Characterization of chemosensitivity and resistance of human cancer cell lines to platinum(II) versus platinum(IV) anticancer agents

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Platinum (Pt)(IV) complexes are thought to function as prodrugs for anticancer Pt(II) drugs. We studied two pairs of Pt(II)/Pt(IV) complexes to explore whether there were differences in their cytotoxic activities, their abilities to cause acquired resistance and their gene expression profiles in the resistant lines. Microtiter methods were used to evaluate the antiproliferative activity of cisplatin, oxoplatin, [*trans-d,l*-(1,2-diaminocyclo-hexane)]dichloroplatinum(II) [DACH-Pt(II)] and *cis,trans*-[*trans-d,l*-(1,2-diaminocyclo-hexane)]-dichlorodihydroxoplatinum(IV) [DACH-Pt(IV)] in a panel of 14 human cancer cell lines. Cisplatin and oxoplatin showed significant similar spectra of cytotoxicity, whereas DACH-Pt(II) and DACH-Pt(IV) did not. DACH-Pt(IV) required more than 24 h to reach full potency, whereas the other three Pt complexes achieved maximal activity in less than 24 h. The SISO cervical cell line was made four- to six-fold resistant to the four Pt complexes by weekly exposure to the respective agent. Glutathione (GSH) levels increased in all resistant lines except for the DACH-Pt(IV) resistant line. The catalytic concentrations of various redox enzymes (GSH transferase, GSH peroxidase, GSH reductase, catalase) were all unchanged in the resistant lines relative to the native line. Multidrug resistance protein 2 expression was detected in the cisplatin-resistant and oxoplatin-resistant cell lines but not in the native line. The transcription of 29 000 genes in the SISO lines resistant to either cisplatin or oxoplatin was studied by DNA-microarray methods and compared with the native line. Overall changes in gene transcription were very different between the cisplatin-resistant and oxoplatin-resistant cell lines. Thus, Pt(IV) complexes seem to have biological actions that distinguish them from their Pt(II) counterparts, even when they show cross-resistance. *Anti-Cancer Drugs* 20:559–572 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The development of platinum (Pt) complexes in general [1] and Pt(IV) complexes in particular [2] as anticancer agents has seen a resurgence of interest. Together with cisplatin, Pt(IV) diamines were among the first Pt complexes, which Rosenberg [3] identified with anti-tumor activity, and since then a number of Pt(IV) complexes have been looked at for cancer therapy [2]. Two early examples of Pt(IV) complexes that underwent clinical trials are iproplatin (CHIP) [4] and ormaplatin (tetraplatin) [4], but both were abandoned because they were either too toxic (ormaplatin) or not effective enough (iproplatin). Oxoplatin [oxoplatinum, *cis,cis,trans*-diammine-dichlorodihydroxoplatinum(IV)], a Pt(IV) analogue of cisplatin, was evaluated in a number of preclinical models as an antitumor agent in the USSR and was found to have superior activity to cisplatin and iproplatin in some tumour models [5,6]. A more recent example of a Pt(IV) complex that has undergone clinical trials is satraplatin (JM216), which was developed in the UK as an orally active drug for the treatment of ovarian cancer [4,7,8] and has just undergone clinical trials for prostate cancer [9].

Evidence has accumulated that Pt(IV) complexes serve as prodrugs for their Pt(II) counterparts; that is, activation takes place *in vivo* when the central Pt(IV) ion is reduced by biological reducing agents and two axial ligands (e.g. hydroxide, chloride, acetate) are lost. In cancer patients treated intravenously with iproplatin, the reduction product *cis*-dichlorobis(isopropylamine)platinum(II) was identified along with the parent drug in the plasma and urine [10]. Likewise, rats treated

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intravenously with oxoplatin were found to eliminate cisplatin along with oxoplatin in their urine [11,12]. Ormaplatin undergoes a facile reduction to the Pt(II) analogue (*d,l-trans*-diaminocyclohexane)-dichloroplatinum(II) in the plasma of rats administered a therapeutic dose of the Pt(IV) complex [13]. When humans were administered satraplatin orally, the main metabolite found in plasma ultrafiltrates was the reduction product *cis*-ammine-dichloro-(cyclohexaneamine)platinum(II) (JM-118) [14].

These studies do not rule out, however, that a Pt(IV) complex could have some direct activity of its own. This is possible, especially for Pt(IV) complexes with axial hydroxo groups, because of the relative high stability compared with Pt(IV) complexes with axial chloride and acetate ligands [2,15,16]. This high stability might allow enough time for such a Pt(IV) complex to act on its own before being reduced to the Pt(II) form. Indeed, in-vitro data indicate that oxoplatin can slowly bind to DNA directly without reduction and this could theoretically result in an additional pharmacological effect not related to those of cisplatin [17]. Furthermore, studies have reported that oxoplatin is not completely cross-resistant with respect to increased life span of mice inoculated with cisplatin-resistant L1210 cells [5], suggesting an additional mode of action for oxoplatin, either directly or through a unique biotransformation product other than cisplatin.

Here we compare and contrast the short-term and long-term biological effects of two pairs of Pt(II)/Pt(IV) complexes in human cancer cell lines; that is, in addition to cisplatin and oxoplatin, (*d,l-trans*-1,2-diaminocyclohexane)dichloroplatinum(II) [DACH-Pt(II)] and *cis,trans*-(*d,l-trans*-1,2-diaminocyclohexane)dichlorodihydroxoplatinum (IV) [DACH-Pt(IV)] (Fig. 1) have also been investigated. First, we have studied short-term effects by determining the kinetics of drug action in cells exposed for either 24 or 96 h to the Pt complex. Next, we compared the selectivity of the four agents in a panel of 14 human cancer cell lines naïve to Pt complexes. Long-term effects have been studied by creating resistant cell lines to all four agents in the SISO human cervical cancer cell line and comparing cross-resistance with each of the other three agents. We characterized each of the four resistant cell lines with respect to glutathione (GSH) content and the cellular activities of various redox enzymes. The expression of multidrug resistance protein 2 (MRP2) was analyzed in the cisplatin and oxoplatin-resistant cell lines. Finally, we performed microarray analysis with the SISO cell lines resistant to cisplatin and oxoplatin to compare transcription profiles of *ca*. 29 000 genes.

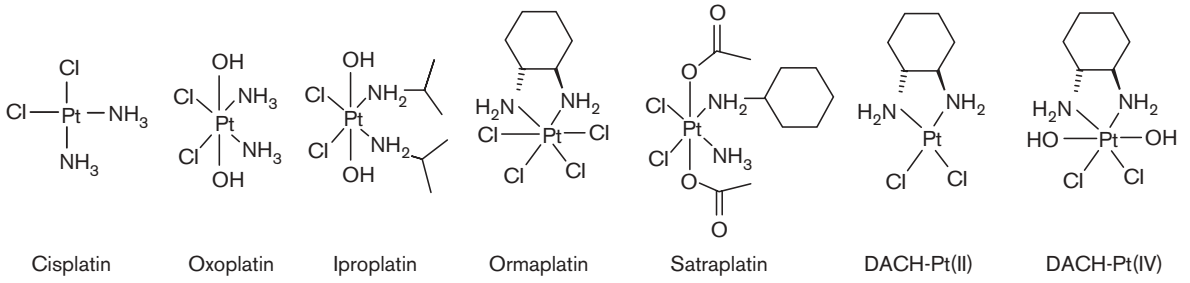
Methods

Materials

Cisplatin was from Chempur (Karlsruhe, Germany) and oxoplatin was a gift from the Riemser Arzneimittel AG (Greifswald-Riems, Germany). DACH-Pt(II) was synthesized as described earlier [18]. DACH-Pt(IV) was obtained by the reaction of 0.29 g (0.75 mmol) of DACH-Pt(II) with 3% hydrogen peroxide in 27.5 ml water at 70°C for 1 h in the dark. After allowing the reaction mixture to cool to room temperature for 24 h with constant stirring, the precipitate was collected by suction filtration, washed with deionized water and diethyl ether, and dried over P_2O_5 in vacuum to give 0.14 g (62%) of a light yellow powder. An elemental analysis gave the composition of carbon, nitrogen and hydrogen to within 0.4% of the expected values.

All reagents for biochemical and cell culture work were from Sigma-Aldrich (Taukirchen, Germany) except for 1-chloro-2,4-dinitrobenzene (Merck, Darmstadt, Germany), glutaraldehyde and sodium azide (Fluka, Buchs, Switzerland), 35% hydrogen peroxide (Baker, Deventer, Holland), and crystal violet (Kallies Feinchemie, Sebnitz, Germany). All reagents for the western blotting were from Roth (Karlsruhe, Germany) except for the antibodies, which were all from Sigma-Aldrich, and the film supplies (Kodak, Darmstadt, Germany). Diionized water was obtained from a Milli-Q-Water System (Millipore, Krefeld, Germany).

Fig. 1



Structures of platinum complexes.

Cell lines, culture conditions and development of resistance in the SISO cell line

All human cancer cell lines were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany) and have been described and referenced in a previous publication [19]. Briefly, the adherent cell lines originated from cervical (SISO), breast (MCF-7), bladder (RT-4, RT-112, 5637), pancreas (DAN-G, YAPC), lung (A-427, LCLC-103H) and oesophagus (KYSE-70, KYSE-510, KYSE-520) cancers. The suspension cell lines HL-60 and U-937 were human leukaemia and lymphoma cell lines, respectively. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/air in RPMI-1640 culture medium supplemented with 10% foetal calf serum and the antibiotics streptomycin and benzylpenicillin, except for the MCF-7 cell line, which was also supplemented with sodium pyruvate and MEM amino acids.

To obtain resistance in the SISO human cervical cancer cell line, treatment of cells began with a concentration of Pt complex half of that of the GI_{50} value (i.e. concentration resulting in 50% growth inhibition) of the respective Pt complex. The concentration was gradually raised over 3 months to one to two times the GI_{50} value for cisplatin/oxoplatin and over 6 months to *ca.* five to 10 times the GI_{50} value for DACH-Pt(II)/DACH-Pt(IV). Treatment with complex was weekly, whereby with the exception of oxoplatin, the medium containing the Pt complex was allowed to remain in contact with the cells for the whole week. In the case of oxoplatin, however, the medium had to be changed after 48 h and replaced with fresh, oxoplatin-free medium until the next treatment 5 days later. This was necessary because the cell line could not tolerate a week-long exposure to oxoplatin.

The level of resistance was determined by dividing the average GI_{50} value of the resistant cell line by the average GI_{50} value of the native SISO cell line to give the resistance factor (RF). GI_{50} values were averages of three to four independent experiments and were determined by the crystal violet method described in the section below.

Cytotoxicity testing

Cytotoxicity testing with the 12 adherent cell lines was carried out by an antiproliferation assay based on the staining of cells with crystal violet, as described earlier [19]. Testing for growth inhibition in the two suspension cell lines was done with the [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] (MTT) assay [19]. Stock solutions of cisplatin, DACH-Pt(II) and DACH-Pt(IV) in DMF (*N*,*N*-dimethylformamide) were diluted 1000 fold into 100 µl/well culture medium to reach the final working concentrations of the complex to be tested. In the case of oxoplatin, which was not completely soluble in DMF, a suspension was used, which was vigorously mixed before dilution in culture medium. Five serial dilutions were used to determine the dose–response relationship. In the case of the crystal violet assay, cells were seeded out (500–1000 cells/well in 100 µl) 24 h before testing and drug treatment took place for 96 h. In the case of the MTT assay, cells were seeded out (5000–10 000 cells/well in 100 µl) and the drug treatment time was 48 h. In the crystal violet staining assay, cell growth was stopped after 96 h by the addition of a 1% glutaraldehyde solution. The cells were later stained with a 0.02% crystal violet solution in water. Excess dye was washed out with water and cell bound dye was taken back into solution with a 70% ethanol/water mixture. The optical density of the crystal violet was determined at $\lambda = 570$ nm with an Anthos 2010 microtiter plate reader (Anthos, Salzburg, Austria) [19]. In the case of the MTT assay, a solution of the tetrazolium bromide in PBS was added to each well after the 48 h incubation to give a final MTT concentration of 300 µg/ml. The plates were returned to the incubator for 4 h to allow for the reduction of MTT to the formazan. Then, 100 µl of a 40 mmol/l HCl solution in isopropanol was added to each well followed by sonication to dissolve the dye. Optical density was read at $\lambda = 570$ nm. The GI_{50} values were calculated by linear regression analysis of the log dose–T/C curves as described in detail earlier [19]. Only concentrations that gave T/C values between 10 and 90% were used in the GI_{50} calculations.

In the case of the time dependency of exposure on the GI_{50} values, cells were exposed to Pt complex for either 24 or 96 h. For the 24-h exposure experiments, after this time the culture medium was carefully aspirated from the adherent cells and replaced with 200 µl fresh medium not containing any Pt complex. The cells were then incubated for an additional 72 h before stopping growth.

Determination of cellular redox status

Intracellular GSH concentrations were determined by a previously described method when the cells were *ca.* 90% confluent [20]. However, instead of lysing the adherent cells directly and counting nuclei as described in the original paper, a cell suspension was first obtained by trypsinisation of the monolayer. In the case of the HL-60 and U-937 cell lines, the cells were used directly. The cells were then counted directly using a Coulter Counter Z2 (Beckman Coulter, Krefeld, Germany), pelleted by centrifugation at 800*g* for 5 min and treated with a 1% Trition solution to obtain the lysate for the GSH determination.

Cellular catalytic concentrations of glutathione-*S*-transferase (GST) [21], glutathione peroxidase (GPx) [21], glutathione reductase (GR) [21], thioredoxin reductase [21] and catalase (CAT) [22] were determined with adherent cells that had reached 80–90% confluence as

described earlier. Suspension cells were harvested when they were in the late exponential growth phase.

Determination of MRP2 status of cisplatin-resistant and oxoplatin-resistant SISO cells

After reaching confluency in 75 cm^2 culture vessels, SISO cells were subjected to a brief trypsinisation and the cell suspension was centrifuged at 800*g* for 5 min. The cell pellet was resuspended in PBS and repelleted twice. The final cell pellet was stored at −20°C until western blotting was performed. The thawed cell pellets were resuspended in 100 µl PBS and through repeated (four to five times) freezing in liquid nitrogen and the thawed cells were lysed. The resulting cell lysate was centrifuged for 30 min at 100 000*g* at 4°C and the pellet was resuspended in 5 mmol/l TRIS buffer (pH 6.8). SDS electrophoresis was performed on 50 µg of cellular protein in 20 µl of TRIS buffer. Protein determination of the resuspended cell membranes was done using a Roti-Quant kit (Roth, Karlsruhe, Germany) with bovine serum albumin asstandard. Prestained marker proteins (Roti Mark prestained; Rot, Karlsruhe, Germany) were run parallel to the lanes with cell lysates. Western blotting and immunodetection for MRP2 were preformed as described earlier [21]. MDCK cells transformed to overexpress MRP2 [23] were used as positive controls.

Genome-wide expression analysis

Cells were expanded in the absence of Pt complex and harvested by trypsination. The cell pellets of approximately 30 × 10^6 cells were stored frozen at −80°C until they were lysed in 2.5 ml extraction buffer (4°C) consisting of 4 mol/l guanidine isothiocyanate, 0.5% sodium *N*-lauroylsarcosinate, 10 mmol/l EDTA, 5 mmol/l sodium citrate and 100 µmol/l β-mercaptoethanol. RNA of the guanidine isothiocyanate lysate was isolated by caesium trifluoroacetate ultracentrifugation (Amersham Biosciences, Little Chalfont, UK) and precipitated using ice-cold 96% ethanol at 4°C for 20 min. After two washes with 70% ethanol, pellets were dissolved in 50 µl autoclaved Millipore water and the RNA content was measured photometrically at $\lambda = 260$ nm (Hitachi, Tokyo, Japan, U 2000).

Gene expression analysis was carried out in duplicate with the Applied Biosystems Human Genome Survey Microarray V2.0 (Applied Biosystems, Foster City, California, USA). Therefore, 2–5 µg mRNA (20–50 µg total RNA) were reversely transcribed to first-strand cDNA and labeled with digoxigenin-dUTP according to the Applied Biosystems Chemiluminescent Reverse Transcription Labeling Kit Protocol (AB1700 RT; MyCycler, BioRad, Hercules, California, USA). For degradation of RNA, 2.5 mol/l NaOH was pipetted into each tube, followed by incubation in the thermocycler at 37°C for 15 min and immediate addition of 1 mol/l Tris-HCl (pH 7.0) to neutralize the reaction. cDNA was purified by centrifugation in a DNA purification column (Pico/Fresco – Heraeus, Hanau, Germany) and the eluate containing the cDNA product was stored at −80°C.

Hybridization of the cDNA and microarray evaluation was performed according to the Applied Biosystems Chemiluminescence Detection Kit Protocol. After blocking of the microarray, cDNA was added to the cartridge and hybridization performed at 100 rpm and 55°C for 16 h (Infors HT Minitron, Bottmingen, Switzerland). After washing, anti-digoxigenin–alkaline phosphatase was added (RT, 20 min), followed by chemiluminescence-enhancing solution and substrate before scanning was performed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer. Further processing of the data was accomplished by using Microsoft Excel software (Microsoft, Oberschleisheim, Germany). Genes showing weak expression in the native cell line (i.e. < 3000 relative pixel intensity) were not included in the analysis.

Statistical analysis

The delta method was used to normalize the GI_{50} data for correlation analysis [24]. For correlation analysis, the software program SPSS (SPSS Inc, Chicago, Illinois, USA) was used to determine the Pearson's correlation coefficient r of the paired GI_{50} delta values as described earlier [19]. Student's two-tailed t-test was used to establish significance.

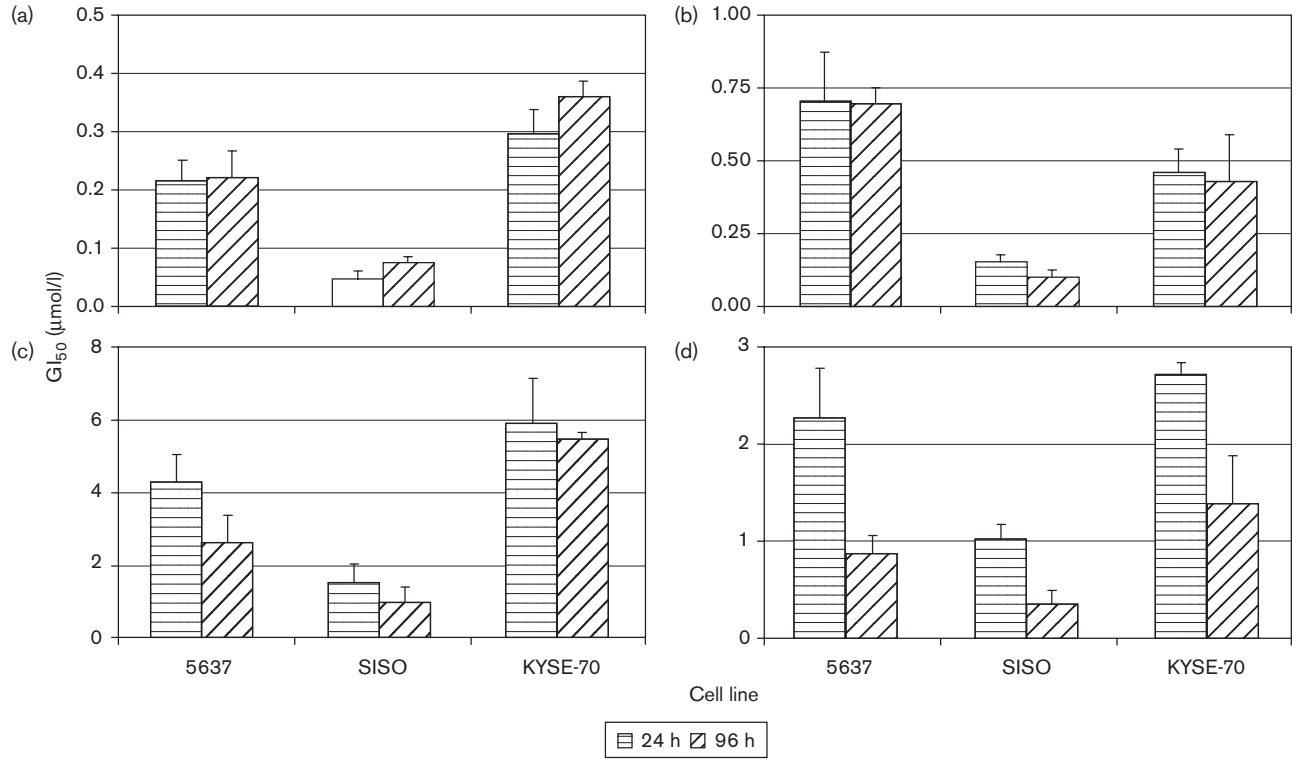
Results

Kinetics of cytotoxicty

If Pt(IV) complexes, such as oxoplatin, are prodrugs for their Pt(II) counterparts (i.e. cisplatin), then as the reduction reaction is a prerequisite for activation, it would be expected that the Pt(IV) complex will take a longer time to achieve maximal cytotoxic activity compared with the Pt(II) complex. Thus, we studied the cytotoxicity of cisplatin, oxoplatin, DACH-Pt(II) and DACH-Pt(IV) as a function of exposure time in three cancer cell lines. For these studies, a microtiter assay based on the staining of cells with crystal violet was used to measure the antiproliferative activity of the complexes [19]. Cells were exposed to the complexes for either 24 or 96 h. When exposure was only for 24 h, medium was removed and replaced with fresh culture medium, and the cells were incubated for an additional 72 h.

Figure 2 reports the GI_{50} values of the four Pt complexes in three cell lines, after either a 24 or 96-h exposure time. In the cases of the two Pt(II) complexes as well as oxoplatin, with one exception, no difference in the activities was observed. For cisplatin in the SISO cell line, a small but statistically significant decrease in potency was observed after 96 h. In contrast, a substantial increase in potency was observed for DACH-Pt(IV) in all three cell lines when the cells were exposed for 96 h as

Fig. 2



Effects of varying exposure times (24 and 96 h) on the potencies (average GI_{50} values ± SD) of the four platinum complexes in three cancer cell lines. (a) cisplatin, (b) DACH-Pt(II), (c) oxoplatin, (d) DACH-Pt(IV). *$P<0.02$, **$P<0.005$.

Table 1 **Average GI_{50} values (µmol/l) ± SD in 14 human cancer cell lines**

	HL-60	U-937	LCLC-103H	5637	A-427	RT-112	SISO	RT-4	DAN-G	YAPC	Kyse-70	Kyse-510	Kyse-520	MCF-7
Cisplatin	0.41 ± 0.08	0.81 ± 0.16	1.1 ± 0.4	0.37 ± 0.08	1.27 ± 0.25	1.22 ± 0.11	0.24 ± 0.05	1.77 ± 0.37	0.53 ± 0.07	4.09 ± 0.62	0.62 ± 0.12	0.44 ± 0.06	5.34 ± 1.04	1.38 ± 0.25
Oxoplatin	1.21 ± 0.30	3.57 ± 0.27	17.2 ± 5.1	4.51 ± 0.80	13.0 ± 2.7	12.6 ± 2.3	1.21 ± 0.20	26.3 ± 8.2	11.8 ± 3.2	20.6 ± 3.0	8.76 ± 1.38	3.06 ± 0.42	31.9 ± 10.8	5.52 ± 1.14
DACH-Pt(II)	0.23 ± 0.07	0.18 ± 0.06	0.49 ± 0.25	0.70 ± 0.22	0.84 ± 0.27	0.56 ± 0.21	0.11 ± 0.05	0.21 ± 0.04	0.27 ± 0.03	0.54 ± 0.35	0.32 ± 0.09	0.59 ± 0.31	1.34 ± 0.09	0.19 ± 0.05
DACH-Pt(IV)	2.76 ± 0.48	2.48 ± 0.25	1.85 ± 0.31	1.62 ± 0.14	2.25 ± 0.15	3.79 ± 0.36	0.96 ± 0.18	1.3 ± 0.2	2.87 ± 0.33	3.09 ± 0.89	1.87 ± 0.25	1.98 ± 0.22	2.59 ± 0.47	2.46 ± 0.23

Data are the results of three to nine independent experiments. The cell lines HL-60 and U-937 were exposed to Pt complex for 48 h, all other cell lines were exposed for 96 h. DACH-Pt(II), (*d,l-trans*-1,2-diaminocyclohexane)dichloroplatinum(II); DACH-Pt(IV), *cis,trans*-(*d,l-trans*-1,2-diaminocyclohexane)dichlorodihydroxoplatinum(IV).

opposed to the 24-h exposure, indicating that this complex requires a longer period of time to act compared with cisplatin, DACH-Pt(II) or oxoplatin.

Cytotoxicity and correlation analysis of cancer cell line sensitivities to Pt(II) and Pt(IV) drugs in 14 cancer cell lines

Table 1 shows the absolute GI_{50} values of the four Pt complexes in 14 cell lines. The best overall potency was found with DACH-Pt(II) followed by cisplatin, although cisplatin was more active in the 5637 cell line. Oxoplatin showed the lowest potency except in the HL-60 cell line, where DACH-Pt(IV) was least potent.

Prodrugs of anticancer drugs differ in the rates with which they attain activity and not in the spectrum of cytotoxic activity. For example, carboplatin, a Pt(II) prodrug for cisplatin, is approximately 10-fold less potent than cisplatin because of its slower activation; however, the spectrum of activity in 14 different cancer cell lines is

Table 2 Correlation analysis of intracellular GSH concentrations, various enyzme activities (GR, GST, GPx, TrxR) and cytotoxicities of four Pt complexes [CDDP, DACH-Pt(II), oxoplatin, DACH-Pt(IV)] in the 14 cell line panel

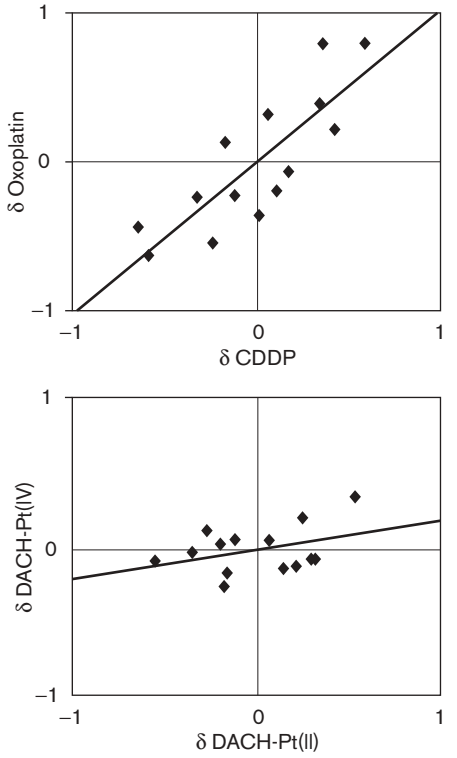
	GSH	GR	GST	GPx	TrxR	Cisplatin	DACH-Pt(II)	Oxoplatin	DACH-Pt(IV)
GSH	1.000								
GR	−0.050 (0.8657)	1.000							
GST	0.391 (0.1671)	0.027 (0.9277)	1.000						
GPx	0.235 (0.4193)	−0.451 (0.1058)	0.197 (0.5007)	1.000					
TrxR	0.159 (0.5882)	0.625 (0.0169)	0.507 (0.0644)	−0.241 (0.4062)	1.000				
Cisplatin	−0.045 (0.8799)	0.468 (0.0912)	0.367 (0.1967)	−0.362 (0.2028)	0.449 (0.1074)	1.000			
DACH-Pt(II)	−0.126 (0.6685)	0.077 (0.7942)	0.482 (0.0808)	−0.023 (0.9383)	0.519 (0.0570)	0.377 (0.1845)	1.000		
Oxoplatin	0.160 (0.5842)	0.100 (0.7344)	0.539 (0.0468)	−0.230 (0.4294)	0.474 (0.0868)	0.824 (0.0003)	0.556 (0.0389)	1.000	
DACH-Pt(IV)	−0.187 (0.5226)	0.268 (0.3545)	0.403 (0.1529)	−0.181 (0.5360)	0.162 (0.5791)	0.491 (0.0748)	0.398 (0.1584)	0.284 (0.3248)	1.000

Upper values represent Pearson's correlation coefficient, lower value in parenthesis represent level of significance. Levels below $P = 0.05$ are significant.
DACH-Pt(II), (*d,l-trans*-1,2-diaminocyclohexane)dichloroplatinum(II); DACH-Pt(IV), *cis,trans*-(*d,l-trans*-1,2-diaminocyclohexane)dichlorodihydroxoplatinum(IV); GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione-S-transferase; TrxR, thioredoxin reductase.

the same as for cisplatin [19]. We used the same delta method to compare the relative GI_{50} values in the same 14 cell lines to identify correlations between the spectrum of potencies. Table 2 reports the Pearson's correlation coefficient obtained by linear regression analysis of the log delta values for each pair of complexes in the 14 cell lines. Consistent with the idea that oxoplatin is a prodrug for cisplatin, oxoplatin showed a strong, highly significant correlation with the activity of cisplatin. Oxoplatin also showed a significant but much weaker correlation with DACH-Pt(II). Interestingly, DACH-Pt(II) showed no correlation with the activity profile of DACH-Pt(IV). As reported previously, cisplatin and DACH-Pt(II) also showed no correlation in their activity profiles [19].

Figure 3 shows the data of the 14 individual cell lines for the correlations of cisplatin versus oxoplatin and DACH-Pt(II) versus DACH-Pt(IV). As expected for a highly significant correlation between cisplatin and oxoplatin, the slope of the regression line is close to 1. In contrast, the nonsignificant correlation between DACH-Pt(II) and DACH-Pt(IV) gave a regression line with a slope of only 0.2, evidence that the complexes act by different mechanisms.

Fig. 3



Linear correlation analysis of the delta GI_{50} values of the four platinum complexes in 14 cancer cell lines. The correlation CDDP versus oxoplatin is significant while the correlation DACH-Pt(II) versus DACH-Pt(IV) is not.

Development of SISO cell lines resistant to cisplatin, oxoplatin, DACH-Pt(II) and DACH-Pt(IV)

If Pt(IV) complexes were merely prodrugs for their Pt(II) counterparts, then it was anticipated that because the

mechanism of cytotoxic action is the same, cells made resistant to either the Pt(II) or the Pt(IV) complex should show complete cross-resistance to each other. Thus, the cervical cancer cell line SISO was made resistant to each of the four Pt coordination complexes by exposure to increasing concentrations of drug for a period of 3 (oxoplatin and CDDP) or 6 months [DACH-Pt(II) and DACH-Pt(IV)]. The initial concentration was chosen to be half of the GI_{50} value in the native cell line (Table 1). Cells were exposed to fresh substance once every week. With the exception of oxoplatin, where exposure was limited to 48 h, the cells were allowed to stay in contact with Pt complex for 96 h, before the medium was changed. At the end of the week, the cells were passaged and 24 h later treated freshly with Pt complex. After reaching the final level of resistance, the cells where expanded for a week in drug-free medium and cryopreserved for future testing.

Table 3 compares the GI_{50} values of the four new resistant cell lines towards all four Pt complexes with the GI_{50} values of the native cell line, tested in parallel with the resistant cell lines. The RF is the quotient of the GI_{50} value in the resistant cell line to the GI_{50} of the native cell line.

The SISO-CDDP and SISO-OXO cell lines showed strong resistance to cisplatin and oxoplatin with RF of 4.03 and 5.03, respectively. Cross-resistance was moderate for cisplatin in the SISO-OXO and for oxoplatin in the SISO-CDDP cell lines (RF 2.95 and 2.40, respectively). Although cisplatin showed strong cross-resistance in the SISO-DACH-II cell line (RF 3.79), the level of resistance in the SISO-DACH-IV cell line was much less. Oxoplatin showed only weak cross-resistance (RF < 2) in both the SISO-DACH-II and SISO-DACH-IV cell lines.

Table 3 Resistant factors of resistant cells relative to native cell line

Drugs/cell line	SISO-CDDP	SISO-OXO	SISO-DACH-II	SISO-DACH-IV
Cisplatin	4.03	2.95	1.74	3.62
Oxoplatin	2.40	5.03	1.78	2.99
DACH-Pt(II)	3.79	1.64	5.85	8.31
DACH-Pt(IV)	2.00	1.82	3.03	5.97

Values are averages of three independent determinations for both the wild and resistant lines.
DACH-Pt(II), (d,l-trans-1,2-diaminocyclohexane)dichloroplatinum(II); DACH-Pt(IV), cis,trans-(d,l-trans-1,2-diaminocyclohexane)dichlorodihydroxoplatinum(IV).

The SISO-DACH-II and SISO-DACH-IV lines also showed strong resistance to DACH-Pt(II) and DACH-Pt(IV) with RF 5.85 and 5.97, respectively. Interestingly, DACH-Pt(II) was very strongly cross-resistant to DACH-Pt(IV) in the SISO-DACH-II cell line (RF 8.31), whereas DACH-Pt(IV) was only moderately cross-resistant to DACH-Pt(II) in the SISO-DACH-II cell line (RF 3.03). The SISO-DACH-IV cell line showed stronger cross-resistance to cisplatin and oxoplatin (RF 3.62 and 2.99, respectively) compared with the SISO-DACH-II cell line, which were weakly cross-resistant (RF 1.74 and 1.76, respectively).

With the exception of resistance of DACH-Pt(II) in the SISO-CDDP cell line, cells made resistant to either cisplatin or oxoplatin were less resistant to DACH-Pt(II) and DACH-Pt(IV), respectively. Cisplatin and oxoplatin showed less cross-resistance in the SISO-DACH-II cell line compared with the SISO-DACH-IV line.

Redox status of wild-type and resistant SISO cell lines

Acquired resistance to anticancer drugs, in particular Pt complexes, can come as a result of changes in the GSH system of the cancer cells [25,26]. Thus, we explored the changes in the intracellular concentrations of GSH as well as the cellular catalytic concentrations of various GSH-dependent enzymes as well as CAT in the resistant and native SISO cell lines.

Table 4 shows the intracellular levels of GSH and the catalytic concentration of GST, GR, GPx and CAT. Of these four parameters, only significant changes in the concentrations of GSH could be observed in three of the four cell lines; the concentrations of GSH approximately doubled in the SISO-CDDP, SISO-OXO and SISO-DACH-II cell lines compared with the native line. No significant increases in the cellular concentrations of GSH were observed in the SISO-DACH-IV cell line.

As reported in an earlier publication [19], no correlations between the GSH concentrations of the 14 cell lines and the GI_{50} values of the two Pt(II) complexes could be

Table 4 Intracellular GSH levels and enzyme catalytic concentrations of various redox enzymes in the SISO cell line and in the cell lines resistant to the four platinum complexes, respectively

Cell line	SISO	SISO-CDDP	SISO-OXO	SISO-DACH-II	SISO-DACH-IV
GSH (mmol/l) ($N=3$)	1.20 ± 0.23	2.72 ± 0.36[a]	2.14 ± 0.08[a]	2.34 ± 0.36[a]	1.47 ± 0.43
GST (IU/ml) ($N=3$)	11.4 ± 1.7	13.0 ± 3.1	16.2 ± 5.9	11.4 ± 3.3	12.6 ± 1.6
GR (IU/ml) ($N=4$)	1.5 ± 0.4	0.8 ± 0.2	1.3 ± 0.3	0.9 ± 0.2	1.0 ± 0.2
GPx (IU/ml) ($N=3$)	2.5 ± 1.0	2.2 ± 0.7	2.1 ± 0.1	2.2 ± 0.3	2.1 ± 0.3
CAT (IU/ml) ($N=3$)	0.6 ± 0.3	0.9 ± 0.5	0.6 ± 0.2	0.5 ± 0.3	0.6 ± 0.3

CAT, catalase; GR, glutathione reductase; GSH, glutathione; GST, glutathione-*S*-transferase; GPx, glutathione peroxidase.
[a]Statistically significant compared with native line ($P<0.05$).

found (Table 2). Likewise, no correlations between GSH concentrations and the potency of the two Pt(IV) complexes were apparent either (Table 2). No correlations were found between the catalytic activities of GR, GST, GPx and thioredoxin reductase and the GI_{50} values of the two Pt(II) complexes, as reported earlier [21]. With one exception, there were no correlations between the activities of these enzymes and the potencies of the Pt(IV) complexes. Only in the case of GST, a statistically significant positive correlation was observed between the catalytic concentration of GST and the potency of oxoplatin in 14 cancer cell lines, indicating that increasing activities of GST lead to decreasing potency of oxoplatin in our 14 cell line panel.

MRP2 status of cisplatin-resistant and oxoplatin-resistant SISO cell lines

Another resistance pathway dependent on GSH is the transport of anticancer drugs out of the cell by various ABC transporters (MRP). For cisplatin, the overexpression of MRP2 transporter has been linked as a cause for resistance in a number of cases [23,27,28]. The expression of the MRP2 protein was investigated by the western blot method in the native SISO cells as well as in the cisplatin and oxoplatin-resistant cell lines. As shown in Fig. 4, the native cell line gave no visible expression of the protein, whereas both SISO-CDDP and SISO-OXO showed noticeable expression of the protein, although not as strong as with the MRP2 positive control line MDCK.

Gene expression in native compared with resistant SISO cell lines

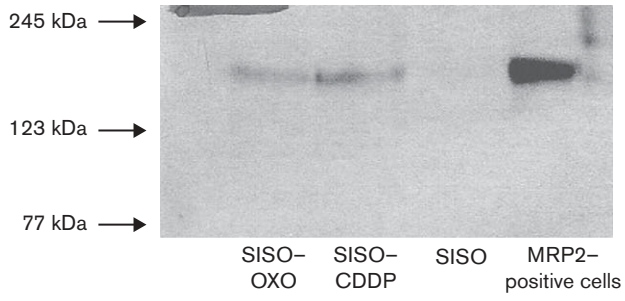
DNA-microarray methods was used to interrogate the transcription of *ca.* 29 000 genes in the native SISO, SISO-CDDP and SISO-OXO cell lines. Transcription of the 30 most highly upregulated and downregulated genes in the two resistant cell lines relative to the native one is shown in Table 5. Changes in gene expression were much stronger in the oxoplatin-resistant cell line compared with the cisplatin-resistant one. Surprisingly, the quality of overall changes in gene transcription was starkly different between the cisplatin and oxoplatin-resistant cell lines. Of the genes that changed their expression by more than three fold, only the genes NOD3 (21.3-fold and 4.3-fold in SISO-CDDP and SISO-OXO, respectively) and SLC1A3 (3.7-fold and 3.9-fold, respectively) were upregulated and only the genes PCDHB8 (142-fold and 250-fold, respectively) and TCN2 (83-fold and 250-fold, respectively) were downregulated in the resistant lines.

Only a few of the top 30 genes in either the upregulated or the downregulated group seemed to be a direct cause of the classical Pt resistance. In the SISO-CDDP cell line, the downregulation of the CDK2 gene by more than 10 fold as well as the upregulation of the oncogene intersectin 2 and heat-shock protein crystallin βB may be important for cisplatin resistance [29–31]. The SISO-CDDP cell line also showed an increased expression of several histone 1 genes (i.e. H2bk, H2bg, H2bc), which may also be important because histone H1 binds tightly to cisplatin-damaged DNA [32]. However, histone 1 H2ag gene expression was depressed in the SISO-OXO cell line. Likewise, cisplatin brought about an eight-fold decrease in the Na^+/K^+-ATPase α1 subunit gene expression, but oxoplatin-resistant cells showed a 27-fold increase in the transcription of the gene for the Na^+/K^+-ATPase β2 subunit. Overexpression of both the α and β subunits of Na^+/K^+-ATPase has recently been associated with cisplatin resistance [33].

The expression of genes affecting GSH metabolism was closely examined, because changes in the GSH levels of both cisplatin-resistant and oxoplatin-resistant cell lines were observed. No changes were observed in either cell lines in the mRNA levels of γ-glutamylcysteine synthetase, the rate-limiting enzyme in the synthesis of GSH [25]. Interestingly, GSH peroxidase 4 transcription was strongly upregulated in the oxoplatin-resistant line but strongly downregulated in the cisplatin line. The expression of most of the other GPxs (i.e. GPX1, 2, 5, 6, 7) was unchanged relative to the native line, but GPX3, a very weakly expressed gene not officially included in the analysis, decreased six-fold in both the lines. As GPX1 has significantly higher levels of expression, our observation that the total GPx enzyme activity in the resistant cell lines remains unchanged relative to the wild type is consistent with the microarray analysis.

The unchanged expression of the CAT and GR genes was also consistent with our enzyme data. Three GST genes did show decreases in expression between the cisplatin-resistant and native cell lines (i.e. GSTT2, GSTM1/M2, GSTM4), but the three GSTs with the highest expression, GSTT1–T3, were unchanged in

Fig. 4



Western blot analysis of MRP2 expression in SISO, SISO-OXO and SISO-CDDP cell lines compared with the expression of MRP2 in transfected MDCK cells.

Table 5 Array data showing 30 genes that changed their transcription levels the most in SISO-CDDP (above) and SISO-OXO (below) cell lines relative to the native SISO cell line

SISO-CDDP upregulated genes			SISO-CDDP downregulated genes		
Gene	Fold change	Protein	Gene	Fold change	Protein
COL8A1	58.8	Collagen, type VIII, α1	*PCDHB8*[b]	0.007	Protocadherin β8
GFR	21.4	Guanine nucleotide exchange factor for Rap1	*TCN2*[b]	0.012	Transcobalamin II; macrocytic anemia
NOD3[a]	21.3	NOD3 protein	*KRT17*	0.012	Keratin 17
CLECSF2	18.9	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 2 (activation-induced)	*GAPD*	0.016	Glyceraldehyde-3-phosphate dehydrogenase
LAMA1	14.4	Laminin, α1	*UBE2S*	0.022	Ubiquitin-conjugating enzyme E2S
MGEA6	14.0	Meningioma expressed antigen 6 (coiled-coil proline-rich)	*CFL1*	0.052	Cofilin 1 (non-muscle)
SPTBN4	10.3	Spectrin, β, non-erythrocytic 4	*KRT14|KRT19*	0.054	Keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)|keratin 19
CPXM	9.2	Carboxypeptidase X (M14 family)	*MDH2*	0.056	Malate dehydrogenase 2, NAD (mitochondrial)
CRYAB	8.7	Crystallin, α B	*RPS2*	0.067	Ribosomal protein S2
CLCN5	8.5	Chloride channel 5 (nephrolithiasis 2, X-linked, Dent disease)	*DAG1*	0.070	Dystroglycan 1 (dystrophin-associated glycoprotein 1)
RARRES1	7.4	Retinoic acid receptor responder (tazarotene induced) 1	*CDK2*	0.072	Cyclin-dependent kinase 2
PLSCR4	7.3	Phospholipid scramblase 4	*PRDX2*	0.078	Peroxiredoxin 2
GDF15	7.1	Growth differentiation factor 15	*ABCF2*	0.080	ATP-binding cassette, subfamily F, member 2
TCN1	6.0	Transcobalamin I (vitamin B12 binding protein, R binder family)	*CALR*	0.082	Calreticulin
PTPRO	5.9	Protein tyrosine phosphatase, receptor type, O	*ILF3*	0.085	Interleukin enhancer binding factor 3, 90 kDa
CENTB5	5.5	Centaurin, β5	*CD81*	0.095	CD81 antigen (target of antiproliferative antibody 1)
VDR	5.0	Vitamin D (1,25- dihydroxyvitamin D3) receptor	*GPX4*[c]	0.095	Glutathione peroxidase 4 (phospholipid hydroperoxidase)
CASP1	4.3	Caspase 1, apoptosis-related cysteine protease (interleukin 1, β, convertase)	*XPO1*	0.097	Exportin 1 (CRM1 homolog, yeast)
ITSN2	4.0	Intersectin 2	*CRTAP*	0.099	Cartilage associated protein
HIST1H2BK	3.9	Histone 1, H2bk	*FLNA*	0.101	Filamin A, α (actin binding protein 280)
COP	3.9	CARD only protein	*TUBA6*	0.102	Tubulin α6
DHRS3	3.8	dehydrogenase/reductase (SDR family) member 3	*TPM3*	0.103	Tropomyosin 3
SLC1A3[a]	3.7	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	*ITGA2*	0.105	Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor)
FRS2	3.7	Fibroblast growth factor receptor substrate 2	*CEACAM8*	0.108	Carcinoembryonic antigen-related cell adhesion molecule 8
HIST1H2BG	3.5	Histone 1, H2bg	*SLC16A6*	0.113	Solute carrier family 16 (monocarboxylic acid transporters), member 6
CNTNAP1	3.4	Contactin associated protein 1	*SMAP-5*	0.115	Golgi membrane protein SB140
DPYD	3.3	Dihydropyrimidine dehydrogenase	*IER2*	0.118	Immediate early response 2
HIST1H2AC	3.2	Histone 1, H2ac	*FLJ43855|SLC6A8*	0.120	Similar to sodium- and chloride-dependent creatine transporter|solute carrier family 6 (neurotransmitter transporter, creatine), member 8
ABCG2	3.1	ATP-binding cassette, sub-family G (WHITE), member 2	*SLC3A2*	0.122	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
ITGA2B	3.1	Integrin, α2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41B)	*ATP1A1*	0.122	ATPase, Na^+/K^+ transporting, α1 polypeptide

Table 5 ***(continued)***

SISO-OXO upregulated genes			SSISO-OXO downregulated genes		
Gene	Fold change	Protein	Gene	Fold change	Protein
ESM1	301.0	Endothelial cell-specific molecule 1	*PCDHB8*[b]	0.004	Protocadherin β8
SLC35B1	227.2	Solute carrier family 35, member B1; UDP-galactose transporter-related	*SAA1*	0.008	Serum amyloid A1
SLC16A9	199.9	Solute carrier family 16 (monocarboxylic acid transporters), member A9	*TCN2*[b]	0.009	Transcobalamin II; macrocytic anemia
TLX1	64.6	T-cell leukaemia, homeobox 1	*CHST4*	0.024	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4
AQP4	61.0	Aquaporin 4	*IL1A*	0.048	Interleukin 1, α
ARHN	44.0	Ras homolog gene family, member N; GTP-binding protein, rho7	*TNF*	0.049	Tumour necrosis factor (TNF superfamily, member 2)
FGG	36.3	Fibrinogen, γ polypeptide	*FGF12*	0.058	Fibroblast growth factor 12
PHGDHL1	31.8	Phosphoglycerate dehydrogenase like 1	*PI3*	0.078	Protease inhibitor 3, skin-derived (SKALP)
PYGM	30.5	Glycogen phosphorylase	*COL16A1*	0.079	Collagen, type XVI, α1
FGA	29.6	Fibrinogen, A α polypeptide	*RAB3A*	0.095	RAB3A, member RAS oncogene family
ATP1B2	27.4	ATPase, Na+/K+ transporting, β-2 polypeptide	*PTGS2*	0.104	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
LAMC2	22.5	Laminin, γ2	*CCL20*	0.112	Chemokine (C-C motif) ligand 20
ADAMTS16	17.9	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 16	*S100A5*	0.120	S100 calcium binding protein A5
FGB	17.6	Fibrinogen, B β polypeptide	*PLA2G4A*	0.133	Phospholipase A2, group IVA (cytosolic, calcium-dependent)
FGFR2	16.3	Fibroblast growth factor Receptor 2	*ABCC4*	0.140	ATP-binding cassette, sub-family C, member 4
TUBA3	13.9	Tubulin, α3	*HIST1H2AG*	0.144	Histone 1, H2ag
EMILIN1	12.6	Elastin microfibril interfacer 1	*S100A9*	0.146	S100 calcium binding protein A9 (calgranulin B)
RTN2	12.1	Reticulon 2	*CXCL1*	0.160	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α)
LCN1	11.0	Lipocalin 1 (tear prealbumin)	*S100A4*	0.168	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)
TENS1	10.4	Tensin-like SH2 domain-containing 1	*SERPINB3*	0.168	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3
PHKA2	9.9	Phosphorylase kinase, α2 (liver)	*SERPINB4*	0.171	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 4
KRT13	9.8	Keratin 13	*MAP17*	0.189	Membrane-associated protein 17
FCN3	9.4	Ficolin (collagen/fibrinogen domain containing) 3 (Hakata antigen)	*SCN10A*	0.194	Sodium channel, voltage-gated, type X, α
GPX4[c]	9.2	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	*CXCL2*	0.204	Chemokine (C-X-C motif) ligand 2
SLC9A1	8.8	Solute carrier family 9 (Na+/H+ exchanger), isoform 1	*GTSE1*	0.209	G-2 and S-phase expressed 1
SCIN	8.6	Scinderin	*TNFRSF13B*	0.222	Tumour necrosis factor receptor superfamily, member 13B
PRKCG	8.3	Protein kinase C, γ	*CXCL3*	0.224	Chemokine (C-X-C motif) ligand 3
ATP6V1C2	8.0	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C isoform 2	*LCN2*	0.245	lipocalin 2 (oncogene 24p3)
PDCD1	7.6	Programmed cell death 1	*C1QTNF1*	0.254	C1q and tumour necrosis factor related protein 1
PGDS	7.6	Prostaglandin D2 synthase, haematopoietic	*PDK4*	0.258	pyruvate dehydrogenase kinase, isoenzyme 4

Relative differences in transcription decrease from top to bottom. Values are averages from two analyses.
[a]Upregulated in both cell lines.
[b]Downregulated in both cell lines.
[c]Upregulated in one cell line and downregulated in the other.

both resistant cell lines, consistent with our enzyme activity data.

Microarray analysis showed that the transporter ABCC2 (MRP2) transcription was unchanged in both resistant cell lines, which is inconsistent with our observation that protein expression was strongly elevated in the SISO-CDDP and SISO-OXO cell lines relative to the wild type. Thus, increases in ABCC2 protein levels are likely to be a result of increased translation or an increase in the

stability of the ABCC2 protein. However, gene expression of other ABC transporters changed by more than three fold. ABCG2 expression increased in the SISO-CDDP cell line but was unchanged in the SISO-OXO cell line, whereas ABCF2 and ABCC4 decreased in the SISO-CDDP and SISO-OXO cell lines, respectively (Table 5).

In general, genes coding for extracellular matrix proteins were affected in both resistant cell lines. The overexpression of the collagen VIII α1 gene could also play a role in cisplatin resistance; overexpression of collagen VI α3 has been attributed to cisplatin [34] and oxaliplatin [35] resistance in ovarian cell lines. Interestingly, the expression of the collagen VIII gene was not changed in the SISI-OXO cell line. In contrast, collagen type XVI was downregulated in the SISO-OXO cell line but was unchanged in the SISO-CDDP cell line. In the SISO-OXO cell line, several fibrinogen genes were upregulated. We observed downregulation of the integrin α-2 gene in the SISO-CDDP cell line; others have reported strong downregulation of the integrin β-1 gene in a cisplatin-resistant ovarian cancer line [34] and strong upregulation of integrin α-1 in an oxaliplatin-resistant cell line [35].

Discussion

Pt(IV) analogues of anticancer Pt(II) complexes are commonly thought to act as prodrugs that are biologically reduced to the active Pt(II) form. Indeed, there is evidence that Pt(IV) complexes can be reduced under biological conditions to the Pt(II) species [10–14]. Thus, Pt(IV) complexes should have the similar short-term and long-term biological effects on cancer cells as their Pt(II) counterparts, differing only in their pharmacokinetics and toxicity profiles. Our studies were designed to compare and contrast short-term and long-term in-vitro biological effects of two pairs of Pt(II)/Pt(IV) complexes to test this idea.

In general, the average antiproliferative potencies of the four Pt complexes over all 14 cell lines decreased in the following order: DACH-Pt(II) > cisplatin > DACH-Pt(IV) > oxoplatin, whereby oxoplatin was typically five- to 10-fold less potent than cisplatin. A very good correlation was found between the cytotoxicity profiles of cisplatin and oxoplatin in the panel of cisplatin-naïve cell lines, indicating that during short-term (i.e. single) exposure to either agent, similar growth inhibitory effects occur. Nonetheless, in a recent study with the H526 small-cell lung cancer cell line exposed to either cisplatin or oxoplatin for short-term (3 days), we found very different gene expression profiles, as determined by the same DNA array method used in this work [36]. Of the 55 genes upregulated in oxoplatin-treated H526 cells, only three were in common with the cisplatin-treated ones. Of the 302 downregulated genes, only 25% were in common. Thus, although the cytotoxicity profiles of cisplatin and oxoplatin may be very similar, these drugs can bring about different changes in cell physiology not picked up in an antiproliferative assay.

For the second pair of Pt(II)/Pt(IV) complexes with DACH ligands, no correlation between GI_{50} values was apparent, suggesting that other short-term mechanisms of growth inhibition may be in place for DACH-Pt(IV). This idea was strengthened by the observation that cancer cells require a longer exposure time to DACH-Pt(IV) (i.e. > 24 h) than DACH-Pt(II).

To study long-term effects, we chose to make resistant lines with a human cervical cancer cell line, SISO, because cisplatin is one of the most effective drugs for this type of cancer although response rates to single-agent chemotherapy are seldom greater than 40% [37]. Resistant SISO lines to each of the four complexes, generated over 3–6 months of weekly exposure to drug, were partially cross-resistant between the pairs of Pt(II)/Pt(IV) complexes. Nevertheless, the degree of resistance was weaker when cisplatin was tested in the line made resistant to oxoplatin and vice versa. In the case of the complexes with the DACH ligands, the Pt(IV) complex also showed weaker resistance in the SISO-DACH-II cell line; however, the SISO-DACH-IV cell line was even more strongly resistant to DACH-Pt(II) than to DACH-Pt(IV). This would seem to indicate that cancer cells become resistant to DACH-Pt(IV) in part by other mechanisms as to DACH-Pt(II).

Our data seemingly indicate that elevated levels of cellular GSH may be important in resistance to cisplatin, oxoplatin and DACH-Pt(II) in the SISO cell line but not to DACH-Pt(IV). However, the catalytic concentrations of key GSH-dependent enzymes (i.e. GST, GR, GPx) and CAT were unchanged in all resistant cell lines compared with the native SISO cell line.

The molecular basis for the resistance of SISO-CDDP and SISO-OXO was investigated in more detail. Overexpression of the ABC membrane transporter protein MRP2 (ABCC2, cMOAT) has been associated with cisplatin resistance [23,38–41]; in fact, this is the only MRP transporter that has been consistently linked with cisplatin resistance [25]. MRP2 could not be detected in the parent SISO cell line by western blotting but appeared equally elevated in the SISO-CDDP and SISO-OXO cell lines, which further evidence that the mechanisms of acquired resistance to cisplatin and oxoplatin may be similar. MRP2 and GSH are closely coupled in cell physiology [42]; MRP2 cotransports substrates with unconjugated GSH, and GSH conjugates of drugs (i.e. cisplatin) are also transported. Thus, the combined elevation of GSH and MRP2 might act

synergistically to decrease the accumulation of cisplatin and oxoplatin in the resistant cells. Future work will aim at measuring the Pt levels in resistant compared with the native cell lines.

Gene transcription analysis of the cisplatin and oxoplatin-resistant cell lines relative to the native SISO line was performed by DNA microarray methods to assess the long-term changes that had taken place in the cells during the 3-month exposure to drug. In this analysis, *ca.* 29 000 genes were interrogated. The SISO-OXO cell line showed greater relative changes in gene transcription compared with the SISO-CDDP cell line (Table 5). The qualitative results of these analyses were unexpected because the profiles of upregulated and downregulated genes were very different for the SISO-CDDP and SISO-OXO cell lines. Only two genes, PCDHB8 and TCN2, were strongly downregulated (> three-fold) in both the SISO-CDDP and SISO-OXO cell lines and two common genes, NOD3 and SLC1A3, were strongly upregulated (> three-fold).

The development of resistance to cisplatin is a multifactoral process and one might have expected changes in the transcription of genes for DNA repair enzymes, high-mobility group proteins, metallothioneines, GSH-dependent enzymes, proteins affecting apoptosis and cell division, oncoproteins and membrane transporters. [25,26,43,44] In only a few cases were the 30 strongest upregulated and downregulated genes associated with the classical resistance to Pt complexes. In the cisplatin-resistant cell line, the downregulation of the CDK2 gene might favour mitosis [29], and upregulation of the oncogenes intersectin 2 and crystalline αB have been associated previously with tumour resistance [30,31].

The profiles of ABC transporters indicated some changes in transcription. In the SISO-CDDP cell line, transcription of the ABCG2 transporter was upregulated by three fold, but the ABCF2 transporter was greatly downregulated in the same cell line. However, there is no evidence that these two transporters are linked to cisplatin resistance. Interestingly, in neither the SISO-CDDP nor the SISO-OXO cell line was the transcription of the ABCC2 (MRP2) gene greatly different from the native cell line, although we observed increased translation of the gene by western blotting in both the resistant lines. This gives rise to the idea that cisplatin resistance may not always be directly related to changes in the transcription of resistance-associated genes but may also affect the stability of resistance-associated proteins. Zhang and coworkers [45] reported no changes in ABCB2 and ABCC2 (MRP2) gene expression in a microarray analysis for a cisplatin-resistant oral squamous cancer cell line, although the cells showed reduced accumulation of Pt. Western blotting did not show changes in the levels of ABCB2 but the authors unfortunately did not check the expression of ABCC2 protein.

In several cases that could be relevant for resistance, gene transcription showed the opposite changes between the SISO-CDDP and SISO-OXO cell lines. For example, several genes for histone 1H were upregulated in the SISO-CDDP cell line but the histone 1 H2ag gene was downregulated in the SISO-OXO cell line [32]. Likewise, the gene for the N^+/K^+-ATPase α subunit was downregulated in the SISO-CDDP cell line but the gene for the β subunit was strongly upregulated in the SISO-OXO cell line [33]. GPX4 was strongly upregulated in the SISO-OXO cell line but strongly downregulated in the SISO-CDDP cell line. GPX3 has recently been associated with resistance to cisplatin in human ovarian cancer cell lines [46], but we saw downregulation of this weakly expressed gene in both cell lines. These changes in the expression of GPX3 and GPX4 had no effect on the overall GPx enzyme activity in the cells (Table 3), probably because the transcription levels of the main GPx, GPX1, were unchanged in the two resistant cell lines compared with the native one.

Our findings of gene expression profiling in the SISO cell line are consistent with what others have observed in cisplatin-resistant cell lines, that is, that many of the differentially expressed genes are not directly related to the classical mechanisms of cisplatin resistance [44]. For example, Whiteside and coworkers [47] followed gene expression in two human lung cancer cell lines over middle-term exposure (i.e. four weekly exposures) to cisplatin, which resulted in low-level resistance (*ca.* two fold). The expression of none of the genes that changed by more than two-fold during this early exposure period were related to classical cisplatin resistance. Johnsson and coworkers [48] studied mRNA expression in the human head and neck cancer cell line by making it six-fold resistant to cisplatin and found the differential expression of 67 genes, most of which were not previously linked with cisplatin resistance. Extensive microarray profiling of gene expression in six pairs of human ovarian cancer cell lines made resistant to cisplatin *in vitro* identified only one gene, metallothionein 2A, previously implicated in cisplatin resistance [49]. However, several genes involved in cell surface interactions and trafficking were differentially expressed. Still, others observed no dramatic changes in genes responsible for classical cisplatin resistance in cisplatin-resistant cell lines, but did observe changes in the expression of genes related to the extracellular matrix [34]. We observed similar effects that the transcription of extracellular matrix proteins in both SISO-CDDP (i.e. collagen type VIII, laminin α1, integrin α2b, integrin α2, protocadherin β8) and SISO-OXO cell lines (i.e. fibrinogen, laminin γ2, emilin1, protocadherin β8, collagen type XVI) were changed relative to the native cell line.

In conclusion, cisplatin and oxoplatin showed very similar cytotoxic responses in 14 human cancer cell lines in short-term exposure, and high levels of cross-resistance to each other in the resistant SISO cell line were observed after long-term exposure. In contrast, not all the cytotoxic activities of DACH-Pt(IV) can be attributed to DACH-Pt(II), as evidenced by the correlation analysis in the 14 cell lines. In both cisplatin and oxoplatin-resistant SISO cell lines, MRP2 and GSH levels were elevated, which is evidence for a similar mechanism of resistance and in keeping with the idea that oxoplatin is a prodrug for cisplatin. However, the gene expression profiles of SISO cell lines resistant to either cisplatin or oxoplatin are not at all similar. This observation illustrates that complex biological effects can occur with cells over long-term drug exposure, many of which may be adaptive processes unrelated to resistance, and may even mask the actual causes of resistance. Thus, Pt(IV) complexes can distinguish themselves biologically from their Pt(II) counterparts, even when the complexes show cross-resistance. Whether the effects are a direct result of the Pt(IV) complex or the result of a unique biotransformation product is unclear.

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