# Reduced drug accumulation is more important in acquired resistance against oxaliplatin than against cisplatin in isogenic colon cancer cells

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**Preclinical studies have indicated that there is only partial cross-resistance between cisplatin and oxaliplatin. The molecular background for this is incompletely known. To investigate the differences in resistance, we rendered a colon cancer cell line (S1) resistant against cisplatin and oxaliplatin and characterized the subclones with regard to cross-resistance, platinum uptake, and gene expression profiles. Four oxaliplatin and four cisplatin-resistant cell lines were produced from S1 by step-wise increasing the concentrations of the drugs in the growth medium. Cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and platinum accumulation in cell lysates and DNA preparations by inductively coupled plasma mass spectroscopy. Gene expression was investigated by cDNA microarrays. The protein expression of the ATP-binding cassette B1 (ABCB1) was measured by immunohistochemistry. The cisplatin-resistant cell lines were 1.5–6.2-fold resistant against cisplatin and the oxaliplatin-resistant sublines 2.6–17-fold resistant against oxaliplatin. There was a limited degree of cross-resistance. Oxaliplatin resistance could be explained to a larger degree by reduced drug accumulation whereas mechanisms for increased tolerance against platinum incorporation in DNA seemed to be of higher importance for resistance against cisplatin. A greater number of ABC transporters were upregulated in the oxaliplatin-resistant cell lines compared with those selected for cisplatin resistance. ABCB1 was highly overexpressed in the three most oxaliplatin-resistant sublines, but significantly underexpressed in the two most cisplatin-resistant cell lines. This was also confirmed by immunohistochemistry. However, functional tests did not show any increase in ABCB1 transport activity in the oxaliplatin-resistant sub-lines compared with S1.**
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## Introduction

Platinum (Pt)-based chemotherapeutic compounds for the treatment of malignant diseases have been routinely used since the early 1980s, when cisplatin was introduced. Cisplatin is still frequently used for the treatment of, for example, testicular, head and neck, bladder, and anal cancer. The clinical use of cisplatin is hampered by extensive side effects, such as nephrotoxicity, neurotoxicity, and nausea/vomiting and cellular resistance, which can be inherent or acquired during treatment. In the following decades much effort was put into developing new Pt-based agents with improved therapeutic index. The first clinically useful follow-up substance was carboplatin, which is less toxic but shows complete cross-resistance with cisplatin. In the late 1990s oxaliplatin was introduced. Oxaliplatin has a favorable toxicity profile compared with cisplatin, with polyneuropathy as a dose-limiting side effect. Oxaliplatin has also been shown to have a different activity profile, with good efficacy in, for example, different gastrointestinal cancers. Preclinical studies have shown only partial cross-resistance between cisplatin and oxaliplatin [1]. The mechanisms of action of Pt drugs are thought to be mediated by the formation of DNA adducts, which leads to hampered DNA replication and RNA transcription, and finally apoptosis [2–4].

Resistance against these compounds has been extensively studied and several factors have been associated with both cisplatin and oxaliplatin resistance, including decreased Pt accumulation, intracellular detoxification by, for example, glutathiones and metallothioneins, and increased DNA damage repair [2,3,5–7].

For the prediction of sensitivity and resistance against cisplatin and oxaliplatin a number of markers have been suggested. Several clinical studies have indicated a correlation between the expression of DNA repair proteins, such as the excision repair gene *ERCC1*, and survival after oxaliplatin or cisplatin treatment [5,8,9].

Thus, there are similarities in the basic mechanisms of action as well as of resistance between cisplatin and oxaliplatin, but given the absence of cross-resistance, it

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seems fair to assume that the mechanisms of resistance differ. However, the molecular background for this is incompletely known. To increase our knowledge on this issue we have rendered a colon cancer cell line (S1) stably resistant against cisplatin and oxaliplatin in a step-wise manner and characterized the resistant subclones with regard to cross-resistance, Pt uptake and gene expression profiles.

## Methods

### Reagents

Cisplatin was obtained from Pharmalink AB (Upplands-Väsby, Sweden), and oxaliplatin (Eloxatin) from Sanofi-Synthelabo (Bromma, Sweden). Elacridar was purchased from Sequoia Research Products Ltd (Pangbourne, UK) and paclitaxel from Sigma-Aldrich (St Louis, Missouri, USA).

### Cell line and growth conditions

A human colon cancer cell line, S1 (derived from the S1 clone of LS-174T colon carcinoma cells [10]) was used. Both the parental cell line and the resistant derivatives were maintained in logarithmic growth as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal calf serum and antibiotic (Invitrogen) (100 units/ml penicillin and 0.1 mg/ml streptomycin), under a humidified 5% $CO_2$ atmosphere at 37°C.

### Development of resistant cell lines

Oxaliplatin or cisplatin-resistant S1 derivatives were developed by serial passage of S1 cells in the presence of step-wise increasing concentrations of oxaliplatin or cisplatin. The initial drug concentration corresponded to approximately 10% of the inhibitory concentration ($IC_{10}$) of the parental S1 cell line. The cells were sub-cultured at 80% confluence. The sub-cultivated cells were allowed to recover for 24 h before the drug was added. This process was repeated and each cell line was exposed at least three times to the same drug concentration. Roughly, each cell line was exposed to one drug concentration during 1 month before the concentration was raised. Before raising the drug concentration, an appropriate amount of cells was frozen and stored in liquid nitrogen. The concentrations of oxaliplatin and cisplatin were 2, 4, 5, and 6 μmol/l yielding the oxaliplatin-resistant cell lines S1-oxa2, S1-oxa4, S1-oxa5, and S1-oxa6 and cisplatin-resistant cell lines S1-cis2, S1-cis4, S1-cis5, and S1-cis6, respectively, where the name of the cell lines indicates the highest drug concentration that they were exposed to. The S1 cells were also serially passaged as an untreated control. Measurements of drug resistance, and all further experiments with the resistant cell lines, were performed after the cells had been allowed to grow in a drug-free medium for 2 weeks.

### Determination of resistance and cross-resistance levels

Cell survival was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see below). In brief, exponentially growing cells were seeded in a 100 μl medium in 96-well plates (1250–1500 cells per well). After 48 h, cisplatin or oxaliplatin was added in DMEM without fetal calf serum and the cells were incubated for 1 h (cisplatin) or 2 h (oxaliplatin) at concentrations ranging from 0 to 128 μmol/l. The incubation times were chosen based on the infusion times usually applied when treating patients with these drugs. Then, the drug-containing medium was removed, and cells were allowed to grow in a drug-free medium for 5 days before the cell survival was determined. The resulting data were fitted to a sigmoidal curve with variable slope using nonlinear regression and the concentration at which the number of metabolically active cells was decreased to 50% ($IC_{50}$) was determined by using the GraphPad Prism 5.01 program (GraphPad Software Inc., LaJolla, California, USA). The resistance factor (R) was calculated as the ratio between the $IC_{50}$ value of the resistant and the parental cell line:

$$R = \frac{IC_{50_{\text{resistant cell line}}}}{IC_{50_{\text{parental cell line}}}}$$

The level of cross-resistance was determined by exposing the oxaliplatin-resistant cell lines to cisplatin and vice versa. Data were collected from at least two replicate experiments.

### Measurement of ATP-binding cassette B1 functionality

The effect of the ATP-binding cassette B1 (ABCB1) inhibitor, elacridar, on oxaliplatin resistance was tested by measuring the $IC_{50}$ values of oxaliplatin, as described above, in the presence of 4 μmol/l elacridar. In addition, the $IC_{50}$ values for the ABCB1 substrate paclitaxel (at concentrations ranging from 0 to 8 μmol/l) were determined for S1, S1-oxa2, S1-oxa4, S1-oxa5, and S1-oxa6; for S1 and S1-oxa5 also with the addition of 4 μmol/l elacridar. The growth conditions were the same as described above and the incubation time with paclitaxel was 3 h.

### MTT assay

Cells were harvested by trypsinization, counted and seeded (1000 cells/well) in 96-well plates. Ten microlitres of the sterile-filtered MTT (MP Biomedicals, Inc., Germany) solution (5 mg/ml in phosphate buffer saline) was added to each well. During a 3 h exposure of the cells to MTT water-insoluble, dark blue formazan crystals are formed because of the action of the mitochondrial enzyme, succinate dehydrogenase. Then, 10% sodium dodecyl sulfate, was added to solubilize the formazane crystals. The optical density of the dissolved material was measured spectrophotometrically on a Multiscan MS

(Labsystems, Finland) at 570 nm, yielding an absorbance directly correlating with the number of metabolically active cells in the well.

### Measurement of platinum accumulation and DNA platination

For the investigation of the intracellular accumulation of cisplatin and oxaliplatin, cells were seeded in 60-mm tissue culture dishes in triplicate. After 48 h the medium was renewed and 24 h later the cells were washed once with DMEM without serum and then exposed to 50 μmol/l oxaliplatin for 2 h or 50 μmol/l cisplatin for 1 h in DMEM without serum. The drug-containing medium was aspirated and the cells were rinsed three times with ice-cold phosphate buffered saline. After trypsinisation the cells were counted using an electrical cell counter, NucleoCounter (ChemoMetec A/S) with the NucleoView software. A portion of the cells, $1.2 \times 10^6$, were saved for DNA extraction. The remaining cells were digested in 1 ml 70% $HNO_3$ at 65°C for 2 h. Extraction of DNA from the remaining cells was performed according to the Wizard Genomic DNA purification Kit (Promega, Madison, Wisconsin, USA) followed by phenol chloroform purification. The isolated DNA was resuspended in 0.1 ml MilliQ water, and the DNA concentration was determined spectrophotometrically at 260 nm using a GeneQuant RNA/DNA Calculator (Cambridge, UK) followed by digestion in 0.6 ml of 70% $HNO_3$ at 65°C for 2 h. Elemental Pt in the cell lysate and in the hydrolyzed DNA was measured by inductively coupled plasma mass spectroscopy (Thermo X7, Thermo Elemental, Winsford, UK). The samples were introduced in a segment-flow mode and analyzed in peak-jumping mode, 100 sweeps and 1 point per peak, 30 ms dwell time for platinum (Pt195) and 10 ms for the internal standard bismuth (Bi209). The detection limit, calculated as three times the standard deviation of the blank, was 0.01 μg/l. All samples were prepared in duplicate and the method imprecision, calculated as the coefficient of variation for duplicate measurements, was 1.6%.

### Genome-wide gene expression analysis

Oligonucleotide microarrays were produced at the SCIBLU Genomics Center, Lund University, Sweden using a set of approximately 35 000 human oligonucleotide probes (Operon, ver. 3.0) as described earlier [11]. Total RNA was extracted from all cells lines using Trizol reagent (Invitrogen, Carlsbad, California, USA) and the RNeasy Midi purification kit (Qiagen, Hilden, Germany) and was quality controlled using a BioAnalyzer 2100 system (Agilent Technologies, Santa Clara, California, USA). Fluorescently labeled cDNA targets for hybridization were prepared according to the manufacturer's instructions using the Corning Pronto Plus System 6 (Corning, Acton, Massachusetts, USA). The resistant cell lines were labeled with Cy3-dCTP (GE Healthcare, Waukesha, Wisconsin, USA) and S1 with Cy5-dCTP. Before hybridization, the arrays were UV cross-linked at 800 mJ/cm$^2$ and then pre-treated and hybridized overnight at 42°C using the Universal Microarray Hybridization Kit (Corning) according to the manufacturer's instructions. Fluorescence was recorded using an Agilent G2565AA microarray scanner (Agilent Technologies).

Tagged image file format (TIFF) images were analyzed using the GenePix Pro 4.0 software (Axon Instruments, Foster City, California, USA), and the quantified data matrix was loaded into a local installation of BioArraySoftware Environment [12]. Positive and nonsaturated spots were background corrected using the median foreground minus the median background signal intensity for each channel. Data were filtered for flagged features from the image analysis, features with signal-to-noise ratio less than 2 in both channels and features without a known gene symbol before Lowess normalization [13]. Reporters not represented at least one time per experiment group were removed (equalling 92% presence required). Ratios of expression in resistant cell lines over parental S1 cells were calculated.

### Immunohistochemistry

The protein expression of ABCB1 was probed by immunohistochemistry on paraffin-embedded cell blocks of all the resistant cell lines and the parental S1 using the primary antibody anti-P-glycoprotein (NCL-JSB1) from Novocastra (Newcastle upon Tyne, UK). To prepare the cell blocks, each cell line was trypsinized, pelleted and treated with the Shandon cytoblock kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The blocks were then formalin fixed, embedded in paraffin and 4-μm-thick slices were prepared. Immunostaining was performed using the Tech-Mate instrument and HRP-LSAB. (DAKO, Glostrup, Denmark) according to the manufacturer's instructions.

Two sections from each cell line were scored independently by two persons in a blinded manner. Immunospecific staining was scored by gross examination of the entire sections containing more than 10 000 cells using the following approximate division: (i) less than 10% visibly stained cells; (ii) 10–50% visibly stained cells; and (iii) $>$ 50% visibly stained cells.

## Results

### Establishing resistant cell lines

By stepwise increasing the concentrations of cisplatin and oxaliplatin, eight resistant sublines were established: four against cisplatin and four against oxaliplatin. The $IC_{50}$ values for the corresponding substance were determined for all cell lines including the parental S1 (Table 1). The cisplatin-resistant cell lines showed a 1.5–6.2-fold increase in resistance against cisplatin and the oxaliplatin-resistant lines were 2.6–17-fold more resistant against oxaliplatin compared with S1.

To assess the long-time stability of the resistance, the cells were grown for 36 days in a drug-free medium and then the $IC_{50}$ values were redetermined. There was no decrease in resistance in any of the cell lines after this time (data not shown).

### Cross-resistance

To investigate the level of cross-resistance, the four oxaliplatin-resistant cell lines were treated with cisplatin and vice versa (Table 1). A certain cross-resistance was detected with the maximum increases in $IC_{50}$ being 2.3 times for S1-cis5 with oxaliplatin and 3.3 times for S1-oxa4 with cisplatin.

### Platinum accumulation

Pt concentrations in the whole cells and in the DNA fractions were determined in the parental S1, S1-cis5 and S1-oxa5 cell lines (Fig. 1). The uptake of Pt in both whole cell and DNA was reduced in the resistant cell lines compared with the parental cell line. The largest decrease was detected for oxaliplatin in S1-oxa5, which was also the most resistant cell line. When the cell lines were compared, the uptake of oxaliplatin was considerably lower in S1-oxa5 compared with S1-cis5 whereas the uptake of cisplatin was lower in S1-cis5. Interestingly, however, the uptake of oxaliplatin was lower than the uptake of cisplatin in S1-cis5, which was more resistant to cisplatin.

To see if this effect could be a consequence of different inherent properties of the substances, the relationship between Pt uptake in DNA and the corresponding $IC_{50}$ values was investigated for each substance (Fig. 2). The effect on Pt uptake with increasing $IC_{50}$ values was much higher for oxaliplatin. In fact, the fitting of the data to power equations indicated that the Pt uptake was almost inversely proportional to the $IC_{50}$ value for oxaliplatin (the exponent being $-0.84$ with a 95% confidence interval between $-1.1$ and $-0.58$), which was not the case for cisplatin (exponent $-0.19$ with a 95% confidence interval between $-0.35$ and $-0.033$).

**Table 1** Resistance against oxaliplatin and cisplatin

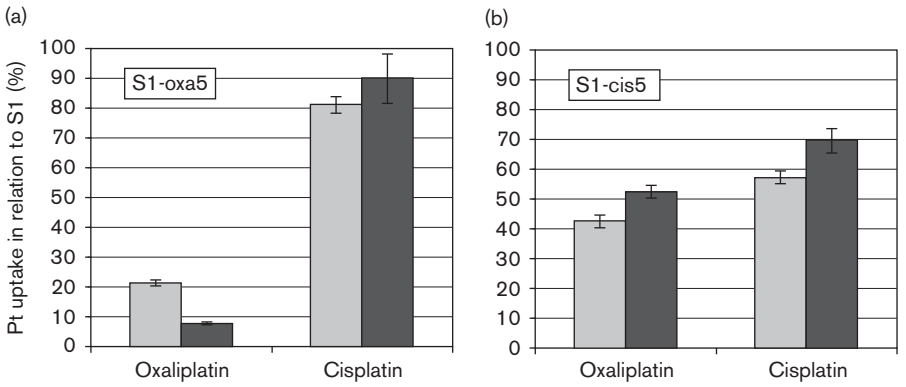
| Oxaliplatin exposure | | | Cisplatin exposure | | |
|---|---|---|---|---|---|
| Cell line | $IC_{50}$ (μmol/l)[a] | *R* | Cell line | $IC_{50}$ (μmol/l)[a] | *R* |
| S1 | 4.0 ± 0.9 | 1.0 | S1 | 7.6 ± 0.8 | 1.0 |
| S1-oxa2 | 11 ± 2 | 2.7 | S1-cis2 | 11 ± 0.5 | 1.5 |
| S1-oxa4 | 11 ± 1 | 2.6 | S1-cis4 | 12 ± 2 | 1.5 |
| S1-oxa5 | 67 ± 2 | 17 | S1-cis5 | 47 ± 7 | 6.2 |
| S1-oxa6 | 42 ± 6 | 11 | S1-cis6 | 33 ± 2 | 4.4 |
| Cross-resistance | | | Cross-resistance | | |
| S1-cis2 | 4.4 ± 0.2 | 1.1 | S1-oxa2 | 10 ± 4 | 1.4 |
| S1-cis4 | 4.5 ± 0.2 | 1.1 | S1-oxa4 | 25 ± 17 | 3.3 |
| S1-cis5 | 9.2 ± 2 | 2.3 | S1-oxa5 | 16 ± 6 | 2.1 |
| S1-cis6 | 6.6 ± 1 | 1.6 | S1-oxa6 | 17 ± 3 | 2.2 |

[a] ± SEM.

### cDNA microarray

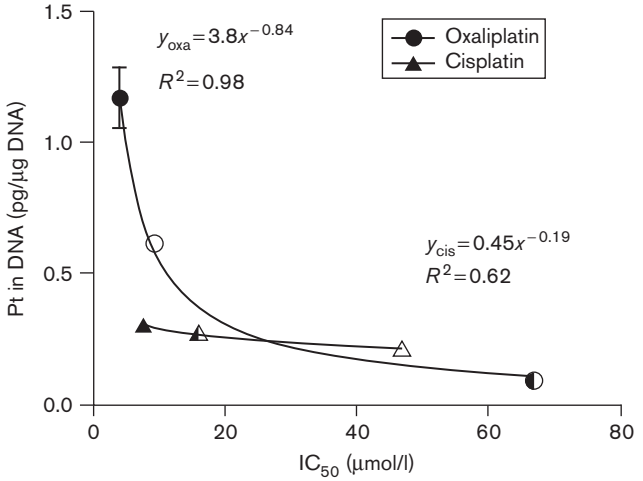
mRNA expression data on selected genes, with known or possible roles in chemotherapy resistance are presented in Fig. 3. Several of the ABC genes were differentially expressed, that is, greater than two-fold upregulated or downregulated, in the resistant cell lines compared with original S1 cells. The most striking finding was that the gene encoding ABCB1 (also known as MDR1 or P-glycoprotein) was highly overexpressed (greater than five-fold) in the three most oxaliplatin-resistant sublines, but significantly underexpressed in the two most cisplatin-resistant cell lines compared with the parental S1 cells. The expression of the remaining 30 ABC genes showed variable patterns among the different cell lines. Several members of the glutathione S-transferase (GST) and metallothionein (MT) families were upregulated or downregulated in the resistant cell lines. However, there

**Fig. 1**




Platinum (Pt) uptake measured in whole cell (grey) and DNA (dark grey) in the oxaliplatin resistant cell line S1-oxa5 (a) and the cisplatin resistant cell line S1-cis5 (b) compared with the corresponding uptake in the parental cell line S1 ± SEM. The cells were treated with 50 μmol/l oxaliplatin for 2 h or 50 μmol/l cisplatin for 1 h. After extraction, elemental Pt was measured with inductively coupled plasma mass spectroscopy.

**Fig. 2**




The platinum (Pt) uptake in DNA, after treatment with either oxaliplatin or cisplatin, as a function of the resistance of the different cell lines to the respective drug ± SEM. Filled symbols, S1, empty symbols, S1-cis5, and half-filled symbols, S1-oxa5. The data were fitted to power equations using the least square method in GraphPad Prism 5.

were no clear-cut expression patterns in these genes that discriminated between cisplatin and oxaliplatin resistance, respectively. Neither was there any apparent relationship between the level of resistance and the expression level of these genes.

### Immunohistochemistry

All eight resistant cell lines were scored for the expression of ABCB1 (Fig. 4 and examples of staining in Fig. 5). Overall, there was a significantly higher expression of ABCB1 in the oxaliplatin-resistant cell lines with an average score of 2.6 compared with 1.4 for the cisplatin-resistant lines ($P < 0.001$). The score for parental S1 cells was 2.0 (not included in Fig. 4).

### Determination of ABCB1 functionality

The ABCB1 protein was overexpressed in oxaliplatin-resistant cell lines compared with cisplatin-resistant lines and the parental S1. To decide whether the overexpressed ABCB1 was active as a drug transporter or not, the oxaliplatin-resistant cell lines and S1 were incubated with paclitaxel, which is a known ABCB1 substrate. In addition, the ABCB1 inhibitor, elacridar [14] was used to investigate whether it was possible to reverse drug resistance.

There were no significant differences in paclitaxel sensitivity between any of the oxaliplatin-resistant cell lines and S1 (Table 2). However, when incubated with paclitaxel in the presence of 4 µmol/l elacridar, the $IC_{50}$ values of S1, S1-oxa5, and S1-cis5 decreased to 14, 11, and 17 nmol/l, respectively, indicating that S1 and the resistant cell lines expressed equal amounts of ABCB1 functionality.

None of the cell lines displayed a decrease in the $IC_{50}$ to oxaliplatin when treated in the presence of elacridar (Table 2).

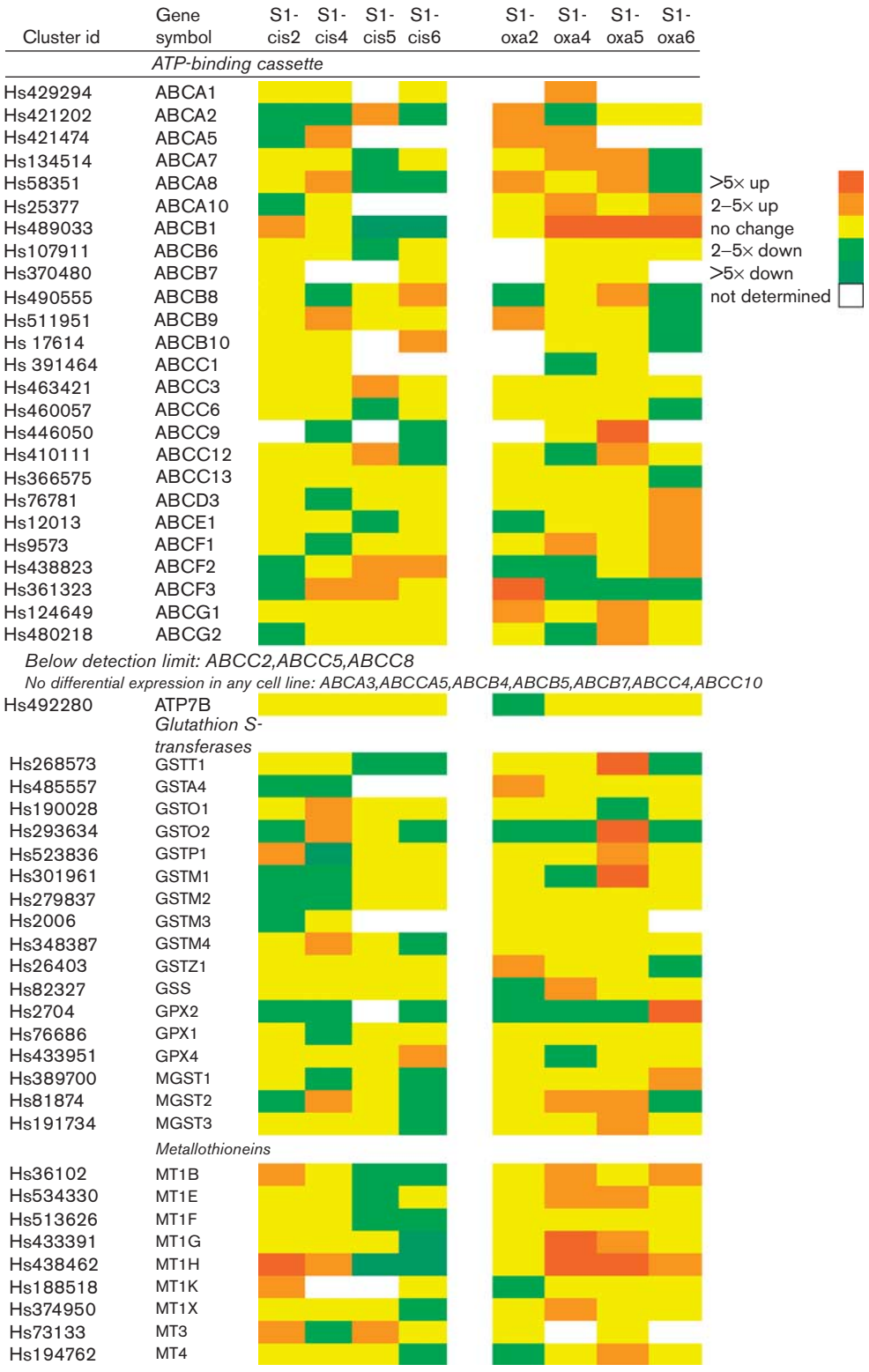
## Discussion

Several mechanisms of resistance against Pt-based chemotherapeutic agents have been identified, such as decreased DNA platination because of reduced cellular influx or increased efflux, intracellular detoxification, DNA repair or tolerance against platinated DNA [3,6,15]. Some resistance factors are common to the two drugs, but others are not. In this study, four cisplatin-resistant and four oxaliplatin-resistant variants of a colon cancer cell line (S1) were produced. A low degree of cross-resistance between the drugs was noted (Table 1); resistance factor up to 2.6 for S1-oxa6 against cisplatin and 3.2 for S1-cis5 against oxaliplatin was noted, which is well in line with earlier studies [16–18].

Drug accumulation after treatment with the two drugs was studied in the two most resistant sublines, S1-oxa5 and S1-cis5. Pt levels were analyzed both in whole-cell lysate and in DNA preparations as a measure of Pt-DNA adducts (Fig. 1). Reduced Pt accumulation was observed in both S1-oxa5 and S1-cis5 compared with the parental S1 cells, after exposure to both oxaliplatin and cisplatin, but the relations were different. After oxaliplatin treatment the Pt accumulation in DNA was clearly reduced in both S1-oxa5 and S1-cis5, the decrease being in the same order of magnitude as the increase in the level of resistance, which was seen as an almost inverse relationship between Pt-DNA and $IC_{50}$ values (Fig. 2), whereas the reduction of drug accumulation after cisplatin treatment was much less pronounced. Thus it seems as if oxaliplatin resistance to a larger degree could be explained by reduced drug accumulation than cisplatin resistance in these cell lines, and that mechanisms for increased tolerance against Pt incorporation in DNA might be of higher importance for resistance against cisplatin. Similar to this, it has earlier been described for ovarian cancer cell lines that tolerance is an important mechanism explaining differences in cisplatin resistance [19,20].

cDNA microarray analysis was performed with the purpose of identifying gene expression alterations that might be involved in acquired resistance, by both comparing all cisplatin-resistant cell lines with all oxaliplatin-resistant cell lines, and cell lines with different levels of resistance against the same drug. Given the results of the Pt accumulation studies, particular interest was paid to genes involved in drug transport and intracellular drug detoxification. When analyzing the gene expression map in Fig. 3, no obvious pattern discriminating between oxaliplatin and cisplatin resistance was apparent, apart from a general observation that a greater number of ABC

**Fig. 3**

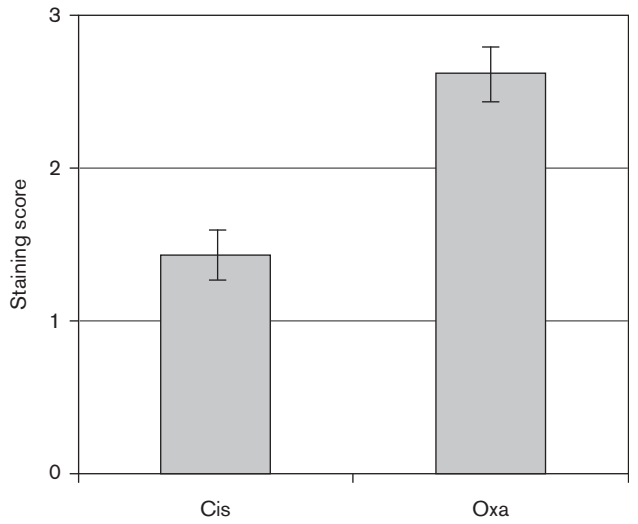



Expression ratios of selected genes derived from a genome wide expression analysis containing approximately 35 000 genes.

transporters were upregulated in the oxaliplatin-resistant cell lines compared with those selected for cisplatin resistance (see discussion below).

The other goal with the microarray experiment was to compare cell lines with different levels of resistance against the same drug. Given the multifactorial nature of drug resistance one could hypothesize that one resistance factor after the other is added when a cell line or a tumor becomes increasingly resistant during drug treatment and that this process would be orchestrated by additional genomic alterations. We found more of the GSTs and the MTs to be overexpressed in the low-grade than in the high-grade cisplatin-resistant cell lines, whereas the opposite was seen in oxaliplatin-resistant sublines. From this one could speculate that intracellular detoxification by thiols is an earlier event during the acquisition of cisplatin resistance compared with the development of oxaliplatin resistance. To determine whether this really is the case needs further studies.

**Fig. 4**



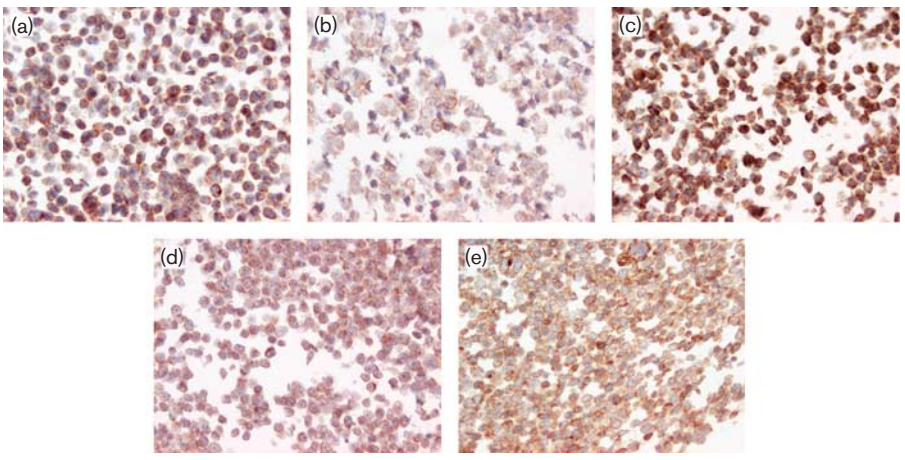

Score of ATP-binding cassette B1 staining. Cell blocks were prepared from each cell line and probed with an anti-P-glycoprotein antibody. The staining was scored and averaged over the cisplatin (Cis) resistant cell lines S1-cis2, S1-cis4, S1-cis5 and S1-cis6 , and the oxaliplatin (Oxa) resistant cell lines S1-oxa2, S1-oxa4, S1-oxa5 and S1-oxa6, respectively ± SEM. Score 1: less than 10% of cells stained, score 2: 10–50% cells stained, and score 3: more than 50% cells stained. $P = 0.0002$ (Mann–Whitney).

We found several of the genes encoding ABC transporters to be differentially expressed in the resistant cell lines, most notably, ABCB1, which was highly upregulated in the three most oxaliplatin-resistant cell lines. This observation was also confirmed on the protein level by immunohistochemistry, which showed a significantly stronger staining in the oxaliplatin-resistant cell lines compared with the cisplatin-resistant counterparts. It should be noted, though, that the parental S1 cells also showed positive immunostaining for ABCB1.

ABCB1 is associated with the multidrug resistance (MDR) phenotype, which confers resistance against a number of cytostatic agents, for example, antracyclins, vinca-alcaloids and taxanes [21], but not against Pt compounds.

To further elucidate the role of the upregulated ABCB1 in our oxaliplatin-resistant cell lines, functional tests were performed. Administration of the MDR reversing agent, elacridar, did not increase the sensitivity against oxaliplatin in the oxaliplatin-resistant cell lines. This indicates that, even if ABCB1 was upregulated in the oxaliplatin-resistant cells, it was probably not a causative step for the

**Fig. 5**




ATP-binding cassette B1 protein expression. Sections of paraffin-embedded cell blocks were stained with anti-P-glycoprotein antibody. Representative views are shown for S1 (a), S1-cis2 (b), S1-oxa2 (c), S1-cis6 (d) and S1-oxa6 (e). All photos were taken with a × 20 objective.

**Table 2 The influence of ABCB1 on oxaliplatin resistance**

| Cell line | Ratio $IC_{50oxa+el}/IC_{50oxa}$ | Paclitaxel $IC_{50}$ (confidence interval)/μmol/l |
|---|---|---|
| S1 | 1.5 | 0.23 (0.17–0.31) |
| S1-oxa2 | 1.6 | 0.16 (0.13–0.20) |
| S1-oxa4 | 1.4 | 0.37 (0.28–0.47) |
| S1-oxa5 | 0.94 | 0.33 (0.27–0.39) |
| S1-oxa6 | 1.1 | 0.21 (0.17–0.25) |

ABCB1, ATP-binding cassette B1; el, elacridar; $IC_{50}$, concentration at 50% inhibition.

development of resistance. This also confirms earlier studies showing that Pt compounds are not substrates for ABCB1.

To test the transport functionality of the ABCB1, the cells were treated with paclitaxel, which is a well-known substrate for ABCB1. There was no difference in sensitivity between parental S1 cells and the oxaliplatin-resistant variants (Table 2), whereas the reversal of MDR by the coadministration of elacridar leads to a substantially increased sensitivity toward paclitaxel in S1, S1-oxa5, and S1-cis5. From this one can conclude that ABCB1 was active in all the cell lines and that the altered ABCB1 levels noted during the induction of resistance against oxaliplatin and cisplatin were not crucial for chemotherapy sensitivity.

With regard to other members of the ABC superfamily, 35 were represented on the microarray. Some of them have been associated earlier with chemotherapy resistance (ABCA2, ABCB4, ABCB11, ABCC1-C6, ABCC10-11, ABCG2) [22], but none of them were overexpressed in this study. Thus, the role of ABC transporters in our resistant cell lines remains unclear.

In recent years it has been shown that the copper transporters, CTR1, ATP7A, and ATP7B, are involved in cellular influx and efflux of both cisplatin and oxaliplatin [21] and alterations in these proteins are associated with resistance to Pt drugs. In this study the expression of the gene encoding ATP7B remained basically unchanged in the resistant cell lines, whereas CTR1, ATP7A were not represented on our cDNA microarrays.

Another known mechanism for Pt resistance is intracellular detoxification by thiols such as MTs or glutathion (mediated by increased activity in GSTs) [6], which in turn may lead to reduced DNA platination. In this study, several members of these gene families were upregulated or downregulated in one or more of the resistant cell lines. There were no obvious expression patterns of these genes that discriminated between cisplatin and oxaliplatin resistance. However, when comparing only the two cell lines that were subjected to Pt accumulation studies, S1-oxa5 and S1-cis5, differences became apparent. In S1-oxa5, several GSTs, and MTs were significantly overexpressed, whereas overexpressions within these gene families were virtually absent in S1-cis5. When S1-oxa5 was exposed to oxaliplatin the relative amount of Pt bound to DNA was very low (Fig. 1), much lower than after cisplatin exposure of the same cell line. The reason for this is unclear. One theoretical explanation could be that the intracellular thiols in S1-oxa5 binds oxaliplatin to a greater extent than cisplatin and prevents DNA-platination. However, this speculation needs further investigations for clarification.

In conclusion, eight resistant subclones of a colon cancer cell line, S1, were created to study mechanisms of resistance against oxaliplatin and cisplatin. There was a limited degree of cross-resistance between the two drugs. Decreased drug accumulation could explain a larger part of the resistance against oxaliplatin than cisplatin. cDNA microarray and immunohistochemical studies showed that ABCB1 was overexpressed in the oxaliplatin- (but not the cisplatin)-resistant cell lines. However, chemical reversal of ABCB1 did not increase sensitivity to oxaliplatin, which indicates that upregulation of ABCB1 was probably not involved in causing the resistance. Our next step will be to investigate other possible mechanisms for decreased drug accumulation in our Pt-resistant cells, such as cupper transporters and intracellular detoxification by thiols.

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