# Involvement of drug transporters in the synergistic action of FOLFOX combination chemotherapy

Dirk Theile [1], Sina Grebhardt [1], Walter Emil Haefeli, Johanna Weiss *

*Department of Internal Medicine VI, Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Im Neuenheimer Feld 410, D-69120 Heidelberg, Germany*

ABSTRACT

FOLFOX is a cytostatic drug combination for adjuvant treatment of colorectal cancer (CRC) consisting of 5-fluorouracil (5-FU), leucovorin, and oxaliplatin. The mechanism of synergistic interaction of these drugs is poorly understood and little is known concerning the role of drug transporters and the impact of oxaliplatin metabolites oxalate and dichloro-diaminocyclohexane platinum. We therefore investigated the influence of FOLFOX components on drug transporter expression by quantitative real-time polymerase chain reaction and on the efficacy of each FOLFOX component by proliferation assay in the CRC model cell line LS180. Control experiments with transporter over-expressing cell lines were used to assess the significance of important transporters for the cytostatic activity of FOLFOX components. Moreover, we assessed the pharmacological contribution of the oxalato-ligand to the effect of oxaliplatin. FOLFOX components led to several alterations in expression of drug transporters. For instance, 5-FU significantly suppressed ATP7B and human organic cation transporter 2 and increased multidrug resistance-associated protein (MRP) 2 mRNA expression (5.8-fold). This was accompanied by a significant sensitisation to oxaliplatin. Over-expression of certain ABC-transporters (BCRP/ABCG2, MRP2/ABCC2 or MRP3/ABCC3) was demonstrated to be beneficial for the efficacy of oxaliplatin. The results obtained indicate that both down- and up-regulations of drug transporters could favour synergistic action of this drug combination. Moreover, oxaliplatin metabolite oxalate seems to positively modulate oxaliplatin's action as elucidated by median effect analysis. In conclusion, we propose as one mechanism for FOLFOX synergism the 5-FU mediated suppression of ATP7B, the over-expression of glutathione exporters such as MRP2/ABCC2 and the decrease of glutathione levels by oxalate.

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## 1. Introduction

The addition of oxaliplatin to standard chemotherapy with 5-fluorouracil (5-FU) and folinic acid (leucovorin) (FOLFOX regimen) has substantially improved the outcome of patients with colorectal cancer (CRC) [1]. The mode of synergistic interaction between these compounds, however, is not well investigated. Moreover, also the potential role of drug transporters limiting access of chemotherapeutic agents and co-factors to CRC cells has been addressed [2,3], but their role in the FOLFOX regimen has not been thoroughly investigated.

The ATP-binding cassette (ABC-) transporter superfamily contains several family members that may confer intrinsic or acquired multidrug resistance (MDR) by extruding anticancer agents or their metabolites from cells [4] and suppression of such transporters may lead to sensitisation to cytostatic agents [5]. Indeed, BCRP/ABCG2 and several members of the multidrug resistance-associated protein (MRP/ABCC) family have been identified as folate export transporters [6] and the activity of both, MRP5/ABCC5 and MRP8/ABCC11 have been linked to MDR to 5-FU [7,8].

In contrast, transport processes may also promote antineoplastic action. The distribution of Pt containing antineoplastic agents is regulated by copper homeostasis components. As an example, human copper transporter 1 (hCTR1/SLC31A1) mediates the cellular uptake of copper, cisplatin, and oxaliplatin [9]. In addition, the two P-type ATPases ATP7A and ATP7B are also associated with transport and resistance to oxaliplatin, either by promoting efflux out of the cell or by sequestration into subcellular compartments [10]. Human organic cation transporter 2 (hOCT2/SLC22A2) was also shown to mediate oxaliplatin uptake [11]. Moreover, glutathione, which inactivates cisplatin and oxaliplatin by conjugation [12,13], is exported by several MRPs/ABCCs, either alone, as co-substrate, or in its conjugated form. Thus, enhanced MRP/ABCC expression can lead to decreased cellular glutathione content [14].

The aim of this in vitro study was to scrutinise the mode of synergistic action of FOLFOX components by evaluation of their effects on both expression of drug transporters and cytostatic

* Corresponding author. Tel.: +49 6221 56 39402; fax: +49 6221 56 4642.
*E-mail address:* johanna.weiss@med.uni-heidelberg.de (J. Weiss).
[1] These authors contributed equally to this work.

properties and to assess the pharmacological contribution of the oxalato ligand to oxaliplatin's effect, because it is well known that oxalate has profound effects on redox status [15], production of radical oxygen species [16], and cytotoxic properties of Pt drugs [17].

## 2. Materials and methods

### 2.1. Materials

Culture media, foetal calf serum (FCS), medium supplements, antibiotics, glutamine, phosphate buffered saline (PBS), and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Invitrogen (Karlsruhe, Germany). Acetylcystein, dimethyl sulfoxide (DMSO), 2-(N-morpholino)ethanesulfonic acid (MES), aprotinin, dichloro-diaminocyclohexane (DACH)-platinum (Cl$_2$-DACH-Pt), DACH, leucovorin, 5-FU, and verapamil hydrochloride were purchased from Sigma-Aldrich (Taufkirchen, Germany) and bromphenol blue from Biomol (Hamburg, Germany). Crystal violet, Triton® X-100, NaCl, Tris, dithiothreitol (DTT), glycerol, sodium dodecylsulfate (SDS), H$_2$O$_2$, and oxalic acid were from AppliChem (Darmstadt, Germany). Methanol was from Roth (Karlsruhe, Germany), p-coumaric acid and luminol from Fluka (Basle, Switzerland). MK571 was from BIOMOL Research Laboratories (Plymouth Meeting, USA). 96-Well microtiter plates were from Nunc (Wiesbaden, Germany) and cell culturing bottles were from Greiner (Frickenhausen, Germany). Pheophorbide A (PhA) was from Frontier Scientific Europe (Carnforth, Lancashire, UK) and collagen R and pefabloc were from Serva (Heidelberg, Germany). Calcein acetoxymethyl ester was purchased from MobiTec (Göttingen, Germany), leupeptin and pepstatin from Biomol (Hamburg, Germany), and rhodamine-123 and Tween-20 from Calbiochem (Darmstadt, Germany). Fumitremorgin C was kindly provided by the National Cancer Institute (Rockville, MD, USA). RNA isolation kit RNeasy Midi-Kit was from Qiagen (Hilden, Germany). Cytotoxicity Detection Kit and materials for real time PCR were from Roche Applied Science (Mannheim, Germany). RevertAid$^{TM}$ H Minus First Strand cDNA Synthesis Kit was from Fermentas (St. Leon-Rot, Germany). Absolute QPCR SYBR Green Mix was from Abgene (Hamburg, Germany). QuantiChrom Glutathione Assay Kit was obtained from BioAssay Systems (Hayward, CA, USA). Oxaliplatin and cisplatin were obtained from the University Hospital's pharmacy.

### 2.2. Cell lines

The cell line LS180 (available at ATCC, Manassas, VA, USA), a cell line derived from human colon adenocarcinoma, was used to investigate changes of drug transporter mRNA expression by induction [18] and to estimate the reciprocal effects on cytostatic properties of the drugs used. Cells were cultured under standard cell-culture conditions as described previously [18] and were seeded 3 days before the assay.

As an in vitro model for human Pgp, BCRP, MRP1, MRP2, and MRP3, we used Madin-Darby canine kidney II cells (MDCKII) over-expressing the corresponding transporter. All cell lines were generated by stable cDNA transfection into MDCKII cells and were provided by Dr. Piet Borst and Dr. Alfred H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Parental MDCKII/Par cells served as a control. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated foetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulphate.

The murine monocytic leukaemia cell line P388 and the corresponding doxorubicin-resistant cell line P388/dx over-expressing mdr1a/1b were used to estimate the Pgp inhibiting

properties of DACH and oxalate as described before. This cell system has proved to be the most suitable for evaluation of Pgp inhibition [19].

### 2.3. Proliferation assay

Cell proliferation was quantified by crystal violet staining [20]. Cells were seeded onto collagen-coated 96-well microtiter plates and preincubated for 24 h. After addition of test compounds, cells were incubated for another 48 h. Cells were then washed with PBS and stained with crystal violet 0.5% as described previously [20]. Absorption was measured using a Multiskan RC photometer with 555 nm excitation. Proliferation was expressed as proliferation index by calculating crystal violet absorption intensity as percentage relative to baseline (absorption intensity of medium-only wells (set to 0%)) and native proliferation (absorption intensity of untreated cells (set to 100%)). Each concentration was tested in octuplets and each assay was performed thrice.

### 2.4. Pretreatment of cells with FOLFOX components

LS180 cells were cultured in culture flasks for 24 h to 70% confluence. Subsequently, cells were incubated for another 72 h with the IC$_{20}$ of the particular drug (leucovorin 1.52 mM, 5-FU 7.14 μM, or oxaliplatin 0.20 μM). After incubation, cells were washed, trypsinised, and seeded onto collagen-coated 96-well microtiter plates. The subsequent proliferation assays were conducted as described above.

### 2.5. Quantification of transporters' mRNA expression

RNA was isolated using the RNeasy Mini-Kit. Quality and concentration was measured spectrophotometrically and isolated RNA was stored at −80 °C until analysis. cDNA was synthesised with the RevertAid$^{TM}$ H Minus First Strand cDNA Synthesis Kit according to the manufacturer's instructions. mRNA expression of the respective genes was quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR) with a Light-Cycler$^{TM}$ (Roche Applied Science, Mannheim, Germany). PCR amplification was carried out in 20 μl reaction volume with the LightCycler-FastStart DNA Master SYBR Green I or with the Absolute QPCR SYBR Green Mix. Primers used are summarised in Table 1. The most suitable housekeeping gene for the cytostatic treatment of LS180 cells was identified using geNorm (version 3.4, Center for Medical Genetics, Ghent, Belgium). Among the housekeeping genes tested (β2-microglobulin (β2 mg),

**Table 1**
Primer sequences.

| Gene | Forward primer 5′-3′ | Reverse primer 5′-3′ |
|------|----------------------|----------------------|
| ABCB1 | CCCATCATTGCAATAGCAGG | TGTTCAAACTTCTGCTCCTGA |
| ABCC1 | ATGTCACGTGGAATACCAGC | GAAGACTGAACTCCCTTCCT |
| ABCC2 | ACAGAGGCTGGTGGCAACC | ACCATTACCTTGTCACTGTCCATGA |
| ABCC3 | AACTATGCCCCCGATGAGGACCAA | AGAGTGGAGATGGCGTTGAAGGAAG |
| ABCC4 | AAGTGAACAACCTCCAGTTCCAG | GGCTCTCCAGAGCACCATCT |
| ABCC5 | AGGGGCAAGAAAGAGAAGGTGAGG | GAGGGGGTCGTCCAGGATGTAGAT |
| ABCG2 | AGATGGGTTTCCAAGCGTTCAT | CCAGTCCCAGTACGACTGTGACA |
| SLC31A1 | AGCTGGAGAAATGGCTGGAG | AGGTGAGGAAAGCTCAGCATC |
| ATP7A | ATGATGAGCTGTGTGGCTTG | TGCCAACCTGAGAAGCAATAG |
| ATP7B | TACCCATTGCAGCAGGTGTC | ACTTGAGCTGCAGGGATGAG |
| SLC22A2 | TCTACTCTGCCCTGGTTGAATTC | ATGCAGCCCAAGGGTAACG |
| β2mg | CCAGCAGAGAATGGAAAGTC | CATGTCTCGATCCCACTTAAC |
| G6PDH | ATCGACCACTACCTGGGCAA | TCTGCATCACGTCCCGGA |
| GU | TTCACCAGGATCCACCTCTG | AGCACTCTCGTCGGTGACTG |
| HPRT | CTGGCGTCGTGATTAGTG | CACACAGAGGGCTACAATG |
| RPL13 | GCTCATGAGGCTACGGAAAC | TATTGGGCTCAGACCAGGAG |
| HUPO | AGCTCTGGAGAAACTGCTG | CAGCAGCTGGCACCTTATTG |

glucose-6-phosphate dehydrogenase (G6PDH), glucuronidase β (GU), hypoxanthine-phosphoribosyltransferase 1 (HPRT), ribosomal protein L13 (RPL13), and 60S (human) acidic ribosomal protein P1 (HUPO) GU proved to be the most stable in LS180 cells under the assay conditions. Data were evaluated by calibrator-normalised relative quantification with efficiency correction using the RelQuant software version 1.01 (Roche Applied Science, Mannheim, Germany) as published previously [21]. All samples were amplified in duplicate or triplicate.

## 2.6. Western blot analysis

To confirm induction of MRP2/ABCC2 after 5-FU treatment and reduction of Pgp/ABCB1 after oxaliplatin treatment at protein level, we analysed whole cell lysates of the corresponding cells by western blot analysis. Trypsinised cells were washed once with PBS. The pellet was homogenised on ice in 500 μl lysis buffer (pH 6.5) containing 25 mM MES, 150 mM NaCl and a combination of protease inhibitors (1 mg/ml pefabloc, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). Protein concentrations in the lysates were determined using BCA protein assay kit from Pierce (Rockford, USA) according to the manufacturer's instructions. 20 μg of protein preheated for 5 min at 99 °C in sample buffer (containing Tris–HCl, SDS, DTT, bromphenol blue, and glycerol) were subjected to a 8% (MRP2/ABCC2) or 10% (Pgp/ABCB1) SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose nitrate membranes (Optitran BA-S 85, Schleicher & Schuell BioScience, Dassel, Germany). Blots were blocked by incubation for 1 h with 5% skim milk (w/v) in phosphate buffered saline containing 0.1% Tween-20. Immunoblot analysis was carried out with a monoclonal antibody raised against Pgp/ABCB1 (Clone F4, Sigma, Saint Louis, USA), MRP2/ABCC2 (Anti-MRP2, Sigma, Saint Louis, USA), or β-actin (Clone AC-74) (Sigma-Aldrich, Taufkirchen, Germany) utilised in a dilution of 1:100 (Pgp/ABCB1), 1:250 (MRP2/ABCC2), and 1:40 000 (β-actin), respectively, in the Tris-buffered saline containing 0.1% Tween-20. The blots were then washed extensively and incubated with horseradish peroxidase-linked secondary antibody (GEHealthcare, Munich, Germany). Bands were visualised by enhanced chemiluminescence using 10 ml 100 mM Tris (pH 8.5), 25 μl 90 mM p-coumaric acid, 50 μl 250 mM luminol, and 3 μl 30% $H_2O_2$.

## 2.7. Functional assay for Pgp (rhodamine-123 efflux assay)

To also exemplify the repression of function in addition to RNA and protein repression Pgp activity was measured in oxaliplatin treated LS180 cells using rhodamine-123 efflux. In brief, cells were harvested after the incubation period and incubated with rhodamine-123 at a concentration of 0.4 μM on a rotary shaker (30 min, 37 °C, 450 rpm). Subsequently, cells were washed with precooled medium containing 2% FCS (4 °C) and incubated another 50 min at 37 °C in rhodamine-123 free medium containing 2% FCS to allow rhodamine123 efflux. After washing the cells with precooled PBS containing 2% FCS they were resuspended in this buffer and rhodamine-123 fluorescence was measured in gated LS180 cells using a 530 bandpass filter in a Becton Dickinson LSRII flow cytometer. In each sample 30,000 cells were counted. Median rhodamine-123 fluorescence was determined in cells treated with oxaliplatin and compared to median rhodamine-123 fluorescence of untreated control cells. We did not use the inhibition ratio as described before [21,22], because Pgp/ABCB1 activity is inherently very low in LS180 cells and after reduction by oxaliplatin the inhibition ratio goes towards 1. A change in intracellular rhodamine-123 fluorescence is only an indication and not a proof for changes in Pgp activity, because rhodamine-123 is also transported by other drug transporters.

## 2.8. Cytotoxicity assay

Oxalate and DACH were screened for cytotoxic effects prior to Pgp/ABCB1 and BCRP/ABCG2 inhibition assays with the Cytotoxicity Detection Kit, a colorimetric assay for the quantification of lactate dehydrogenase activity released from the cytosol of damaged cells. Cytotoxicity was expressed as a percentage of the effect obtained with the positive control (total cell lysis). Neither oxalate nor DACH exhibited relevant cytotoxic effects.

## 2.9. Impact of oxaliplatin metabolites on Pgp activity (calcein uptake assay)

For evaluation of possible Pgp inhibiting properties of the oxaliplatin metabolites oxalate (4–4000 μM) and DACH (1–2000 μM) were tested in a calcein assay. The assay was conducted and validated as described previously [19,23]. Each experiment was performed in duplicate on different days.

## 2.10. Impact of oxaliplatin metabolites on BCRP/ABCG2 activity (PhA efflux)

The BCRP/ABCG2 inhibition assay using PhA as fluorescent BCRP/ABCG2 substrate was performed for oxalate (1 μM to 4000 μM) and DACH (1 μM to 2000 μM) as described and validated previously [24]. Each experiment was performed at least in triplicate or in duplicate (if no inhibition was observed) on different days.

## 2.11. Impact of oxaliplatin metabolites on MRP1 activity

For evaluation of possible MRP1 inhibiting properties of the oxaliplatin metabolites oxalate and DACH, both compounds were studied in a MRP1 inhibition assay as described elsewhere [25] with minor modifications. In brief, MDCKII cells over-expressing MRP1 ($6 \times 10^5$ cells) or the corresponding parental cell line were seeded on coverslips in a closed miniperfusion chamber directly before the experiment and preincubated for 30 min with or without the test compound in darkness at 37 °C in 1 ml cell culture medium. CMFDA in a final concentration of 50 nM was subsequently added and incubated for 10 min. The selective MRP inhibitor MK571 (BIOMOL Research Laboratories, Plymouth Meeting, PA) served as positive control at a concentration of 50 μM.

## 2.12. Glutathione assay

Glutathione concentrations were measured using the QuantiChrom Glutathione Assay Kit. The assay was conducted in duplicate according to the manufacturer's instruction with the minor modification of using $2 \times 10^7$ cells.

## 2.13. Effects of combinations of oxaliplatin components

Briefly, either oxalate and $Cl_2$-DACH-Pt or oxalate and cisplatin were concurrently added to MDCKII cells at a fixed ratio of concentrations that were previously applied in the corresponding single drug assays. Proliferation assays were performed as described above. Data were subsequently analysed by the median effect method of Chou and Talalay [26]. Calculated combination indices (CIs) indicate synergism, summation, or antagonism of the two drugs, if CI < 1, CI = 1, or CI > 1, respectively.

We determined $IC_{50}$-values and slopes of the concentration-response curves according to the standard Hill equation. All deduced $D_x$ (=$IC_x$) values were calculated using GraphPad QuickCalcs. For each level of inhibition in this study ($IC_{10}$, $IC_{30}$,

$IC_{50}$, $IC_{70}$, $IC_{90}$), the CI was calculated under the assumption that the two agents were mutually non-exclusive.

## 2.14. Effect of acetylcysteine treatment on glutathione level and resistance

To further clarify the relation between glutathione levels and resistance to $Cl_2$-DACH-Pt, LS180 cells were incubated with 1 mM, 5 mM, or 10 mM of acetylcysteine for 72 h. After incubation, cellular glutathione levels were measured as described above. For proliferation assays, cells were seeded in 96-well plates containing the respective amount of acetylcysteine and were subsequently treated as described above.

## 2.15. Statistical analysis

Data are presented as means ± S.D. and were analysed using GraphPad Prism® Version 5.0 and InStat Version 3.05 (GraphPad Software, San Diego, CA, USA). Differences in resistance or mRNA expression following drug incubation compared with the respective vehicle control were tested using two-tailed unpaired student's t-test compared to the corresponding untreated control. A p-value of $\leq 0.05$ was considered significant.

## 3. Results

### 3.1. Differential effects of FOLFOX components on resistance/sensitivity of LS180 cells

After incubation with oxaliplatin or 5-FU for 72 h, LS180 cells were significantly more sensitive to oxaliplatin. $IC_{50}$ values were approximately 50% of vehicle treated cells. The effect of 5-FU preincubation on 5-FU activity was even greater but did not reach statistical significance. In contrast, leucovorin did not influence the vulnerability of the cells to any of the drugs applied (Fig. 1).

### 3.2. Differential effects of FOLFOX components on mRNA expression of drug transporters

With the exception of hOCT2/SLC22A2, which was suppressed during leucovorin treatment, leucovorin had no impact on drug
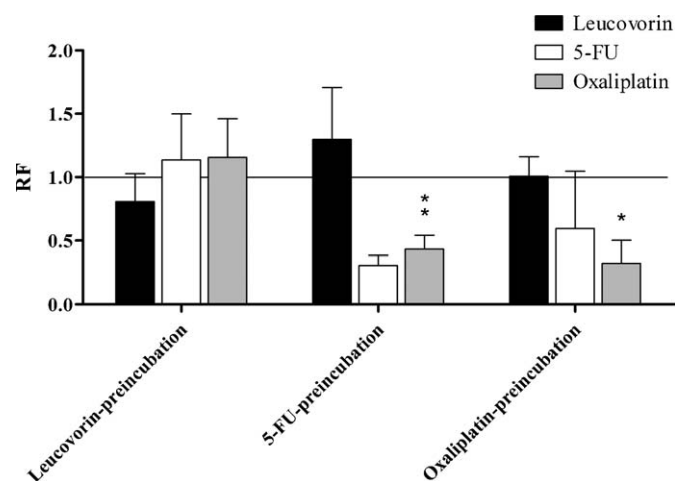
**Fig. 1.** Impact of incubation of LS180 cells with leucovorin, 5-FU, or oxaliplatin for 72 h on resistance to leucovorin (black bars), 5-FU (white bars), and oxaliplatin (grey bars), respectively. Resistance factors (RF) were calculated by division of $IC_{50}$ of preincubated cells with $IC_{50}$ of native cells. RF values are means ± S.D. of at least three independent experiments. Statistical significance was tested by Student's unpaired, two-tailed t-test. $^*p < 0.05$, $^{**}p < 0.01$.
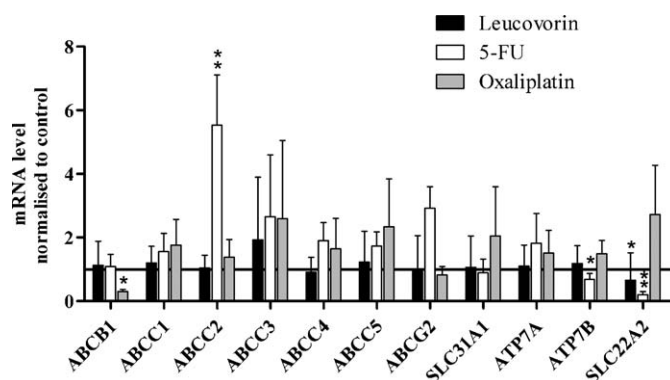
**Fig. 2.** mRNA expression of drug transporters in LS180 cells after incubation with leucovorin (black bars), 5-FU (white bars), or oxaliplatin (grey bars) for 72 h, respectively. Expression levels were related to glucuronidase-β mRNA expression and normalised to vehicle treated control. Statistical significance was evaluated using Student's unpaired, two-tailed t-test (compared to the respective vehicle control). Data are expressed as mean ± S.D. for $n = 5$–9. $^*p < 0.05$, $^{**}p < 0.01$.

transporter mRNA expression. 5-FU treatment significantly suppressed ATP7B and hOCT2/SLC22A2 and increased MRP2/ABCC2 (5.8-fold) mRNA expression. Oxaliplatin treatment only led to a significant suppression of Pgp/ABCB1 (Fig. 2).

### 3.3. Effects of oxaliplatin on Pgp/ABCB1 protein expression and activity and impact of 5-FU on MRP2/ABCC2 protein expression in LS180 cells

To verify whether the changes in mRNA expression of Pgp/ABCB1 and MRP2/ABCC2 observed in LS180 cells after treatment with oxaliplatin and 5-FU translate into protein changes we also analysed the protein expression by western blot. MRP2/ABCC2 protein expression was significantly increased in LS180 cells after incubation with 5-FU (Fig. 3) and Pgp/ABCB1 protein expression and activity was significantly reduced in LS180 cells after incubation with oxaliplatin as demonstrated by western blot (data not shown) and rhodamine-123 efflux assay (decrease of Pgp activity by 16%; $p = 0.02$).

### 3.4. Impact of selected human ABC-transporters on cytostatic activity of FOLFOX components

To estimate the impact of individual ABC-transporters on the cytostatic effect of FOLFOX components, proliferation assays with MDCKII cells over-expressing single human ABC-transporters were conducted. The corresponding parental cell line was used as a control. None of the investigated ABC-transporters mediated

**Fig. 3.** Western blot analysis of whole cell lysates of LS180 cells treated for 72 h with 7.14 μM 5-FU. Three independent experiments (#1–3) were conducted. MDCKII (low expression of canine MRP2) and MDCKII-MRP2 (over-expression of human MRP2) cells served as controls. β-actin served as a marker for approximately similar protein content of all samples.
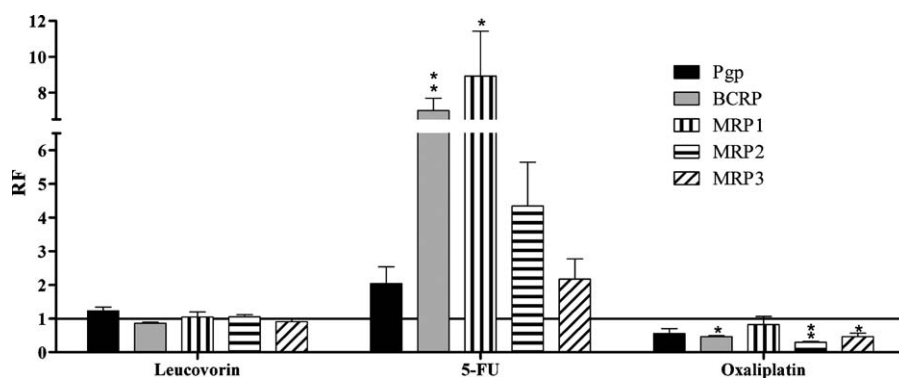
**Fig. 4.** Effect of leucovorin, 5-FU, and oxaliplatin on proliferation of MDCKII cells over-expressing Pgp/ABCB1, BCRP/ABCG2, MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3, respectively. Resistance factors (RF) were calculated by division of $IC_{50}$ of ABC-transporter over-expressing cells with $IC_{50}$ of parental cells. RF values are means $\pm$ S.D. of at least three independent experiments. Statistical significance was evaluated using Student's unpaired, two-tailed $t$-test (compared to the respective vehicle control). $^*p < 0.05$, $^{**}p < 0.01$.

resistance to leucovorin. Cells over-expressing BCRP/ABCG2 or MRP1/ABCC1 were extraordinarily resistant to 5-FU, whereas BCRP/ABCG2, MRP2/ABCC2, and MRP3/ABCC3 over-expressing cell lines were hypersensitive to oxaliplatin (Fig. 4).

### 3.5. Impact of selected human ABC-transporters on cytostatic activity of oxaliplatin metabolites, cisplatin, and combinations of compounds

MDCKII cells over-expressing BCRP/ABCG2 or MRP2/ABCC2 were partially resistant to cisplatin. All investigated ABC-transporters mediated resistance to oxalate. In addition, cells over-expressing Pgp/ABCB1, MRP1/ABCC1, MRP2/ABCC2, or MRP3/ABCC3 were also resistant to DACH. MDCKII cells over-expressing Pgp/ABCB1 were partially resistant and those expressing MRP1/ABCC1 were hypersensitive to $Cl_2$-DACH-Pt. When oxalate and $Cl_2$-DACH-Pt were combined, MDCKII cells over-expressing Pgp/ABCB1, MRP1/ABCC1, or MRP2/ABCC2 were resistant to this combination. In contrast, MDCKII cells over-expressing BCRP/ABCG2 or MRP3/ABCC3 were hypersensitive. Control experiments with the combination of oxalate with cisplatin revealed MDCKII-MRP1 cells to be partially resistant and MDCKII cells over-expressing MRP3/ABCC3 to be hypersensitive to this combination (Fig. 5).

### 3.6. Measurement of glutathione content and correlation with cytostatic activity of $Cl_2$-DACH-Pt

Normalised to parental MDCKII cells, MDCKII cells over-expressing Pgp/ABCB1 revealed significantly higher glutathione values, whereas MDCKII cells over-expressing MRP1/ABCC1 or MRP3/ABCC3 contained significantly less glutathione. The difference in glutathione content between MDCKII cells over-expressing MRP2/ABCC2 or BCRP/ABCG2, and their parental counterpart was approximately 10%, but did not reach statistical significance (data not shown). However, there was a strong correlation (Spearman correlation coefficient $r = 0.94$) between glutathione content and $IC_{50}$ of $Cl_2$-DACH-Pt (Fig. 6).

### 3.7. Evaluation of interaction between oxalate and $Cl_2$-DACH-Pt

Analysis of combination indices revealed that the combination of oxalate and cisplatin acts additively (Fig. 7), whereas the interaction of oxalate and $Cl_2$-DACH-Pt was synergistic with CI values generally <1 (Fig. 8). Different extents of proliferation inhibition resulted in different degrees of synergism. However, in any cell line tested the combination indices for the combination of oxalate and $Cl_2$-DACH-Pt were significantly lower than the
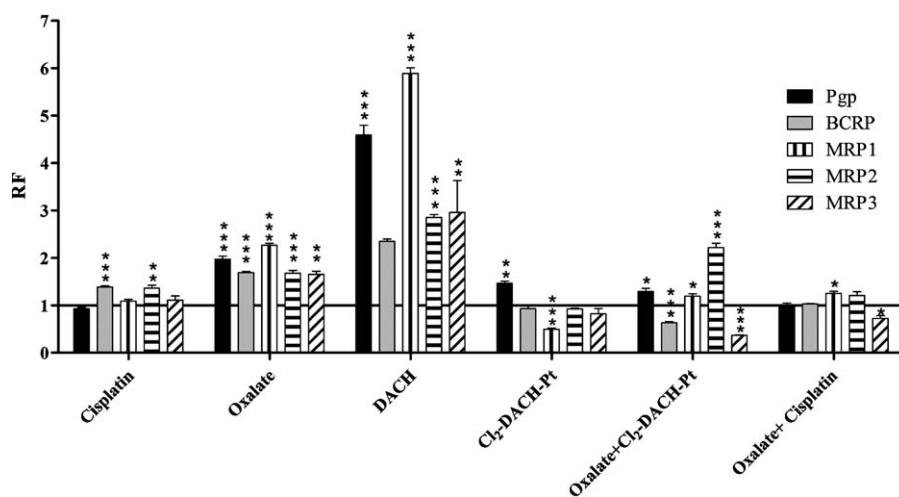


**Fig. 5.** Effect of cisplatin, oxalate, DACH, $Cl_2$-DACH-Pt, combined application of oxalate with $Cl_2$-DACH-Pt, and combined application of oxalate with cisplatin on proliferation of MDCKII cells over-expressing Pgp/ABCB1, BCRP/ABCG2, MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3, respectively. Resistance factors (RF) were calculated by division of $IC_{50}$ of human ABC-transporter over-expressing cells with $IC_{50}$ of parental cells. RF values are means $\pm$ S.D. of at least three independent experiments. Statistical significance was evaluated using Student's unpaired, two-tailed $t$-test (compared to the respective vehicle control). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$.
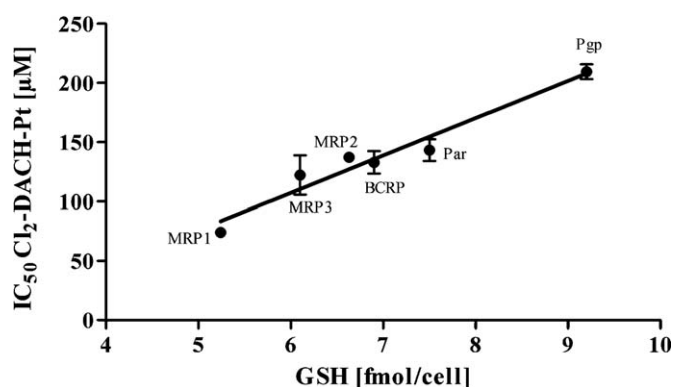
**Fig. 6.** Correlation of intracellular glutathione content and $IC_{50}$ of $Cl_2$-DACH-Pt among MDCKII cells over-expressing Pgp/ABCB1, BCRP/ABCG2, MRP1/ABCC1, MRP2/ABCC2, MRP3/ABCC3, respectively, and the corresponding parental cell line. Spearman correlation coefficient ($r = 0.94$) was calculated with six XY pairs; $p$-value = 0.0167.
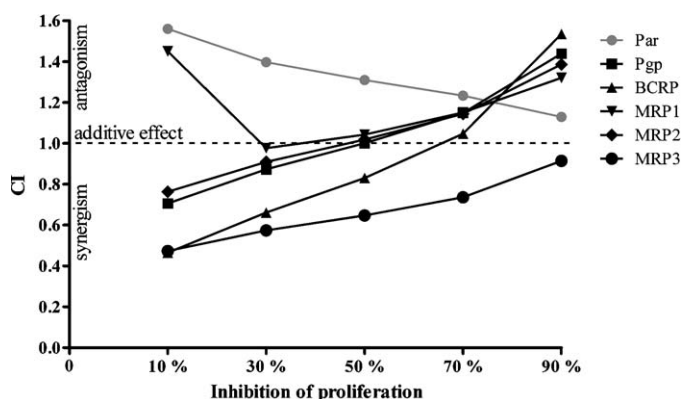


**Fig. 7.** Median effect analysis of combined cytostatic properties of oxalate and cisplatin in MDCKII cells over-expressing Pgp/ABCB1, BCRP/ABCG2, MRP1/ABCC1, MRP2/ABCC2, MRP3/ABCC3, respectively, and the corresponding parental cell line.

corresponding values in the control experiment comprising the combination of oxalate and cisplatin suggesting oxalate to be a pharmacodynamically relevant ligand that specifically contributes to oxaliplatin's action. In addition, the most profound synergistic interaction was always observed in MDCKII over-expressing MRP3/ABCC3 (Fig. 7,8).
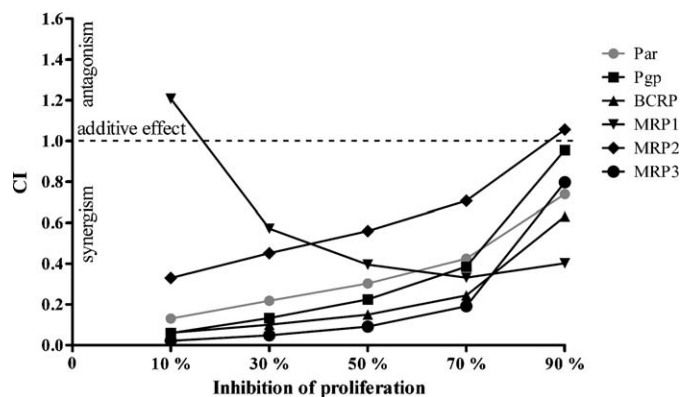


**Fig. 8.** Median effect analysis of combined cytostatic properties of oxalate and $Cl_2$-DACH-Pt in MDCKII cells over-expressing Pgp/ABCB1, BCRP/ABCG2, MRP1/ABCC1, MRP2/ABCC2, MRP3/ABCC3, respectively, and the corresponding parental cell line.

### 3.8. Impact of oxalate and DACH on Pgp/ABCB1, BCRP/ABCG2, and MRP1/ABCC1 activity

To exclude that FOLFOX synergism can be attributed to drug accumulation mediated by ABC-transporter inhibition by oxaliplatin metabolites, both oxalate and DACH were screened for Pgp/ABCB1, BCRP/ABCG2, and MRP1/ABCC1 inhibiting properties up to concentrations of 4 mM and 2 mM, respectively. Neither oxalate nor DACH had profound impact on Pgp/ABCB1 or BCRP/ABCG2 activity. Only at 2 mM DACH showed slight BCRP/ABCG2 and MRP1/ABCC2 inhibiting properties (data not shown).

### 3.9. Effect of acetylcysteine treatment on glutathione level and resistance

To increase the cellular glutathione levels, cells were treated with different concentrations of acetylcysteine. Only 10 mM acetylcysteine caused a significant increase in glutathione content ($1.8$-fold $\pm 0.6$, $p < 0.05$). Cells being cultured in media containing 5 or 10 mM acetylcysteine were significantly more resistant to $Cl_2$-DACH-Pt than their respective untreated counterparts (resistance factor for 5 mM: $18.2 \pm 0.4$, $p < 0.0001$; for 10 mM: $22.1 \pm 3.9$, $p < 0.0001$).

## 4. Discussion

Multidrug resistance increasingly requires anti-cancer combination therapy. The aim of this in vitro study was to elucidate the transcriptional alterations of drug transporters' mRNA expressions and functional alterations following the exposure of LS180 cells to individual components of the FOLFOX regimen. Functionally, preincubation with oxaliplatin or 5-FU both sensitised LS180 cells to oxaliplatin. Incubation with oxaliplatin was accompanied by a decrease of mRNA and protein expression of Pgp/ABCB1. In addition, oxaliplatin led to a significant increase of accumulation of rhodamine-123 in LS180 cells indicating diminished Pgp function.

Although we could not demonstrate leucovorin to be a substrate of Pgp in MDCKII cells (Fig. 4), it is plausible that suppression of this drug transporter can lead to leucovorin accumulation with subsequently enhanced efficacy of 5-FU (Fig. 1) as Pgp was formerly reported to efflux folate derivatives such as methotrexate [27].

5-FU treatment led to several statistically significant mRNA alterations. First, mRNA expression of one of oxaliplatin's importers (hOCT2/SLC22A2) [11] and of the exporter ATP7B [28] was suppressed. Secondly, 5-FU increased mRNA and protein expression of MRP2/ABCC2. These data raised the question of whether expressional alterations of drug transporters–especially MRP over-expression – could generally increase drug efficacy. To further clarify the relationship between ABC-transporter expression and cytostatic properties of FOLFOX components proliferation assays with MDCKII cells over-expressing individual human ABC-transporters and the corresponding parental cell line were performed. In contrast to other reports [6], none of the investigated ABC-transporters mediated resistance to leucovorin, which might be attributable to a different in vitro cell model used by us. Over-expression of BCRP/ABCG2 and MRP1/ABCC1 was related to resistance to 5-FU, as it was previously demonstrated for BCRP [29]. To date, there are no reports demonstrating MRP1 to be a MDR-mediating transporter of 5-FU. In addition, BCRP/ABCG2 and MRP1/ABCC1 mediated resistance to 5-FU could also be the result of their ability to extrude folates [6] and thereby bypassing the biochemical synergism between folates and 5-FU. Over-expression of BCRP/ABCG2, MRP2/ABCC2, and MRP3/ABCC3 was associated with hypersensitivity to oxaliplatin suggesting that 5-FU mediated induction of MRPs/ABCCs in LS180 cells provoked hypersensitivity

to oxaliplatin. These results are in contrast to those of Ceckova et al. [30], who demonstrated MDCKII cells transfected with BCRP transporter and enhanced GFP (EGFP) to be resistant to oxaliplatin in comparison to the non-transfected parental cells suggesting BCRP to be an oxaliplatin resistance mediating ABC-transporter. To date, there is no obvious rationale for this discrepancy. However, the authors also attribute their findings with Pt-drugs at least partly to the high expression of EGFP in their cell system.

Oxaliplatin is non-enzymatically metabolised into several distinct moieties such as oxalate and the cytostatically active remnant $Cl_2$-DACH-Pt [31]. Proliferation assays with these compounds indicate that oxalate is a substrate of all ABC-transporters investigated. DACH seems to be a substrate of at least Pgp/ABCB1, MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3. The only moderate increases of $IC_{50}$ values (<50%) suggest that $Cl_2$-DACH-Pt is not a substrate of Pgp/ABCB1, BCRP/ABCG2, MRP2/ABCC2, or MRP3/ABCC3. In contrast, over-expression of MRP1/ABCC1 was associated with hypersensitivity to this oxaliplatin metabolite suggesting better efficacy of oxaliplatin in tumours over-expressing this particular ABC-transporter.

Because oxaliplatin is a much more effective cytostatic agent than its metabolite $Cl_2$-DACH-Pt, we applied the mathematical procedure introduced by Chou and Talalay to investigate oxalate's contribution to the cytostatic properties of oxaliplatin [25]. By combining oxalate and $Cl_2$-DACH-Pt oxaliplatin was supposed to be reconstituted in vitro. We combined the same concentrations as in the corresponding single substance experiments to draw unbiased conclusions from this combination experiment. Comparison with control experiments comprising the combination of oxalate and cisplatin revealed that oxalate synergistically amplified $Cl_2$-DACH-Pt action highlighting its relevance for oxaliplatin. As shown in Fig. 6, sensitivity to $Cl_2$-DACH-Pt correlates with glutathione content in MDCKII cells and increasing glutathione concentration significantly increased resistance against $Cl_2$-DACH-Pt. These findings therefore suggest that the oxalato ligand of oxaliplatin contributes to oxaliplatin's cytostatic effect by a further decrease of cellular glutathione content [15] thus attenuating the detoxification capacity of the cells.

We therefore propose the following mechanism that might contribute to FOLFOX synergism: 5-FU mediated suppression of ATP7B leads to diminished sequestration capacities for oxaliplatin. Concurrently, over-expression of MRPs increases efflux of reduced glutathione and thereby reduces the cellular capacities

of detoxifying oxaliplatin or its active metabolite (Fig. 9). In addition, oxalate also decreases glutathione levels hence further amplifying oxaliplatin action. In contrast, the impact of the observed down-regulation of the oxaliplatin importer hOCT2/SLC22A2 remains open as it did not cause oxaliplatin resistance in our experimental setting.

Moreover, our results offer an explanation for the observation by Langer et al. showing high pretherapeutic MRP1 expression in locally advanced adenocarcinomas of the oesophagus to be beneficial for the treatment with Pt drugs like cisplatin [32].

In conclusion, this in vitro study yields further insight into the potential mechanism of synergistic action of FOLFOX combination chemotherapy. First, anti-cancer drugs can lead to several alterations of drug transporters expression potentially favouring reciprocal accumulation of drugs leading to superior efficacy. Secondly, both down- and up-regulation of ABC-transporter expression can lead to higher drug efficacy confirming earlier findings [33–35].

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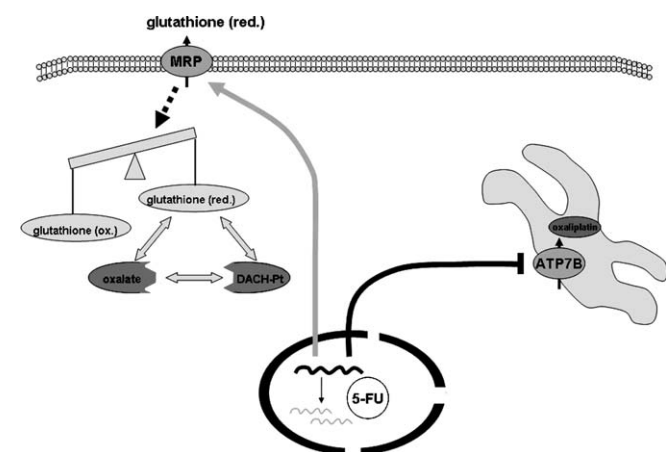


**Fig. 9.** Proposed main mechanism for the synergism of 5-FU and oxaliplatin in the context of FOLFOX chemotherapy: 5-FU mediated suppression of ATP7B leads to diminished sequestration capacities for oxaliplatin into the endoplasmatic reticulum. In addition, induced over-expression of MRPs promotes efflux of reduced glutathione resulting in cellular glutathione depletion, which in turns reduces detoxification capacities for oxaliplatin and its active metabolite. In addition, oxalate can further decrease glutathione levels.

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