of three separate experiments. Comparable results were obtained using a trypan blue staining method (data not shown).

DNA content and cell cycle distribution analysis

The distribution of cells in different phases of the cell cycle was determined by flow cytometry, according to methods described previously [16]. Acquisition of data was performed within 1 h using a FACSCalibur (BD Biosciences, Oxford, UK). Five thousand cells were analysed for each data point, and the percentage of cells in sub-G1 (apoptotic fraction—cells with a reduced DNA content but similar morphology), G1, S and G2/M phases were determined using the cell cycle analysis program WinMDI v2.8 (http://facs.scripps.edu/). Cell cycle analysis was performed on all three pairs of cell lines treated with cisplatin at the EC_{50} concentration over 24 and 72 h. The effects of all three platinum analogues were then compared in the Susa cell line pair over a 72-h incubation period.

Immunoblot analysis

Cell extracts were separated on NUPAGE 4–12% bis–tris precast gels (Invitrogen, Paisley, UK). Primary antibody probing was performed with P53 (1/1,000 clone DO-7 Dako, Ely, Cambs, UK), MDM-2 (1/200 clone D-12 Santa Cruz Biotech, CA, USA) and P21 antibodies (1/200 clone Ab-1 Oncogene Research Products, Boston, MA, USA). Anti-β-actin was used as a loading control (1/15,000 clone Ab-1, Calbiochem, San Diego, CA, USA). Bands were visualized using the ECL detection system (Amersham Biosciences Ltd, Little Chalfont, UK).

P53, p21 and MDM2 were investigated after cisplatin treatment for 24 and 72 h, and were compared in all three sensitive and resistant paired cell lines. Additionally the effect of an EC_{50} concentration of each platinum analogue on p53 protein expression was also studied in the Susa cell line pair after 72 h.

Results

Cell viability

EC_{50} values for cell viability (μM, with 95% confidence interval) for the cisS parental lines, and resistant (cisR) lines are summarised in Table 1 and Fig. 1a–c. Sensitivity

Table 1 EC_{50} values (with 95% confidence intervals) of platinum analogues in paired TGCT cell lines determined by Vialight ATP assay

Cell line	Cisplatin (μM)	Oxaliplatin (μM)	Satraplatin (μM)
Susa	0.51 (0.46–0.56)	0.79 (0.67–0.93)	1.26 (1.06–1.49)
Susa cisR	1.94 (1.61–2.33)	1.54 (1.25–1.90)	3.39 (2.81–4.09)
Resistance factor at EC_{50}	3.8	1.9	2.7
Resistance factor at EC_{80}	6.5	2.1	1.8
GCT-27	1.25 (1.17–1.32)	0.60 (0.50–0.73)	1.19 (1.00–1.42)
GCT-27 cisR	3.34 (3.08–3.63)	2.65 (2.20–3.20)	3.05 (2.25–4.13)
Resistance factor at EC_{50}	2.7	4.4	2.6
Resistance factor at EC_{80}	6.1	5.1	2.5
833K	0.27 (0.26–0.28)	0.52 (0.49–0.55)	0.31 (0.26–0.37)
833K cisR	0.48 (0.46–0.51)	0.49 (0.41–0.60)	1.14 (1.02–1.28)
Resistance factor at EC_{50}	1.8	0.9	3.7
Resistance factor at EC_{80}	1.6	2	3.6

The resistance factor is the fold difference in sensitivity between the sensitive and resistant EC_{50} or EC_{80} values

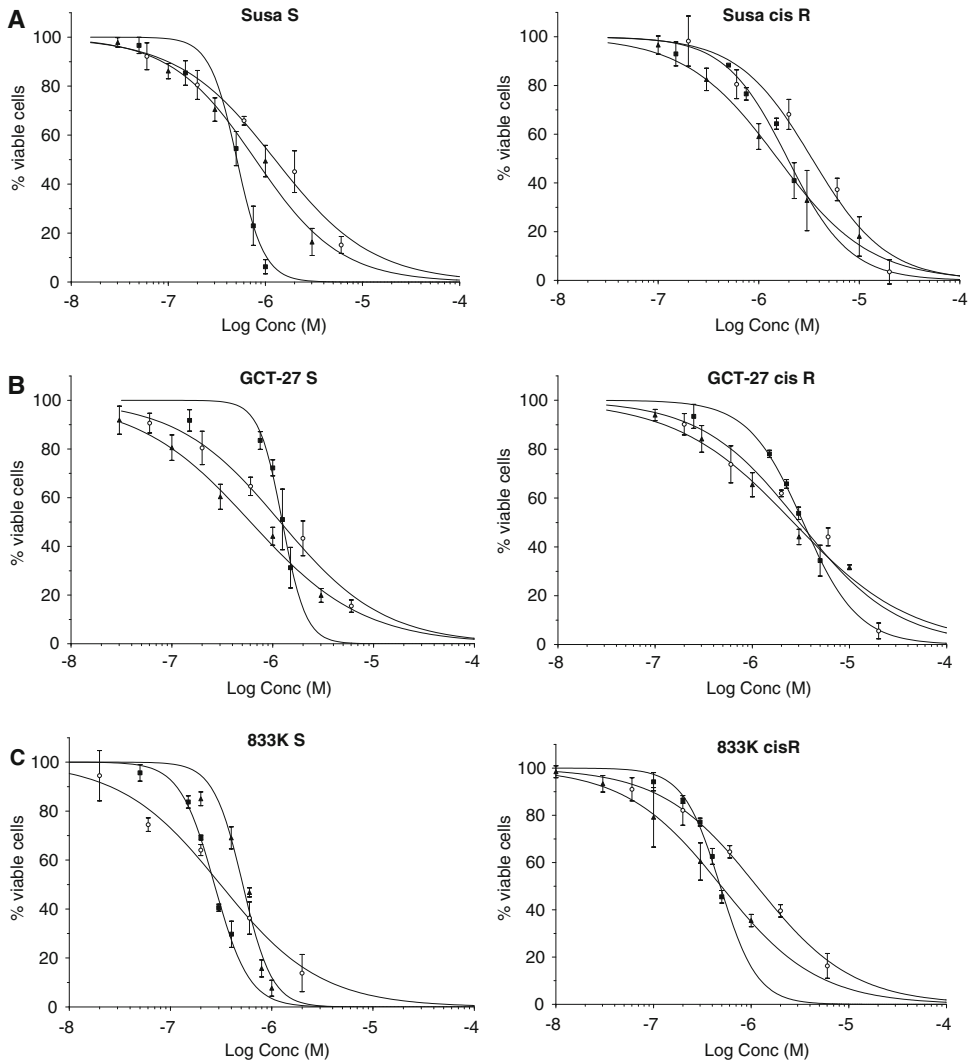

Fig. 1 The effect of platinum analogues on paired TGCT cell lines (*filled square* cisplatin, *filled triangle* oxaliplatin, *open circle* satraplatin. **a** Susa cells, **b** GCT27 cells, **c** 833K cells). Data points are the mean ± SD of three independent experiments

to the three platinum compounds was broadly similar in the cisS lines. EC_{50} values for cisplatin in the three cisR sub lines were 1.8- to 3.8-fold higher than in the sensitive parental lines, confirming previous reports [12, 28].

Satraplatin showed a similar decrease in activity in all three cisR lines, with EC_{50} values between 2.6- and 3.7-fold higher than in parental cells. Oxaliplatin activity was reduced in Susa cisR and GCT-27 cisR cell lines, with EC_{50}

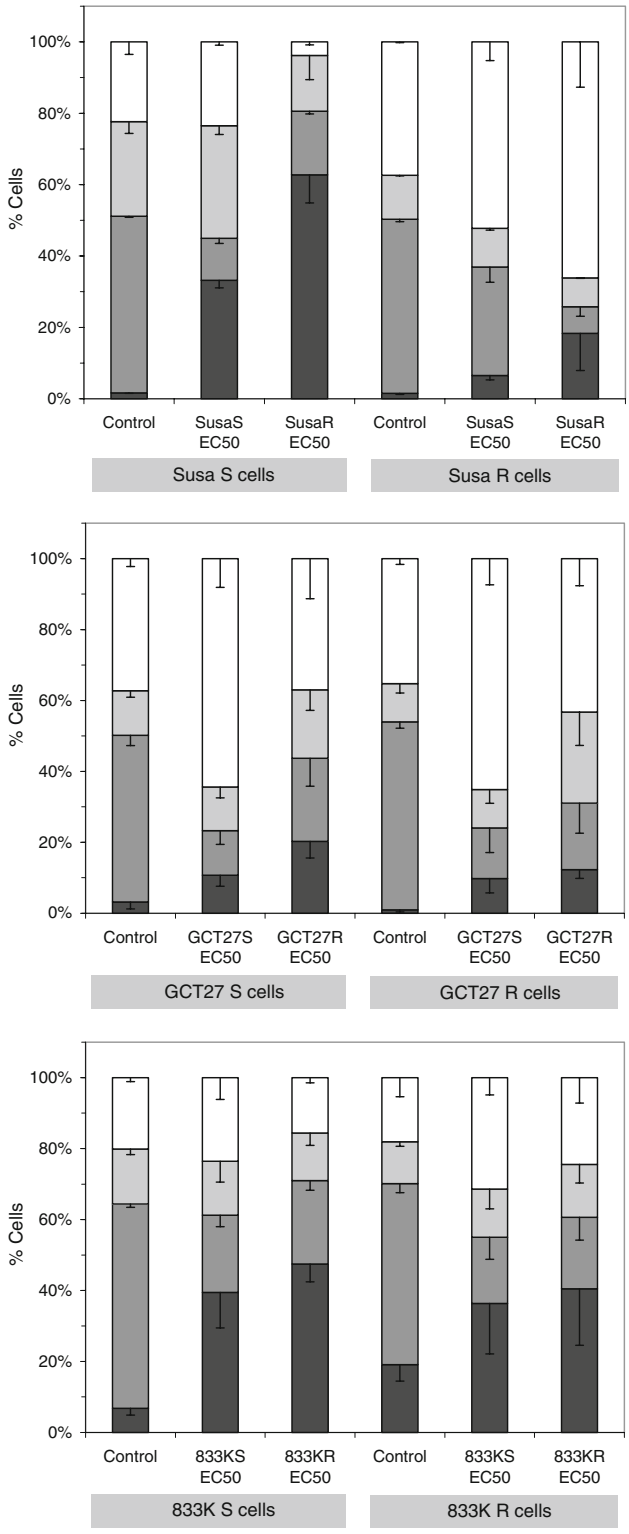

Fig. 2 The effect of cisplatin on cell cycle distribution in TGCT cell line pairs after 72-h exposure at the EC_{50} concentration of the sensitive line (S EC_{50}) and the resistant line (R EC_{50}), (*white* G2/M cells, *light grey* S phase, *dark grey* G1/G0, *black* apoptotic). Data shown is the mean and SD of three separate experiments

values 1.9- and 4.4-fold higher than in the parental lines, but notably was unaltered at the EC_{50} concentration in 833K cisR cells (0.52 vs. 0.49 μM, respectively). Figure 1a–c also demonstrates the difference in the shape of the concentration–effect curve between the three compounds, with cisplatin typically showing a steeper concentration–effect relationship than that seen with oxaliplatin or cisplatin (sigmoid slope factor 1.4–3.3 for cisplatin with 4 of 6 values >2.0, 0.72–3.0 for oxaliplatin with 1 of 6 values >1.2, 0.86–1.18 for satraplatin). Consequently, at higher drug concentrations resistance was often more apparent, particularly for cisplatin in the Susa and GCT27 cell lines (Table 1, Fig. 1), but also for 833K cisR cells treated with oxaliplatin in which drug activity was similar at the EC_{50} concentrations.

Cell cycle distribution

In all lines a common response to cisplatin was a decrease in the percentage of cells in the G1/G0 phase of the cell cycle (Fig. 2). However, this was associated with other cell cycle changes which differed between cell lines and between parental and resistant cells within the same cell line pair. In Susa S and both 833K lines the decrease in G1/G0 cells was accompanied by an increase in the apoptotic population, whereas in Susa cisR and GCT-27 cells the apoptotic response was smaller but a clear increase in the G2/M population was observed. The cell line pair with the biggest difference in cisplatin sensitivity (Susa, 3.8-fold difference between S and R cells) also showed the clearest difference in the apoptotic response to cisplatin. A difference in apoptosis was less clear for the 833K cisR line (1.8-fold resistance), although this line had a larger apoptotic fraction in control cells than the 833K parental line, such that the difference in apoptosis between control and treated cells was less marked than in 833K cisS cells.

There was also a difference in cell cycle changes between different platinum compounds, as shown in Fig. 3 for Susa cells. In Susa S cells the apoptotic response to cisplatin is apparent with a clear increase in the sub-G1 fraction, whereas oxaliplatin and satraplatin treatments result in a marked increase in the G2/M population, particularly at the higher concentration. In Susa cisR cells the clearest response to all three platinum agents was an increased G2/M population. This difference in the apoptotic response at 72 h is shown graphically in Fig. 4.

Immunoblot analysis

Immunoblot analysis of P53, MDM2, and P21 in the three paired cell lines in response to cisplatin in shown in Fig. 5. All cell lines showed an increase in p53 after cisplatin expo-

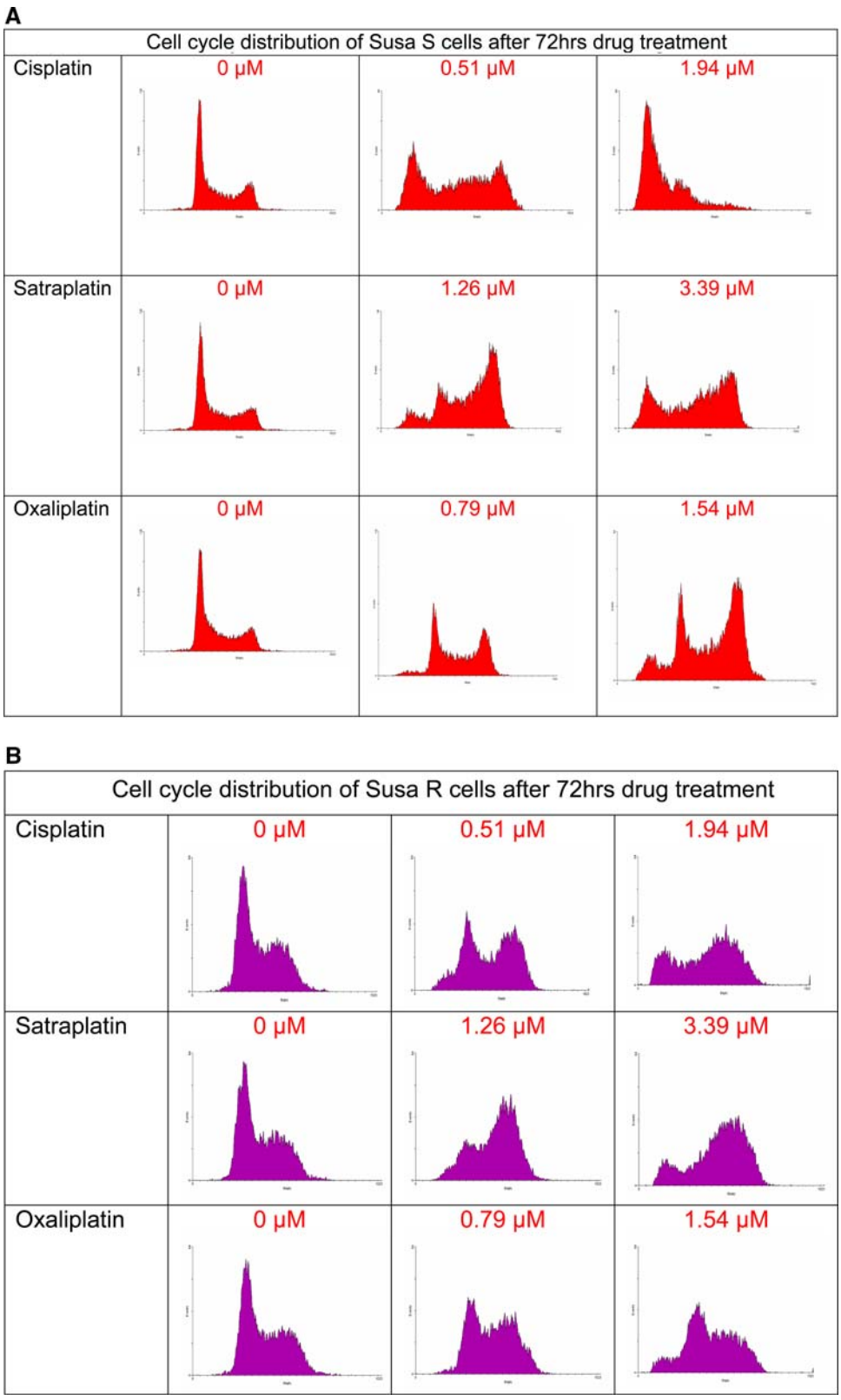

Fig. 3 The effect of platinum analogues on cell cycle distribution in Susa cisS (**a**) and cisR (**b**) cell lines

sure, suggesting all cells had functional p53. This increase was typically less pronounced in cisR cells, particularly when treated at the EC_{50} concentration of the sensitive cell line. These increases were apparent at 24 h, sustained until 72 h, and were typically associated with an increase in p21 staining that again persisted out to 72 h. Cell lines also showed an increase in MDM-2 after 24 h that returned to baseline, or lower than baseline, levels by 72 h.

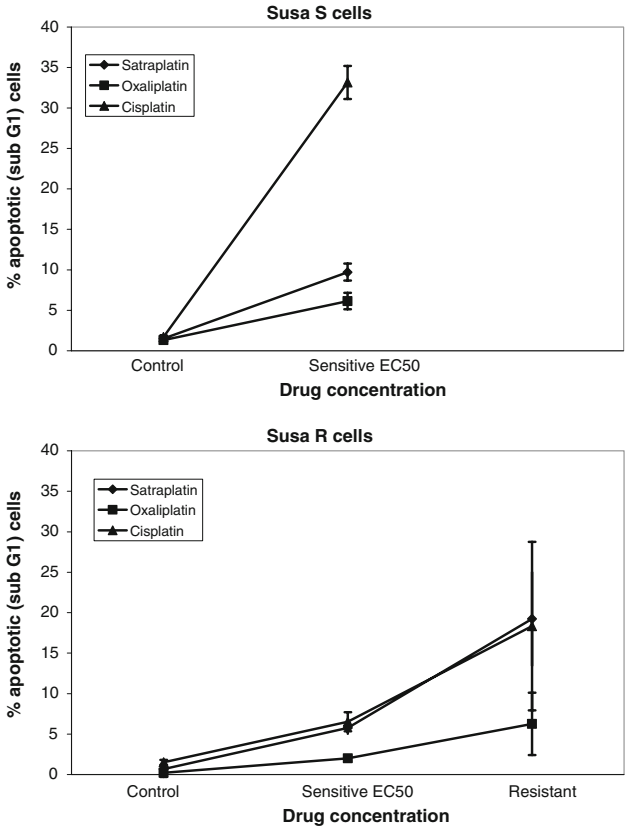

Fig. 4 Apoptosis (sub-G1 population by flow cytometry) in Susa S and Susa R cells after 72-h incubation at the EC_{50} concentration of each platinum compound. Data shown is the mean ± SD of three separate experiments

Similar analyses in Susa S and cisR cells with EC_{50} concentrations of all three platinum compounds again showed an increase in p53 that was concentration dependent in the sensitive line (Fig. 5b). In contrast the increase in p53 in the cisR cells was similar at the cisS and cisR EC_{50} concentrations.

Discussion

The treatment of GCTs has been one of the success stories of combination chemotherapy, largely because of the sensitivity of these tumours to platinum compounds. This is due, at least in part, to the reduced nucleotide excision repair of platinum-DNA adducts in such cells (including GCT27 and 833K cells), attributable to decreased expression of XPA, ERCC1 and XPF proteins involved in this repair activity [13, 29]. Despite this a minority of patients are either refractory to, or relapse after, cisplatin containing therapies. Such cisplatin-resistant tumours have been shown to have increased nucleotide excision repair capacity, as reported in the GCT27 cisR [12] and Susa cisR [9] cell lines used in this study, and in other tumour types [7]. New approaches are needed in such patients, and the in vitro evaluation of novel platinum compounds in sensitive and resistant GCTs is a useful model system in which to investigate these agents.

Using such a model system the data presented in this report confirm the decreased activity of cisplatin in the resistant cell lines used compared to the cisS parental lines (1.8- to 3.8-fold resistance at EC_{50} concentrations). Although representing low level resistance to the drug, the two to fourfold difference in sensitivity is likely to be clinically relevant [19]. In a comparison of cisplatin activity in TGCT and bladder cancer cell lines, tumours known to differ in their clinical sensitivity to cisplatin, there was a similar two to fourfold difference in EC_{50} values between the two tumour types [27]. These cell lines may therefore be a better model system in which to investigate the activity of novel agents than cell lines with marked cisplatin resistance. The data presented here also highlights a difficulty in comparing the relative resistance of cell lines between reported studies in that the fold resistance is dependent in part on the primary assay endpoint used to determine drug activity. For example, resistance to cisplatin based on results from the ATP assay was confirmed by flow cytometric analysis of cell cycle distribution (based on the apoptotic (sub-G1) population) for Susa cisR and 833K cisR cells, but not for GCT 27 cells in which only a small increase in the apoptotic fraction was seen at EC_{50} concentrations in the cisR or sensitive cells (Fig. 2).

While not intending to investigate the mechanism of resistance to cisplatin in these cell lines the more marked increase in p53 in the cisS cells (Fig. 5a) at the sensitive EC_{50} concentration suggests differences in the generation or repair of DNA damage, rather than an altered apoptotic response to the same amount of DNA damage. The GCT27 resistant cell line has previously been reported to demonstrate increased removal of cisplatin adducts from DNA [12]. The Susa cisR cell line also showed a marked increase in the G2/M arrested fraction at EC_{50} concentrations, rather than the clear apoptotic response seen in the cisS Susa cells (Fig. 2a). The induction of p53 after 24-h exposure to cisplatin was also typically associated with an increase in the p53 target genes p21 and MDM2. However, although p53 and p21 remained elevated or even increased at 72 h, this was usually associated with a marked decrease in MDM2 protein. Although MDM2 may be expected to reflect the continued increase in p53 recent reports have described a marked decrease in MDM2 stability in response to DNA damage, mediated both by kinases and MDM2 auto-ubiquitination, thereby prolonging the p53 response [3, 25]. This may explain the decreased MDM2 protein level at 72 h in our studies and may also explain the increased sensitivity of these cells to DNA damaging agents as this effectively prolongs the p53 response. Moreover, MDM2 also promotes

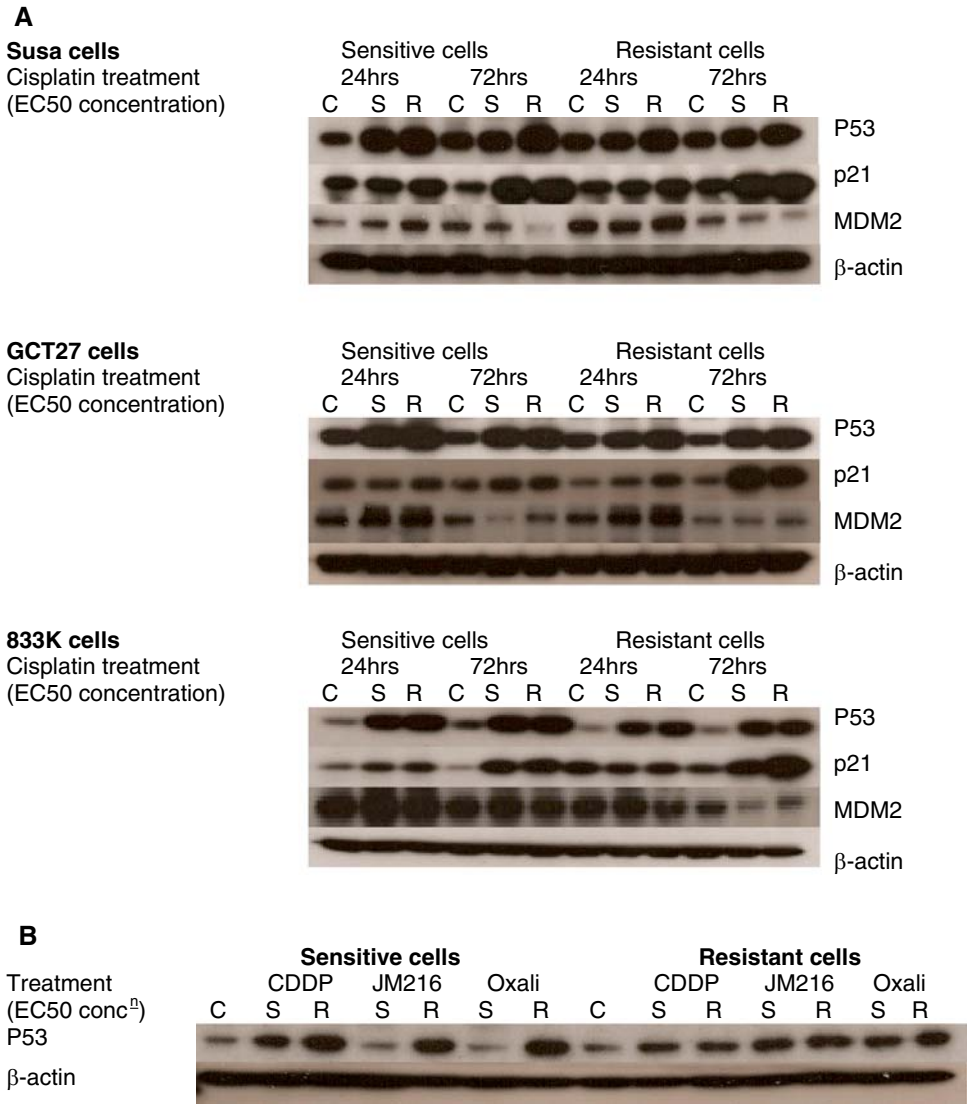

Fig. 5 **a** The effect of cisplatin on P53, P21 and MDM2 in the three pairs of TGCT cell lines. Sensitive and resistant cells were each treated at the EC_{50} concentration of the sensitive line (*S*) and the resistant line (*R*) for 24 and 72 h. **b** The effect of cisplatin, satraplatin and oxaliplatin on p53 expression in Susa S and R cell lines after 72 h

the degradation of the p21 protein in a non-ubiquitinated manner [10], such that the marked decrease in MDM2 at 72 h may contribute to the increase in p21 observed relative to that seen at 24 h. There was not a clear relationship between p21 levels at 72 h and cell cycle arrest.

These results show that oxaliplatin and satraplatin have activity similar to cisplatin in both cisS and cisR GCT cell line pairs. However, the data do not show one platinum compound to be uniformly superior to the other two in this setting after a 72-h exposure. It is noteworthy that oxaliplatin did not exhibit clear cross resistance to cisplatin in all three resistant sub lines, showing similar activity in both cisS and resistant 833K cells at EC_{50} concentrations, but twofold resistance at higher drug concentrations. These findings confirm the results of a previous study investigating oxaliplatin and cisplatin in GCT lines that reported similar sensitivity to the two agents in some lines, but increased potency of oxaliplatin in others [4]. Satraplatin has been reported to be more potent than cisplatin in both cisS and resistant GCT cells (Tera cell line) by Fokkema et al. [8], although the resistance factor was similar for both cisplatin and satraplatin (3.7 and 2.7, respectively). In prostate cancer cell lines the relative sensitivity to these three platinum compounds also differed between lines, with no one compound being clearly superior [30]. There are no other data describing the relative activity of these compounds in the GCT cell line panel used in our own studies.

For different reasons satraplatin and oxaliplatin can overcome cisplatin resistance. The activated intracellular oxaliplatin species has a bulky diaminocyclohexane substituent which is not recognised by the mismatch repair proteins that recognise cisplatin adducts, while satraplatin is an orally bioavailable platinum with differences in its transmembrane transport compared to cisplatin [11]. Recent reports describe differences in platinum uptake after a short exposure to the 3 platinum compounds studied here and

increased Pt-DNA adduct formation with satraplatin [18]. These subtle differences in the pharmacology of the three agents is demonstrated in our own data in the biological responses of the cell lines studied: for all cell lines the dose response curve was steeper for cisplatin than for the other two agents, markedly so in some cases (Fig. 1); at the EC_{50} concentration of each agent the number of apoptotic cells at 72 h was more marked with cisplatin in the Susa cisS cell line (Figs. 2, 4); at EC_{50} concentrations based on the ATP assay the cell cycle response with satraplatin and oxaliplatin showed arrest in the G2/M phase, rather than clear apoptosis (Fig. 3); an increase in p53 was observed at 72 h in Susa cisS cells at the sensitive EC_{50} concentration of cisplatin, but not at the equipotent concentrations of oxaliplatin or satraplatin (Fig. 5).

Early reports of oxaliplatin activity suggested that it could overcome acquired cisplatin resistance in vitro [23]. However, a recent review of the relative activity of oxaliplatin and cisplatin in both clinical and in vitro studies has suggested that the non-cross resistance of oxaliplatin is only seen in the setting of marked (>10-fold) cisplatin resistance, not low level resistance, and that as a single agent oxaliplatin has limited activity in cisplatin refractory/resistant disease [26]. Oxaliplatin has shown activity in TGCT patients with cisplatin refractory disease [14] and may be easier to use in combination with other cytotoxic drugs.

Previous studies have shown that substituting cisplatin with carboplatin in chemo-naïve GCT patients with metastatic disease adversely effects outcome [1]. However, carboplatin and cisplatin have an identical activated species, which is not the case for either oxaliplatin or satraplatin. This is particularly important in the relapsed setting. Carboplatin was not included in the studies reported here because of this inferior activity in good risk TGCT patients and the known cross resistance to cisplatin in vitro [15].

The data presented here suggest that the two novel platinum analogues satraplatin and oxaliplatin could theoretically be used in chemo-naive GCTs and support the further clinical evaluation of these agents in this setting.

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