Molecular aspects of 5-Fluorouracil and Oxaliplatin activity

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Chapter 5

Oxaliplatin activity in selected and unselected human ovarian and colorectal cancer cell lines

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Abstract

Oxaliplatin is used for treatment of colon cancer in combination with 5-fluorouracil or Irinotecan. Oxaliplatin has similar, but also different resistant mechanisms as cisplatin. We studied the activity of oxaliplatin in ovarian and colon cancer cells with different resistance patterns to cisplatin. The 40-fold cisplatin-resistant cell line ADDP was only 7.5-fold resistant to oxaliplatin. The gemcitabine-resistant AG6000 cell line, 9-fold resistant to cisplatin, was not cross-resistant. LoVo-175X2, with mutant-p53 showed no resistance compared to the empty vector control. However, LoVo-Li, with inactive p53, was 3.6-fold resistant corresponding to decreased accumulation and Pt-adducts. Accumulation and DNA adducts formation showed no significant correlation with oxaliplatin sensitivity. Cell cycle distribution after exposure to oxaliplatin showed arrest in G2/M (A2780) or in S-phase (LoVo-92) for wt-p53 cells. ADDP and LoVo-Li showed G1 arrest followed by S-phase arrest and no changes in distribution, respectively. The cell cycle related proteins cyclin A and B1 and (p)CDC25C were marginally affected by oxaliplatin. Expression of hCTR1 was decreased in ADDP, LoVo-Li and AG6000, OCT1 decreased in ADDP and AG6000 and OCT3 in LoVo-175X2, compared to the parental cell lines. In ADDP and LoVo-175X2 ATP7A and B were decreased but was increased in AG6000.

From this study it can be concluded that changes in cell cycle distribution were cell line dependent and not related to changes in expression of Cyclin A or B1. Oxaliplatin accumulation was related to hCTR1 and, at low concentration, ATP7A to DNA adducts formation while the retention was related to hCTR1, OCT2 and ATP7B.

Introduction

Cisplatin is one of the most frequently used drugs in cancer therapy and shows activity against testicular cancer, ovarian cancer and cancer of the head and neck but not in colon cancer(1). Oxaliplatin is a third generation analog of cisplatin that does show activity in colon cancer. Because of the activity of oxaliplatin in intrinsically cisplatin resistant colon cancer and the rather different toxicity pattern during treatment it is increasingly used in cancer therapy. The dose limiting toxicity for oxaliplatin is neurotoxicity. The most successful combination of oxaliplatin is with other drugs such as 5-fluorouracil with leucovorin and irinotecan (CPT11), for which response rates up to around 60% were found(2).

The activity of oxaliplatin is, like cisplatin, thought to be mediated by the formation of platinum DNA adducts after which cells go either in growth arrest to repair the damage or undergo apoptosis. However, besides induction of apoptosis as down stream effect

of platinum-DNA adducts formation oxaliplatin can induce a mitochondrial apoptotic response in enucleated cells indicating that it exerts its effects both at nuclear and cytoplasmic level(3). Additional targets of oxaliplatin that affect the apoptotic and antiproliferative effect of oxaliplatin may involve specific proteins like the inhibitor of apoptosis (IAP) family member survivin. Recent studies show that oxaliplatin decreases the expression of survivin which is further enhanced in combination with taxol or cyclooxygenase-2 inhibitors(4, 5).

Oxaliplatin is active in cell lines(6, 7) with intrinsic and acquired cisplatin resistance. As for cisplatin the activity of oxaliplatin may be hampered by resistance mechanisms such as decreased accumulation, reduced DNA adduct formation, increased DNA repair, increased adduct tolerance and reduced response to the platinum DNA adducts. Accumulation of oxaliplatin is a net result of uptake and efflux. Recent studies have described that the uptake of oxaliplatin can be mediated by the human copper transporter hCTR1 or the organic cation transporters OCT1-3 and the efflux via the p-type ATPases ATP7A and ATP7B (8-11). It has also been found that cell lines resistant to oxaliplatin show increased cellular levels of glutathione[12] (GSH) which might form a complex with oxaliplatin and subsequently can be exported by efflux pumps like the multidrug resistance protein 2 (MRP2)[13, 14]. Reduced platinum DNA adduct formation might be a result of lower accumulation of oxaliplatin and increased repair of the DNA adducts. Recent studies have shown that oxaliplatin forms less DNA adducts compared to cisplatin but these adducts seem to have a bigger impact(15, 16). In vitro DNA repair studies have shown that the nucleotide repair (NER) system has no difficulties in repairing oxaliplatin DNA adducts(17). Excision repair cross-complementing 1 (ERCC1) is one of the key factors of NER and its gene expression has been shown to correlate with oxaliplatin sensitivity in vitro(7) and was also an independent predictive marker for survival after treatment of colorectal cancer patients with 5-FU and oxaliplatin(18). However, the mismatch repair (MMR) system hardly recognizes oxaliplatin DNA adducts as compared to cisplatin(19, 20). It has been demonstrated that oxaliplatin has substantial activity in MMR defective cell lines(21, 22). Activity of oxaliplatin in MMR deficient cells may be one of the reasons that oxaliplatin shows activity in colon cancer which is characterized by high incidence of microsatellite instability and defects in the MMR genes MSH2 and MLH1(23-26). An intact MMR system is necessary for maximal cisplatin activity because detection of DNA adducts by the MMR leads to activation of signal transduction pathways that can lead to induction of apoptosis(27). Oxaliplatin is active in MMR deficient cells and therefore the signaling to the apoptotic machinery must be different than that of cisplatin including the effect of oxaliplatin on the mitochondria(3). Detection of platinum DNA adducts of cisplatin and oxaliplatin may therefore be one of the major

differences in the activity of both drugs. In the apoptotic response p53 plays an important role and it has been demonstrated that inactivation or mutated p53 alters the cytotoxicity of cisplatin(28-30). Although targeted inactivation of p53 in HCT1116 cells decreased the activity of oxaliplatin it was not possible to predict sensitivity based on p53 status in a group of 30 colorectal cancer cell lines(31).

In this study we describe the activity of oxaliplatin in a panel of ovarian and colon cancer cell lines with different p53 status. The activity of oxaliplatin was studied in relation to accumulation of the drug, formation and repair of platinum DNA adducts and to expression of uptake and efflux transporters. Furthermore the effect on the cell cycle and proteins involved in cell cycle progression were studied.

Materials and Methods

Chemicals

Oxaliplatin (l-OHP) was kindly provided by Sanofi-Synthelabo (Paris, France). Platinol®, cisplatin (CDDP) saline injection solution was obtained from Bristol-Myers Squib B.V. (Woerden, The Netherlands) in a concentration of 0.5 mg/ml. RPMI and DMEM culture medium, phosphate buffered saline (PBS) and Trypsine were obtained from BioWhittaker (Cambrex BioSience, Verviers, Belgium) and the fetal calf serum (FCS) was from GIBCO (Paisley, UK) and G418 antibiotics was from Roche Applied Science (Almere, The Netherlands). RNase A (50 U/mg), Antibodies against CDC25C (5H9, rabbit mAb) and pCDC25C (63F9, rabbit mAb) were obtained form Cell Signaling Technology (Danvers, MA, USA). Cyclin A (6E6, mouse mAb) was from Novacastra (Vision BioSystems, New Castle Upon Tyne, UK), Cyclin B1 (D-11, mouse mAb) was from Santa Cruz Biotechnology (Heidelberg, Germany) and ß-actin (AC-15, mAb) from Sigma (Saint Louis, Missouri, USA). The secondary polyclonal Rat anti Mouse immunoglobins/HRP was from DAKO (Glostrup, Denmark) and the Donkey anti Rabbit immunoglobins/HRP was obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). Dried milk powder, Protifar Plus, was from Nutricia. All other chemicals were of analytical grade. Solutions were made in water purified by a Millipore Reagent Q system (Millipore, Bedford, USA).

Cell culture

Eleven human tumor cell lines originating from different tissues were used in this study. The colon carcinoma cell lines LoVo-92 and their variants were kindly provided by Dr. Poupon(32). LoVo-92 (wild-type (wt) p53) is the parental cell line of LoVo-175X2, which is transfected with mutant (mt) p53 (mutation at codon 175 (Arg $\rightarrow$ His)) and

of LoVo-B2, which is the empty vector control for LoVo-175X2. Lovo-Li is also derived from Lovo-92 and has acquired functionally inactive p53 without p53 mutations. The p53 functional activity of LoVo-Li was determined as described previously(32, 33). The presence or absence of the mutations were confirmed by sequencing after transfer to our laboratory and were stable after culturing(34). WiDr is a colon carcinoma cell line with mutant (missense mutation at codon 273) p53. Various ovarian cancer cell lines were used. A2780 was the parental cell line of ADDP(35) with induced resistance to CDDP (>40-fold resistance, mt p53, mutation at codon 272 (Gln → Lys)) and of AG6000(36) with induced resistance against gemcitabine (> 30000 fold resistance) and cross-resistant to CDDP (3.7 fold)[37]. The ovarian cancer cell line 2008 is another ovarian cancer cell line. All cell lines were cultured in RPMI + 10% heat inactivated FCS except for A2780 and AG6000, which were cultured in DMEM + 10% heat inactivated FCS, and at 37°C in a 5% CO_2 humidified atmosphere. The culture medium for LoVo-B2 and LoVo-175X2 was supplemented with G418 (500 µg/ml) for selection and maintenance of the transfected phenotype.

Growth inhibition experiments

Growth inhibition experiments were performed in triplicate using the sulforhodamine B (SRB, (Sigma)) assay as described before(38, 39). Briefly, cells were plated in 100 µl in their specific culture medium in triplicate in 96 wells flat bottom plates (Costar, Cambridge, MA, USA) in densities related to their growth(40). After 24 hours, 100 µl culture medium was added to the control wells and 100 µl drug containing culture medium was added to the other wells and subsequently cells were cultivated for another 72 hours. The drug concentration range used for CDDP was from 0.1 to 500 µM and for Oxaliplatin it was from 0.01 to 200 µM. At the end of the drug exposure the cells were fixed with trichloro-acetic acid (TCA) and stained with the SRB protein dye. The optical density (OD) was measured at 540 nm and the results were expressed relative to the control growth and corrected for the optical density at the start of the incubation: Relative growth = $(OD_{72hr} - OD_{0hr})/(OD_{control} - OD_{0hr})$ and if <0 than $(OD_{72hr} - OD_{0hr})/(OD_{0hr})$ was used. From the growth curves the 50% growth inhibitory concentration (IC_{50}) and the total growth inhibitory concentration (TGI, IC_{100}) were determined via interpolation.

Cell cycle distribution

A2780, ADDP, Lovo-92 and LoVo-Li cells were plated in 6 well plates (250000 cells/well/2 ml). Cells were exposed for 48 and 72 hours to l-OHP at their IC_{100} concentration, 10.4, 160, 21.6, and 22.3 µM, respectively, in order to have a comparison at equitoxic concentrations. Except for the IC_{100} of the resistant cell line, these concentrations are achievable in patients. After incubation control and treated cells were harvested and

resuspended in buffer containing 0.1% tri-sodium citrate, 0.1% Triton X-100, 0.1 mg/ml RNase A and 50 µg/ml propidium iodide. Cell cycle distribution was measured on a FACScan flow cytometer (Becton and Dickinson).

Expression of cell cycle related proteins (p)CDC25C and Cyclin A and B1

Frozen cell pellets of A2780, ADDP, LoVo-92 and LoVo-Li cells that were treated for 72 hours at their IC_{100} concentration were lysed and the protein content was determined using the BioRad Protein Assay. 20 µg of protein was loaded on a 12% SDS-PAGE gel (30% acrylamide/bis, BioRad, Veenendaal, The Netherlands) and run for 2 hours at 120 V. Subsequently proteins were transferred to a PVDF membrane (Polyscreen, PerkinElmer Life Sciences, Groningen, The Netherlands) and blocked with Tris-buffered Saline Tween-20 (TBST) + 5% dried milk powder for 1 hour. After washing with TBST the membranes were incubated o/n at 4°C with the primary antibodies diluted in 5% BSA (Sigma) in TBST (CDC25C 1:1000, pCDC25C 1:1000, Cyclin A 1:500, Cyclin B1 1:1000) washed again with TBST and incubated with the secondary antibody diluted in TBST+2% dried milk powder (1:2000) for 1 hour. Subsequently detection of the proteins was performed using ECLplus (GE Healthcare Bio-Sciences, Uppsala, Sweden). To detect the reference protein, ß-actin, the procedure was repeated with primary (1:10000) and secondary antibody (1:2000) incubation for this protein. Detection was performed with ECL (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Oxaliplatin accumulation and DNA adducts formation

For determination of Oxaliplatin accumulation and DNA adducts formation, cells were exposed to 200 µM l-OHP for 24 hours followed by 3 hours drug free medium for determination of the retention of accumulated l-OHP and the formed Pt-DNA. The cells were subsequently washed 3 times with ice-cold PBS and harvested on ice by trypsinization, counted (accumulation) and stored as pellets at -20°C until processing. For determination of accumulated l-OHP cells were resuspended in 500 µl 2 M NaOH and incubated overnight at 55°C. Subsequently 1 ml 1 M HCl was added and samples were measured using flameless atomic absorption spectrometry (FAAS) as described previously for CDDP(41, 42) including a calibration curve ranging from 0.20 µM to 3.0 µM. To determine the Platinum-DNA adducts formation DNA was isolated using a QIAmp DNA Mini Kit (Westburg, Leusden, The Netherlands) and the DNA concentration was determined by measuring the optical density at 260 and 280 nm with a Nanodrop ND-1000 (Isogen Life sciences BV, IJselstein, The Netherlands). To 250 µl DNA sample or Pt-standard solution (ranging from 0.25 µM to 1.5 µM) 25 µl 1.68 M NaCl was

added and samples were subsequently measured using FAAS as described above.

Gene expression measurement

To determine gene expression measurements RNA was isolated from cell pellets using Trizol (Invitrogen, Paisley, UK). After quantification with a Nanodrop ND1000, 500 to 1500 ng of RNA was used for cDNA synthesis as described previously(43). Subsequently quantitative gene expression was measured using Taqman gene expression assays and using human reference RNA (Stratagene, Amsterdam, The Netherlands) to establish a calibration curve. Used Taqman gene expression assays were: Hs00427554_m1 (SLC22A1, OCT1), Hs00533907_m1 (SLC22A2, OCT2), Hs00222691_m1 (SLC22A3, OCT3), Hs00156229_m1 (hCTR1), Hs00163707_m1 (ATP7A), Hs00163739_m1 (ATP7B), Hs00157415_m1 (ERCC1) and Hs99999903_m1 (ACTB, ß-actin) which was used as endogenous control.

Results

Growth inhibition

The results of the growth inhibition experiments are shown in Table 1 and example curves for LoVo-92 and A2780 in Fig 1a and 1b. The colon carcinoma cell line LoVo-92 was most sensitive to Oxaliplatin with an IC_{50} value of 0.21 µM whereas the cisplatin resistant variant of A2780, ADDP, was least sensitive with an IC_{50} of 2.43 µM. A similar pattern was found for cisplatin with an IC_{50} of 1.5 µM for LoVo-92 and 20.33 µM for ADDP. When expressed as resistance factors, ADDP, as expected, showed the highest resistance factor of 40.9 for cisplatin but only 7.5 for oxaliplatin. The gemcitabine resistant cell line AG6000, cross resistant to CDDP, showed a comparable sensitivity to oxaliplatin as the parental cell line A2780. The LoVo-Li cell line, with inactive p53, showed a moderate resistance factor both for cisplatin (2.8) and oxaliplatin (3.6) compared to the wild-type LoVo-92. However, introduction of mutant p53 in LoVo-175X2 did not influence the activity of oxaliplatin and CDDP in this cell line with resistance factors similar to the empty vector control cell line LoVo-B2. The difference in activity of cisplatin and oxaliplatin in A2780 was only marginal (CDDP/l-OHP=1.5) but more pronounced in the colon cancer cell lines as well as in the ovarian cancer cell line 2008, 5.6 to 7.6 fold. The gemcitabine resistant ovarian cell line AG6000 showed a somewhat higher ratio between CDDP/l-OHP (11) which was even higher than that found in the cisplatin resistant cell line ADDP (8.4). The differences in resistance factors for cisplatin and oxaliplatin also led to the highest ratios between the drugs in colon cancer cells compared to ovarian cancer cells.

TABLE 1. Growth inhibitory effect (IC50), total growth inhibition (IC100) values and resistance factors (RF) in colon and ovarian cancer cell lines after 72 hours exposure to l-OHP and CDDP.

Cell line	P53	MMR	l-OHP IC_{50} (μM)	l-OHP IC_{100} (μM)	CDDP IC_{50} (μM)	RF l-OHP	RF CDDP	CDDP/l-OHP
LoVo-92	wt	D	0.21 ± 0.04	14.3 ± 1.6	1.50 ± 0.40			7.1
LoVo-Li	i.a.	D	0.75 ± 0.08	22.3 ± 3.8	4.23 ± 1.65	3.6	2.8	5.6
LoVo-B2	wt	D	0.39 ± 0.09	14.0 ± 4.0	7.50 ± 1.70	1.8	5.0	19.2
LoVo-175X2	wt	D	0.41 ± 0.07	21.3 ± 5.2	7.40 ± 2.40	1.9	4.9	18.2
WiDr	wt	N	0.91 ± 0.14	88.3 ± 21.7	6.90 ± 1.7			
A2780	wt	N	0.32 ± 0.03	10.4 ± 2.6	0.50 ± 0.14			1.6
ADDP	mt	L	2.43 ± 0.35	160 ± 25	20.3 ± 2.7	7.5	40.9	8.4
AG6000	wt	N	0.40 ± 0.06	11.6 ± 2.6	4.50 ± 1.00	1.2	9.0	11.3
2008	wt	N	0.25 ± 0.15	13.7 ± 0.3	1.45 ± 0.13			5.8

Values are given as mean ± SEM of at least 3 separate experiments. P53 status is depicted as: wt = wild type, mt = mutant, i.a. = in active and mismatch repair (MMR) status as: D = deficient, L = lower and N = normal.

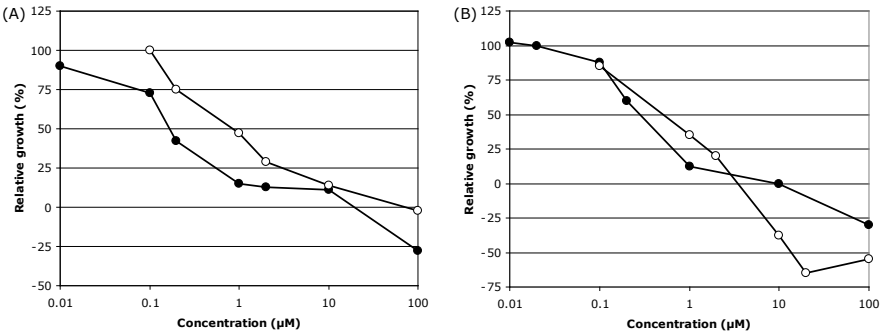

Figure 1. *Representative drug response curves for l-OHP and CDDP in LoVo-92 (A) and A2780 (B) cells.*

Cell cycle distribution

To determine whether the differences in oxaliplatin sensitivity might be related to effects on the cell cycle, four paired cell lines were selected, LoVo-92, Lovo-Li, A2780 and ADDP, and treated with oxaliplatin at IC_{100} concentration for a period of 48 and 72 hours. The distribution of the different phases of the cell cycle after exposure to oxaliplatin is shown in Fig 2. Both cell lines expressing wild-type p53, A2780 and LoVo-92, showed a decrease in time of the G1 phase. However in A2780 this was

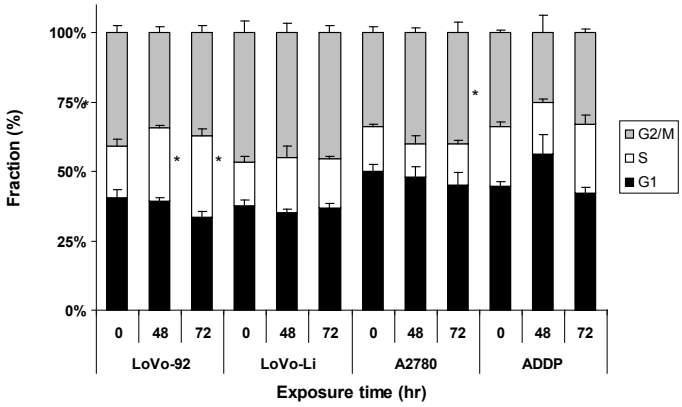

Figure 2. *Cell cycle distribution after exposure to oxaliplatin at IC100 concentration for 48 and 72 hours compared to control. Results are the mean values of 3 experiments ± SEM. * = significantly different compared to control.*

accompanied with an increase in G2/M whereas, in LoVo-92 an increase in S phase was observed. The increase in G2/M found in A2780 was significant compared to control after 72 hour exposure. In Lovo-92 the observed increase in S-phase was significant both at 48 and 72 hours after oxaliplatin exposure. In contrast, the ADDP cell line showed an initial increase in G1 after 48 hours but was subsequently followed by decrease in G1 and an increase of the S-phase fraction. The smallest change in cell cycle distribution was observed in LoVo-Li cells. After 48 hours a small decrease in G1 and increase in S-phase was found similar to the parental cell line LoVo-92 but after 72 hours the G1 was back to control level again and only a small increase in S-phase was still visible.

Expression of cell cycle related proteins (p)CDC25C and Cyclin A and B1

In order to determine whether the changes in cell cycle distribution were accompanied with changes in protein expression of the cell cycle related proteins (p)CDC25C and the Cyclins A and B1 western blot analysis was performed after exposure of the cells for 72 hours to IC_{100} concentration of oxaliplatin. Representative blots are shown in Fig 3. Differences in expression were mainly observed between cell lines and were most pronounced for Cyclin B1, which was much lower in A2780 and also for pCDC25C which showed lower expression in LoVo-Li and A2780. Cyclin A which is involved in S-phase progression in a complex with CDK2, showed an increased expression in LoVo-92 and ADDP cells whereas a decrease was observed in LoVo-Li and A2780 cells.

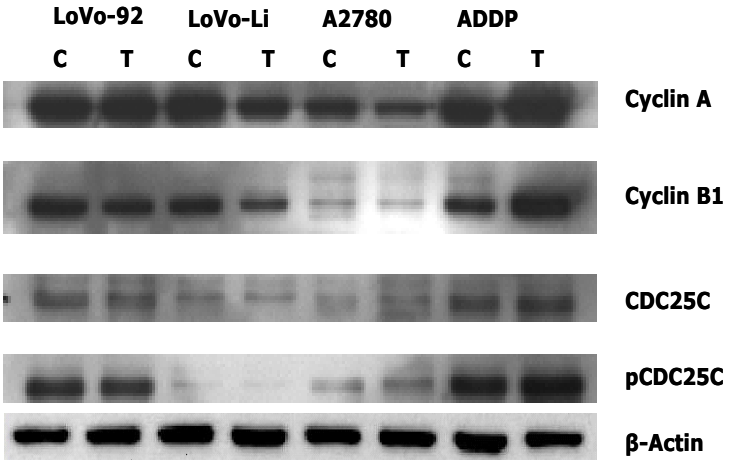

Figure 3. *Protein expression of cell cycle related proteins. Cells were treated for 72 hours at IC100 concentration of oxaliplatin. Shown blots are representative of the multiple passages (at least 3) tested for each cell line, demonstrating the striking difference between LoVo-92 and Lovo-Li, and between A2780 and ADDP, for pCDC25C. Equal loading was confirmed with β-actin as a control. C = Control and T = Treated.*

Cyclin B1, involved in G2/M transition in complex with CDK1, showed a decreased expression for all cell lines except ADDP where an increase was observed. For CDC25C expression hardly any change could be observed except ADDP which showed an increase. Phospho CDC25C, indicative for DNA damage at the G2/M phase, showed a similar pattern as Cyclin B1. Expression decreased in all cell lines upon exposure to oxaliplatin except ADDP where an increase was observed.

Oxaliplatin accumulation and DNA adducts formation

Oxaliplatin accumulation after 24 hour exposure to 200 μM oxaliplatin and the formation of oxaliplatin DNA adducts after 24 hour exposure to 20 and 200 μM are shown in Table 2. Accumulation of oxaliplatin was determined in the same subset in which cell cycle effects were studies. Accumulation ranged from 226 pmol/10^6 cells for LoVo-Li to 767 pmol/10^6 cells for LoVo-92 cells. Retention of accumulated oxaliplatin after 3 hours incubation in drug free medium, indicative for efflux activity, was about 80% for A2780, LoVo-92 and LoVo-Li. Only the cisplatin resistant cell line ADDP showed a somewhat lower retention, 73%.

The DNA adduct formation ranged from not detectable in the 2008 cell line, at 20 μM, to 9.53 pmol Pt/μg DNA at 200 μM oxaliplatin in ADDP cells. A2780 showed the largest difference in adduct formation between 20 and 200 μM oxaliplatin, 40 fold, whereas in LoVo-92 the difference was around 3 fold. Also in the other ovarian cancer

TABLE 2. Platinum accumulation and DNA adducts formation after 24 hr exposure to 20 or 200 μM oxaliplatin and 24 hours followed by 3 hour in drug free medium.

Cell line	Total platinum accumulation		Platinum DNA adducts			Retention
	24 hr, 200 μM (pmol Pt/10^6 cells)	24+3 hr, 200 μM (pmol Pt/10^6 cells)	24 hr, 20 μM (pmol Pt/μg DNA)	24 hr, 200 μM (pmol Pt/μg DNA)	24+3 hr, 200 μM (pmol Pt/μg DNA)	(%)
LoVo-92	767 ± 141	612 ± 102	1.92 ± 0.32	5.41 ± 1.08	3.90 ± 0.65	72.0
LoVo-Li	226 ± 69	185 ± 82	0.20 ± 0.09	2.07 ± 0.33	1.79 ± 0.65	86.8
LoVo-B2			0.35 ± 0.15	1.40 ± 0.38	0.67 ± 0.20	47.8
LoVo-175X2			0.33 ± 0.03	1.34 ± 0.45	0.82 ± 0.22	61.1
WiDr			0.09 ± 0.06	0.99 ± 0.25	0.92 ± 0.17	92.9
A2780	523 ± 41	416 ± 82	0.18 ± 0.05	7.25 ± 0.72	6.50 ± 1.16	89.7
ADDP	258 ± 31	189 ± 55	0.33 ± 0.15	9.53 ± 0.70	6.97 ± 0.90	73.1
AG6000			0.17 ± 0.07	2.60 ± 0.24	2.15 ± 0.26	82.6
2008			n.d.	1.21 ± 0.27	n.d.	n.d.

Retention (%) is the relative level of Pt-DNA adducts after 3 hour in drug free medium. Values are means of at least 3 experiments ± SEM. n.d. = not detectable.

cell lines a high ratio was found; 28.9 in ADDP and 15.3 in AG6000. The other cell lines showed a much lower difference in the formation of platinum DNA adducts.

The retention of the formed DNA adducts, indicative for DNA repair, ranged from 48 %, after a 3 hour drug free period, for LoVo-B2 to 93 % for WiDr. Comparison of adduct formation in LoVo-92 and LoVo-Li showed that the inactivation of p53 was accompanied with lower DNA adduct formation. This difference was not found after transfection of mutant p53 in LoVo-175X2 that showed similar DNA adducts formation as its parental empty vector control LoVo-B2.

Gene expression measurement

Formation of platinum DNA adducts as described above is dependent on transport of oxaliplatin into the cells and sequestering or efflux out of the cells. Uptake of oxaliplatin might be mediated via the human copper transporter hCTR1 or the organic cation transporters OCT1-3 (SLC22A1-3) while efflux might be mediated via ATP7A and B. Also repair of the DNA adducts by ERCC1 might determine the amount of adduct formation. The mRNA expressions of the transporters and ERCC1 are shown in Fig 4a and 4b.

Figure 4a shows that there was a large variation in expression of genes that might mediate oxaliplatin uptake. For all cell lines the expression of hCTR1 was the lowest ranging from not detectable in A2780 to 0.05 in the LoVo-B2 cell line. OCT1 expression was lowest in the cisplatin resistant cell line ADDP and LoVo-Li, 0.25 and 0.12, respectively, whereas in the ovarian cancer cell lines 2008 and the gemcitabine resistant cell line AG6000 the expression was about 100 fold higher. OCT2 was not

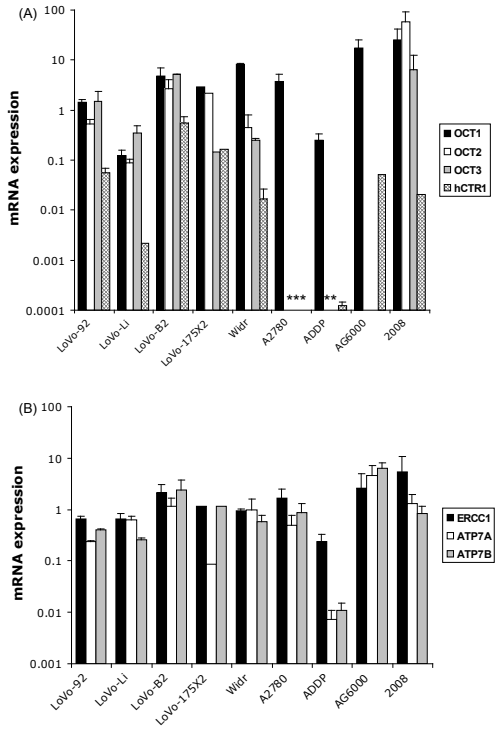

Figure 4. *mRNA expression of influx (A) and efflux transporters and DNA repair gene ERCC1 (B). Results are means of 2 experiments in triplicate and are expressed relative to expression of the housekeeping gene β-actin. * = not detectable.*

detectable in A2780 and its cisplatin resistant variant ADDP, low in LoVo-Li (0.09) and more than 600 fold higher in the ovarian cell line 2008. OCT3 varied from not detectable in A2780 and ADDP to 6.4 in 2008. The pattern of OCTs expression in LoVo-92 was similar but lower than its empty vector variant LoVo-B2. Transfection of mt-p53 (LoVo-175X2) or inactivation of p53 (LoVo-Li) led to decreased expression of the OCT transporters.

In contrast to the influx transporters the variation in expression of the efflux transporters and ERCC1 as shown in Fig 4b was less pronounced. Except for ADDP in which the expression of ATP7A and B was 100 fold lower and AG6000 which showed 2 fold higher expression no large differences in expression of the ATP7A and B were found between the cell lines. Comparison of the parental cell lines LoVo-92 and LoVo-B2 with its corresponding derivatives LoVo-Li and LoVo-175X2, respectively, showed decreased expression of ATP7B while for ATP7A LoVo-Li shows increased expression and LoVo-175X2 a reduced expression. Highest ERCC1 expression was

TABLE 3. Pearson and Spearman correlation analysis of IC50 values, Total Platinum accumulation and retention, Platinum DNA adducts formation and retention and mRNA expression of transporters and ERCC1.

		Spearman		Pearson's		n
		Correlation	p value	Rho	p value	
IC_{50} l-OHP	IC_{50} CDDP	0.912	<0.001	0.683	0.042	9
TotalPt200	hCTR1			0.997	0.053	4
TotalPt200R	hCTR1			0.999	0.025	4
PtDNA20	PtDNA200R%			-0.743	0.035	9
	OCT1			-0.647	0.083	9
	ATP7A			-0.707	0.050	9
PtDNA200	PtDNA200R	0.985	<0.001	0.905	0.002	9
PtDNA200R	ATP7B			-0.690	0.058	9
PtDNA200R%	OCT2	-0.919	0.027	-0.900	0.037	9

TotalPt200 = total platinum accumulation, TotalPt200R = retention of total platinum accumulation, Pt adducts at 20 μM (PtDNA20), Pt adducts at 200 μM (PtDNA200), Retention of Pt adducts at 200 μM (PtDNA200R), Relative retention of Pt adducts at 200 μM (PtDNA200R%). n = number cell lines tested. Platinum accumulation was only evaluated in LoVo-92, LoVo-Li, A2780 and ADDP.

found in the 2008 cell line, lowest in the cisplatin resistant cell line ADDP. Inactivation of p53 did not alter the expression of ERCC1 but the mutant p53 cell line LoVo-175X2 showed lower expressions as its empty vector control.

Statistical evaluation

Pearson's and Spearman correlation coefficients were calculated from the IC_{50} values, Total platinum accumulation, DNA platinum adduct formation and the mRNA expression of transporters and ERRC1. The correlations that were significant or showed a trend to significance are shown in Table 3.

The results showed that the sensitivity to cisplatin and oxaliplatin were highly correlated. None of the investigated parameters showed a significant correlation with either cisplatin or oxaliplatin sensitivity. Although the accumulation and retention of oxaliplatin in A2780, ADDP, LoVo-92 and LoVo-Li were highly correlated to the expression of the copper transporter hCTR1, no significant correlation was found with the formation of platinum DNA adducts. However, the formation of DNA adducts was significantly related to the (relative) retention of the adduct formation at 200 μM and to the expression of OCT1 and ATP7A. The retention of the DNA adducts formation at 200 μM was further correlated to the ATP7B expression. The relative retention showed a significant correlation with OCT2, hCTR1 and also ATP7B expression.

Discussion

In this paper we describe that activity of oxaliplatin in a panel of cell lines of different origin, with various corresponding variants and different p53 status has a better cytotoxicity profile than cisplatin. This is possibly related to the different structure of oxaliplatin DNA adducts compared to cisplatin DNA adducts which affects the recognition and repair as shown previously(44, 45). Also differences in uptake and efflux may determine the activity of the drug(8-11).

The cytotoxicity profile of oxaliplatin in the cell line panel tested showed that, except for the cisplatin resistant cell line ADDP, they are all in the sub micromolar range. The results for cisplatin show a much higher variation resulting in cisplatin/oxaliplatin sensitivity ratios ranging from 1.5 to 20, with a mean sensitivity ratio of 8.6 which is somewhat higher as found previously (46). This might be the result of the longer exposure time in our experiments. Introduction of mt-p53 did not alter sensitivity as can be concluded from similar sensitivity towards both oxaliplatin and cisplatin in LoVo-175X2 cells compared to the empty vector control LoVo-B2. However, the LoVo-Li cell line with inactive p53 showed a 2 fold lower sensitivity. Lower expression of p53 combined with an increased expression of MDM2 compared to the parental LoVo-92 cells might explain the reduced activity of both cisplatin and oxaliplatin(34). These results are in agreement with a study of Arango et al in which they show that in HCT116 cells targeted inactivation of p53 significantly increased resistance towards oxaliplatin although in a panel of 30 colorectal cell lines the status of p53 could not predict the response to oxaliplatin(31).

It has been demonstrated previously that oxaliplatin can affect the cell cycle in different stages [7, 31, 47, 48]. In our panel of cell lines, both G1 arrest and delay of S-phase progression were observed but also to some extent arrest in G2/M phase. Since there is not a clear picture for cell cycle arrest it can be concluded that in this panel of cell lines oxaliplatin is not very cell cycle specific and that it is dependent on schedule and the cell line tested. This is in line with the small oxaliplatin induced decrease of the Cyclins A and B1 and of (p)CDC25C. The decreased expression of the Cyclins A and B1 might not be enough to affect cell cycle progression in S and G2/M phase in complex with CDK1 and CDK2, respectively. Also the minor change in (p)CDC25C would probably not be enough to affect CDK1 activation by dephosphorylation of Tyr^{15}. This is in contrast to data of Voland et al (47), who clearly demonstrated that treatment of HCT116 cells with oxaliplatin at 5 μM decreased the protein expression of the Cyclins A and B to 3% and 2%, respectively, compared to untreated control cells and of CDC25C to 38%, which was associated with G2/M block. The increased expression of Cylin A, Cyclin B1 and (p)CDC25 in the ADDP cell line compared to A2780, might be related to the high resistance to cisplatin and to

some extent also to oxaliplatin.

It is generally believed that the activity of platinum compounds is mediated via accumulation and the subsequent formation of DNA adducts followed by activation of DNA damage pathways leading to induction of apoptosis. The levels of platinum adducts formation in LoVo-92 cells are in agreement with the results found by Arnould et al [46] although they were found at a 5 times lower concentration than in our experiments. Furthermore they showed that oxaliplatin formed less DNA adducts as compared to cisplatin. This was also observed in A2780 and AG6000 cells, in which we previously reported 2 and 6 fold higher adduct formation, respectively, for cisplatin compared to the present data on oxaliplatin(41). However in ADDP, 40 fold resistant to cisplatin and 7.5 fold to oxaliplatin, the formation of oxaliplatin DNA adducts was similar to the parental cell line A2780 although the accumulation of oxaliplatin was 2 fold lower. This indicates that ADDP has increased tolerance towards oxaliplatin. The same phenomenon was previously observed with AG6000 cells for cisplatin. The accumulation of cisplatin in AG6000 was about 60 fold lower but adduct formation was similar as the parental cell line A2780(41). Increased tolerance towards cisplatin induced DNA damage has been shown to be associated with resistance(49) and the results in ADDP indicates that this might be similar for oxaliplatin. Comparison of the wt-p53 cell line LoVo-92 and its derivative LoVo-Li, with inactive p53, showed that the 3.6 fold resistance of LoVo-Li corresponded with a similar decrease in oxaliplatin accumulation and 2.6 fold decrease in DNA adducts formation. The decreased accumulation and DNA adducts formation together with the low expression of p53 and increased expression of MDM2 as described above might therefore explain the reduced activity of oxaliplatin in this cell line. In the panel of cell lines tested no significant relation was found between DNA adducts formation and sensitivity towards oxaliplatin. This might be another indication that other mechanisms are involved in the activity of oxaliplatin, such as the induction of a mitochondrial apoptotic response without activation of DNA damage response pathways(3) or specific proteins that influence the apoptotic response like the IAP family member survivin(4, 5)

Recent papers demonstrated that the human copper transporter, hCTR1, mediates uptake of oxaliplatin while also organic cation transporters may play a role, however, the relative contribution is not clear(8, 10, 11). In the cell lines tested for oxaliplatin accumulation, a strong correlation was found with hCTR1 gene expression. Since it has recently been shown that at a concentration of 10 μM oxaliplatin loss of hCTR1 did not result in decreased accumulation(8) this correlation was unexpected because in our experiments a concentration of 200 μM was used. It might be that this correlation is not representative for all cell lines in the panel but only for the selected cell lines.

The gene expression of the organic cation transporters, frequently expressed in colorectal tumor tissue(11), did not correlate with accumulation of oxaliplatin neither did the expression of the efflux transporters ATP7A and ATP7B. However, there was a significant correlation between the hCTR1, OCT2 and ATP7B gene expression and the relative retention of oxaliplatin DNA adducts. Apparently, these transporters are playing a role in the relative retention of oxaliplatin after removal of the drug. Unexpectedly, the expression of ERCC1 showed no relation with the absolute or relative retention of the DNA adducts nor with the sensitivity towards oxaliplatin as shown before[7]. The lack of significant correlations between the gene expression levels and the sensitivity towards oxaliplatin might be hampered by the use of many paired cell lines and and could have reduced the power of the statistical evaluation.

In conclusion: our data show a variable effect of oxaliplatin on the cell cycle and a cell cycle related proteins which may play a role in the resistance of ADDP cells. The expressions of influx and efflux transporters were not significantly correlated with activity of cisplatin and oxaliplatin but were significantly related to the accumulation of oxaliplatin and might be related with DNA adduct formation. Insight in the determinants of oxaliplatin activity and resistance is of crucial importance since the drug is now increasingly used in the treatment of colorectal cancer in combination with 5FU/LV or Irinotecan.

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