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Defining the role of MRP-mediated efflux and glutathione in detoxification of oxaliplatin

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Dedicated to Professor Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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Albeit platinum complexes are widely used in cancer chemotherapy, their cellular processing has not been completely elucidated so far. In this study the effects of modulating multidrug resistance-associated protein (MRP)-mediated efflux and glutathione (GSH) depletion on the cytotoxicity of oxaliplatin were assessed in a human ileocecal colorectal adenocarcinoma cell line and its oxaliplatin-resistant variant. Upon oxaliplatin exposure, DNA platination was elevated by co-incubation with Gü83, a MRP1 and MRP2 inhibitor, but cytotoxicity was not increased. Addition of oxaliplatin did not alter the cellular GSH content. Following GSH depletion, platinum accumulation was unchanged but cytotoxicity was increased in oxaliplatin-sensitive cells. In conclusion, modulation of MRP-mediated efflux did not affect oxaliplatin cytotoxicity in the investigated cell lines. Intracellular GSH depletion seems to sensitize the cells but does not overcome resistance.

1. Introduction

Chemotherapy regimens based on the platinum complexes cisplatin, carboplatin and oxaliplatin are used in the treatment of diverse solid tumors. Cisplatin and carboplatin are effective in the same tumor entities, on the contrary oxaliplatin can be used in the treatment of tumors which are intrinsically resistant to cisplatin and carboplatin, such as colorectal cancer (Kelland 2007). Besides toxicity, acquired or intrinsic drug resistance is the main obstacle in successfully treating cancer patients with platinum complexes. Resistance derives from a multitude of altered cellular processes resulting in reduced cellular platinum accumulation, such as reduced uptake or increased efflux, and in increased inactivation of platinum complexes. Moreover, increased repair of platinum-DNA adducts and increased tolerance to platinum-DNA adducts contribute to platinum resistance (Galluzzi et al. 2012). Also the apoptotic response resulting from the formation of platinum-DNA adducts (or other platinum compound induced damage) can be reduced. In this context the role of genes regulating DNA damage, apoptosis and survival signaling has been discussed (Stewart 2007).

With regard to reduced accumulation, it is by now accepted that active transport is involved in platinum uptake and efflux and hence contributes to reduced platinum accumulation found in resistant cells. The copper transporter CTR1 plays a role in the uptake of platinum complexes and CTR1-deficient cells were described to be cisplatin-resistant (Stewart 2007). The copper-transporting P-type adenosine triphosphatases ATP7A and ATP7B are discussed in the context of increased efflux (Kalayda et al. 2008; Stewart 2007) as well as the multidrug resistance-associated proteins (MRP) (Galluzzi et al. 2012).

MRP are known to be involved in resistance to various drugs and in the phenomenon of multidrug resistance in cancer. They belong to the superfamily of ATP-binding cassette (ABC) transporters (Jedlitschky et al. 2006) and are expressed in tissues that require protection from exogenous substances including liver, intestines and kidney. However, beside toxins they also transport endogenous substances (Leitner et al. 2007). Experiments in Madin-Darby canine kidney (MDCKII) cells stably expressing either MRP1 or MRP2 have indicated that cisplatin is transported by both, MRP1 and MRP2 (Wortelboer et al. 2008). The results of cell culture experiments, however, predominately suggest an association of MRP2 but not MRP1 overexpression with cisplatin resistance (Bracht et al. 2007; Burger et al. 2011; Cnubben et al. 2005; Noma et al. 2008). The role of MRP in efflux of and resistance to oxaliplatin has not been investigated intensively but increased levels of MRP2 and unchanged levels of MRP1 were seen in oxaliplatin-resistant colon cancer cells (Liu et al. 2010). We recently reported an increase in platinum accumulation after incubation with oxaliplatin and simultaneous inhibition of MRP1 and MRP2 in an ileocecal colorectal adenocarcinoma cell line (Mohn et al. 2010).

Glutathione (GSH) is described to contribute to platinum resistance by binding and inactivating platinum complexes and reducing platinum complex-induced oxidative stress (Stewart 2007). Increased cellular GSH levels have been reported in cells resistant to platinum complexes but overall GSH levels do not correlate with sensitivity towards platinum complexes (Boubakari et al. 2004; Bracht et al. 2006). Besides GSH, the rate-limiting enzyme in intracellular GSH synthesis, γ-glutamylcysteine synthetase (γGCS), and the enzyme catalyzing reactions of GSH with substrates, glutathione *S*-transferase

Table 1: Sensitivity of the cell lines used towards cisplatin and oxaliplatin investigated using an MTT-based cytotoxicity assay (mean pEC_{50} ± SEM, n = 3–11)

Cell line	pEC_{50} (EC_{50})	
	Cisplatin	Oxaliplatin
HCT-8	4.96 ± 0.10 (11.0 μM)	5.56 ± 0.06 (2.8 μM)
HCT-8ox	4.53 ± 0.12 (29.5 μM)	4.47 ± 0.05 (33.9 μM)
A2780	5.68 ± 0.08 (2.1 μM)	6.03 ± 0.26 (0.93 μM)
A2780cis	4.93 ± 0.07 (11.7 μM)	5.43 ± 0.14 (3.7 μM)

(GST), are probably involved in platinum resistance (Galluzzi et al. 2012). It was postulated by many authors that inside the cell the reactive aqua species of the platinum complexes form platinum–GSH adducts that are excreted from the cell by transporters such as MRP (Galluzzi et al. 2012; Hall et al. 2007; Ishikawa and Ali-Osman 1993; Kelland 2007). Oxaliplatin–GSH adducts were described but have not been identified in tumor cells so far (Luo et al. 1999; Mohn et al. 2010).

Considering that GSH is a substrate of MRP1 and MRP2 itself and that it was found to stimulate MRP-mediated transport of substances, different mechanisms of platinum efflux are possible (Ballatori et al. 2009). In general, four scenarios are likely: A drug–GSH adduct could be transported, drug efflux could be stimulated by GSH, there could be GSH drug co-transport or a drug could stimulate GSH efflux but is not a substrate itself (Cnubben et al. 2005). According to the literature, in case of platinum complexes the first scenario is most likely. Nevertheless, currently available reports are contradictory as after experiments in cell models it was suggested that GSH does not play a role in cisplatin transport (Wortelboer et al. 2008).

The mechanism of GSH-mediated platinum resistance, however, is more complex and may be different in distinct cells. On the one hand, GSH is involved in signal transduction and on the other hand the results of *in vitro* experiments showing the formation of platinum–GSH adducts cannot be easily transferred to intracellular processes in animals or humans. After incubation of cell lysate produced from cisplatin-resistant ovarian cancer cells, mainly macromolecular and no GSH adducts with cisplatin were identified (Kasherman et al. 2009). Thus, the role of GSH in platinum resistance is still a matter of research and its clinical impact needs to be assessed.

To better comprehend platinum resistance and involved cellular processes, the aim of this project was to reveal whether the inhibition of MRP1 and MRP2 has an impact on the cytotoxicity of oxaliplatin. As an efflux of platinum–GSH adducts via MRP was postulated, the impact of GSH on oxaliplatin cytotoxicity was also investigated.

2. Investigations and results

2.1. Cytotoxicity

Experiments were performed using human cancer cell lines such as the human ileocecal colorectal adenocarcinoma cell line HCT-8 and the oxaliplatin-resistant variant HCT-8ox as well as the ovarian carcinoma cell line A2780 and the cisplatin-resistant variant A2780cis. The sensitivity of the cell lines towards cisplatin and oxaliplatin was determined using the MTT assay (results are shown in Table 1). On the basis of the EC_{50} values, the resistance factors of the cell lines were calculated. Both resistant cell lines were not only resistant to the platinum complex used for inducing resistance but were also cross-resistant to the second platinum complex tested (resistance factor HCT-8ox/HCT-8: cisplatin 2.7, oxaliplatin 12.1; resistance factor A2780cis/A2780: cisplatin 5.6, oxaliplatin 4.0).

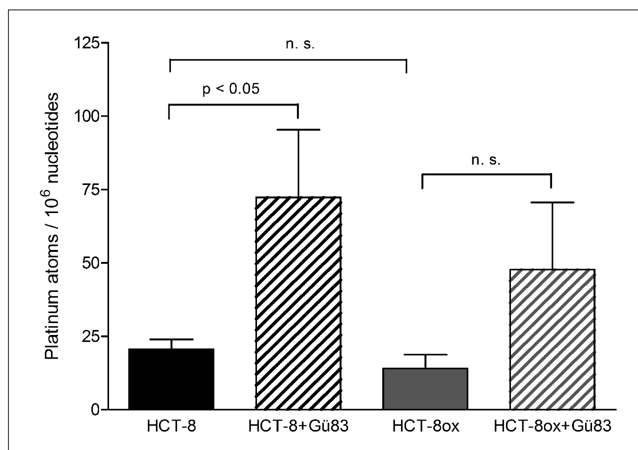

Fig. 1: DNA platination after 3 h incubation with 200 μM Gü83 and 100 μM oxaliplatin (mean ± SD, n = 3, unpaired t-test; n.s.: not significant).

2.2. MRP1 and MRP2 inhibition

Gü83, a 4-aminobenzoic acid derivative recently shown to inhibit MRP1 (IC_{50} = 1.2 μM) and MRP2 (IC_{50} = 21.5 μM) and to increase oxaliplatin accumulation in HCT-8 and HCT-8ox cells (Leyers et al. 2008; Mohn et al. 2010), was used to explore the impact of MRP inhibition on DNA platination and oxaliplatin cytotoxicity.

DNA was isolated using a QIAmp™ DNA Mini Kit for solid phase extraction and platinum was determined by ICP-MS. The results illustrate that Gü83 led to an increase in DNA platination (Fig. 1). The increase was statistically significant in the sensitive HCT-8 cells only.

As MRP modulators have an effect on cellular platinum accumulation and DNA platination, they could as well alter the sensitivity of cells towards platinum complexes. To test this hypothesis the MTT assay was performed with oxaliplatin alone and in the presence of Gü83. It was found that EC_{50} values increased when cells were coincubated with 100 μM Gü83 (Table 2). The effect was found to be significant in HCT-8 cells.

2.3. Cellular glutathione content

The GSH content of the two corresponding sensitive/resistant cell line pairs HCT-8/HCT-8ox and A2780/A2780cis was fluorometrically determined after cell lysis and derivatization with naphthalene-2,3-dicarboxaldehyde (NDA). As can be seen in Fig. 2 the GSH content was the same in HCT-8 and HCT-8ox cells but was about 2.5 times higher in A2780cis compared to A2780 cells. The GSH content in the sensitive ovarian carcinoma

Table 2: Cytotoxicity of oxaliplatin with or without coincubation with 100 μM Gü83 (mean pEC_{50} ± SEM, n = 4, paired t-test)

Cell lines	pEC_{50} (EC_{50})		
	+ Gü83	− Gü83	p value
HCT-8	5.35 ± 0.09 (4.5 μM)	5.45 ± 0.08 (3.5 μM)	0.03
HCT-8ox	4.62 ± 0.22 (24.0 μM)	4.84 ± 0.11 (14.4 μM)	0.15

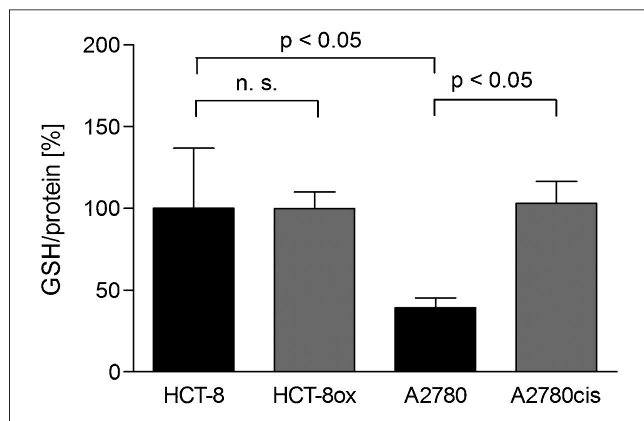

Fig. 2: Cellular GSH content related to the protein content in untreated cells (mean ± SD, n = 3, unpaired t-test; n. s.: not significant). The mean value in HCT-8 cells was set to 100%.

A2780 cells was significantly lower than in the sensitive ileocecal colorectal adenocarcinoma HCT-8 cells. The cell line with the lowest GSH content (A2780) was most sensitive towards cisplatin as well as oxaliplatin. Exhibiting similar GSH levels the three cell lines with higher GSH content differed in their sensitivity towards the two platinum complexes investigated.

2.4. Effect of platinum incubation on the cellular glutathione content

To investigate the effect of platinum complexes on the cellular GSH content, cells were incubated with platinum complexes for 24 h. Control cells were incubated with PBS only. Samples were taken after 0, 4, 8, 12 and 24 h and the GSH and protein content was assessed. A small increase in the cellular GSH content was observed in HCT-8 and HCT-8ox cells with a faster onset in the resistant variant. In A2780 cells the increase in cellular GSH was of a higher magnitude than in A2780cis cells. A two-way ANOVA was performed for statistical analysis of the results describing the effect of oxaliplatin or cisplatin on GSH content over time. According to the test results, incubation of cells with the platinum complexes did not significantly influence the cellular GSH content in the cell lines investigated over time. Only in A2780cis cells the factor 'time' affected GSH content significantly (see Fig. 3).

2.5. GSH depletion

To further elucidate the role of GSH in respect of platinum cytotoxicity and accumulation, intracellular GSH was depleted. For this, buthionine sulfoximine (BSO), a GSH analogue reversibly inhibiting γ-glutamylcysteine synthetase (γGCS), the rate-limiting enzyme in GSH synthesis (Anderson 1998), was used. The amount of BSO used in these experiments was not cytotoxic over the time studied as confirmed by the MTT assay. In preliminary experiments the concentration–effect relationship of BSO over the relevant time periods was studied. After 12 h incubation with 100 μM BSO, GSH content was $\leq$ 30% and after 72 h incubation with 50 μM BSO $\leq$ 15% of the baseline level.

GSH depletion led to slightly higher pEC_{50} values of oxaliplatin (corresponding to lower EC_{50} values) but the effect was only statistically significant in HCT-8 cells (see Table 3). The cellular platinum accumulation was not affected by GSH depletion after 12 h incubation with 100 μM BSO as can be seen in Fig. 4.

3. Discussion

3.1. Role of MRP-mediated oxaliplatin efflux

To achieve a better understanding of oxaliplatin detoxification, one part of this project focused on the effect of inhibition of the transport of oxaliplatin by MRP1 and MRP2 on oxaliplatin cytotoxicity. The observed increase in platinum accumulation after addition of the MRP1 and MRP2 inhibitor Gü83 in HCT-8 and HCT-8ox cells described earlier (Mohn et al. 2010) led to an increase in DNA platination. However, when the impact of Gü83 on oxaliplatin cytotoxicity was investigated the expected increase of oxaliplatin cytotoxicity was not observed. In contrast, slightly higher EC_{50} values indicated a reduced cytotoxic effect. These results suggest that MRP-mediated efflux takes place but is not decisive for cytotoxicity in the cells investigated. Although it is widely recognized that reduced cellular platinum accumulation is associated with resistance, it has also been described that this correlation is not necessarily seen in all types of cancer cell lines (Johnson et al. 1997). However, as platinum accumulation is reduced in the oxaliplatin-resistant cell line HCT-8ox compared to its sensitive counterpart HCT-8, an association of oxaliplatin sensitivity and cellular platinum accumulation is likely (Mohn et al. 2010). One possible explanation is that MRP1 and MRP2 inhibition leads to increased cellular accumulation of oxaliplatin metabolites, such as mono-GSH adducts, which still bind to DNA but are not capable anymore to form inter- or intrastrand crosslinks.

A limitation of our results is that the conditions of the MTT assay differed from the conditions used for measuring DNA platination. Whereas DNA platination was assessed after 3 h oxaliplatin incubation, the protocol of the MTT assay included an incubation period of 72 h. Thus, it is possible that the MRP inhibition is only effective during a relatively short incubation period. Modifications in the protocol of the MTT assay or further methods should be applied for confirming that MRP inhibition does not affect oxaliplatin cytotoxicity in the cells investigated. Moreover, there are hints that the mechanism of action of platinum complexes involves more than the processes initiated by DNA platination. For cisplatin involvement of the endoplasmic reticulum and for oxaliplatin involvement of mitochondria in apoptosis was shown in enucleated cells (Gourdier et al. 2004; Mandic et al. 2003). Thus, an increase in DNA platination as facilitated by Gü83 not necessarily leads to an increased sensitivity towards oxaliplatin. Also, there might be other cellular targets, e.g. in mitochondria, that were not affected by the MRP modulator. With regard to oxaliplatin the achieved increase in DNA platination by Gü83 is obviously not helpful in sensitizing cells.

3.2. Role of glutathione

An increased cellular GSH content has been observed in cisplatin-resistant and oxaliplatin-resistant cells repeatedly (Ballatori et al. 2009; Chen et al. 1995; Chen and Kuo 2010;

Table 3: Impact of GSH depletion induced by 12 h pre- and 72 h coincubation with 50 μM BSO on oxaliplatin cytotoxicity determined by the MTT assay (mean pEC_{50} ± SEM, n = 4–5, paired t-test)

Cell lines	pEC_{50} (EC_{50})		
	+ BSO	− BSO	p value
HCT-8	5.74 ± 0.17 (1.8 μM)	5.57 ± 0.19 (2.7 μM)	p = 0.02
HCT-8ox	4.47 ± 0.05 (33.9 μM)	4.37 ± 0.10 (42.7 μM)	p = 0.24

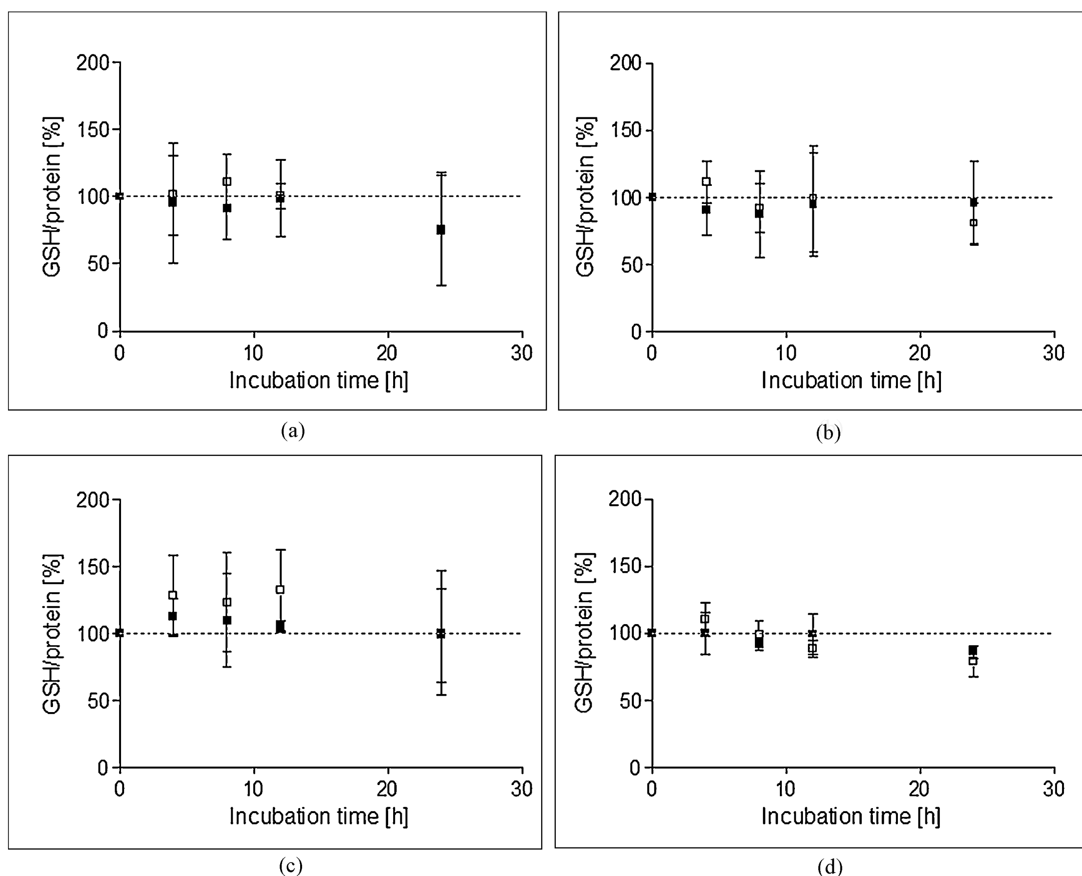

Fig. 3: Cellular GSH content related to protein relative to baseline value in **(a)** HCT-8 and **(b)** HCT-8ox cells upon incubation with (□) or without (■) 100 nM oxaliplatin and in **(c)** A2780 and **(d)** A2780cis cells upon incubation with (□) or without (■) 100 nM cisplatin (mean ± SD, n = 3).

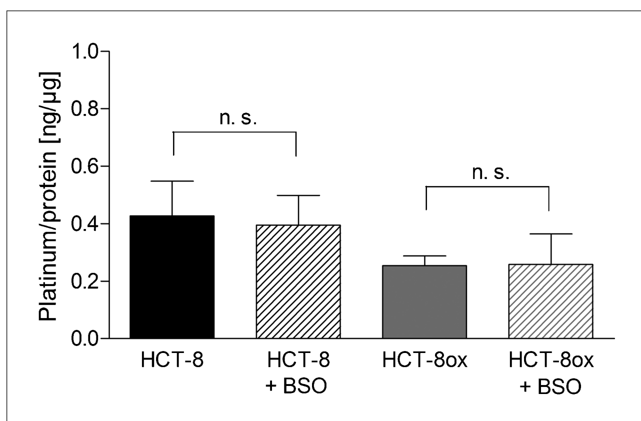

Fig. 4: Cellular platinum accumulation related to protein content after 2 h incubation with 100 µM oxaliplatin with or without 12 h preincubation with 100 µM BSO (mean ± SD, n = 5, paired t-test; n.s.: not significant).

El-Akawi et al. 1996; Hector et al. 2007). But considering cells of different tumor entities it was also reported that sensitivity to cisplatin and oxaliplatin did not correlate with the cellular GSH content as it was the case for the cells used in our project (Arnould et al. 2003; Boubakari et al. 2004; Bracht et al. 2006; Johnson et al. 1997). It is, however, noteworthy that the cells with the lowest GSH content, A2780, were most sensitive to cisplatin and oxaliplatin.

The GSH content did not increase after addition of a platinum complex. In human melanoma cells and in MDCKII cells stably overexpressing MRP1 and MRP2 a change in reduced GSH content was not seen after addition of cisplatin either (Pendyala et al. 1997; Wortelboer et al. 2008). In HCT-8 variants resistant to cisplatin a 6-fold increase in GSH content has been reported after cisplatin incubation (Goto et al. 1995). These results reflect the heterogeneity in data on GSH response to platinum complexes. The effect is probably dependent on cell type and baseline GSH content but could also differ among individual platinum complexes. Moreover, we preferred to use non-toxic concentrations of the platinum complexes which could have been too low to observe a distinct effect on GSH content. In general, comparability of GSH levels between the cells employed in this and other research projects is rather limited since methods used for cell lysis and for GSH determination differed. Some authors have measured total GSH by reducing oxidized GSH as we did but often only reduced GSH has been determined.

GSH depletion was used to further clarify the role of GSH in oxaliplatin accumulation and cytotoxicity in HCT-8 and HCT-8ox cells. In different cell models accumulation of cisplatin was not altered after GSH depletion (Benedetti et al. 2011), corresponding to the findings of unchanged platinum accumulation after incubation with oxaliplatin in HCT-8 and HCT-8ox cells. Thus, the cellular GSH content does not seem to be relevant for platinum accumulation after oxaliplatin exposure in the cells investigated. Assuming that GSH is an important cytoprotectant in cancer cells, an augmented toxicity of platinum complexes would be the logical consequence of GSH depletion and has been observed in cell culture experiments (Benedetti et al. 2011; Meijer et al. 1992; Pendyala et al. 1997). In our study HCT-8 cells were sensitized by GSH depletion possibly because less intracellular platinum is bound to GSH. The same tendency was also observed in the oxaliplatin-resistant cell line HCT-8ox but the effect was smaller and not significant. Hence the intracellular GSH content seems to affect oxaliplatin cytotoxicity but its depletion does not overcome resistance.

The differences in basal GSH content among different cell types found in this and other projects (Boubakari et al. 2004; Bracht

et al. 2006) could be due to varying activities of intracellular pathways like mitogen-activated protein kinase (MAPK) signaling pathways. Activation of activator protein 1 (AP-1) mediated by extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK), two major MAPK, was found to produce increased GSH levels. The change was described to be caused by altered expression of γGCS, the rate limiting enzyme in GSH synthesis (Ballatori et al. 2009). Reversible glutathionylation of proteins is important in signal transduction and is probably changed in diseases like cancer (Mieyal et al. 2008). In the context of platinum cytotoxicity it has to be considered that GSH and related enzymes like GST are not only involved in detoxification but could also function in regulating proteins in pathways that mediate cytotoxicity and resistance.

In conclusion, the results suggest that oxaliplatin or its metabolites are transported by MRP1 and/or MRP2. Inhibition of MRP-mediated efflux did not result in an increase of sensitivity to oxaliplatin suggesting that MRP-mediated efflux does not play a major role for the cytotoxic activity of oxaliplatin. Intracellular GSH depletion did not affect platinum accumulation but increased oxaliplatin cytotoxicity. However, our results also suggest that GSH depletion does not overcome oxaliplatin resistance.

4. Experimental

4.1. Chemicals

Cisplatin and oxaliplatin were purchased from Sigma-Aldrich, Steinheim, Germany. RPMI-1640® medium, L-glutamine solution, penicillin-streptomycin solution, fetal calf serum and trypsin–EDTA solution were obtained from Sigma-Aldrich, Steinheim, Germany. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and dithiothreitol (DTT) were obtained from AppliChem, Darmstadt, Germany. Dimethyl sulfoxide (DMSO), perchloric acid 70%, glutathione (GSH), naphthalene-2,3-dicarboxaldehyde (NDA), and buthionine sulfoximine (BSO) were obtained from Sigma-Aldrich, Steinheim, Germany, nitric acid 65% (Suprapur) from Merck, Darmstadt, Germany. The Novagen® BCA Protein Assay Kit was obtained from EMD Chemicals Inc., Gibbstown, USA. Ultrapure water was obtained using a Purelab Plus™ system from ELGA Labwater, Celle, Germany.

4.2. Cell culture

In this project the human ileocecal colorectal adenocarcinoma cell lines HCT-8 and HCT-8ox and the ovarian carcinoma cell lines A2780 and A2780cis (European Collection of Cell Cultures, United Kingdom) were used. Cells were cultivated as monolayers in RPMI-1640® medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (37 °C, 5% CO_2). For A2780 and A2780cis cells the medium was additionally supplemented with 0.6 mM L-glutamine. Cells were used over a period of 12 passages at most. Level of resistance of the resistant variants was monitored by the MTT-based cytotoxicity assay described in section 4.4.

4.3. Glutathione (GSH) quantification

Cells were seeded out in 6-well plates and allowed to attach. When incubation steps were finished the medium was removed and cells were washed with ice-cold phosphate buffered saline (PBS). Cells were detached using trypsin-EDTA and resuspended in medium. The suspension was centrifuged and the cell pellet washed with PBS twice. Subsequently, the cell pellet was lysed using ice-cold perchloric acid (3.3%). The lysate was centrifuged and the supernatant stored at -80 °C.

For GSH determination in the supernatant a fluorogenic microplate assay was used (Lewicki et al. 2006). To reduce oxidized GSH (GSSG) samples were incubated with 4 mM dithiothreitol (DTT) for 2 min. After derivatization of GSH with 0.5 mM naphthalene-2,3-dicarboxaldehyde (NDA) for 15 min the fluorescence signal was detected using a Fluoroskan Ascent® microtiter plate reader (Thermo Fisher Scientific, Germany) (λ_{exc} = 485 nm and λ_{em} = 538 nm). GSH content was calculated by means of a calibration curve ranging from 1 to 20 μM. To exclude effects of different cell sizes and cell growth the GSH content was related to the protein content determined by the bicinchonic acid (BCA) assay (Novagen® BCA Protein Assay Kit, EMD Chemicals Inc., Gibbstown, USA). The intracellular GSH content was expressed relative to the respective mean value for HCT-8.

4.4. Cytotoxicity

Cytotoxicity of platinum complexes was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)-based assay performed in 96-well plates (Alley et al. 1988). In each well $3 \cdot 10^3$ cells (HCT-8, HCT-8ox) or $10 \cdot 10^3$ cells (A2780, A2780cis) were seeded out and allowed to attach over 12 to 14 h. Platinum complexes were then added and cells incubated for 72 h (37 °C, 5% CO_2). The plates were then incubated with MTT solution for 1 h (37 °C, 5% CO_2). The medium was removed and the formazan crystals built from MTT by living cells were dissolved in DMSO. The absorbance at 570 nm with background subtraction at 690 nm was measured using a Multiskan Ascent® microtiter plate reader (Thermo Fisher Scientific, Langenselbold, Germany). EC_{50} values were calculated by non-linear regression using the software GraphPad Prism® (GraphPad Software, San Diego, USA). Resistance factor was calculated by dividing the EC_{50} value of the resistant cells by the EC_{50} value of the sensitive cells.

4.5. Platinum accumulation

To investigate the cellular platinum accumulation, 10^6 HCT-8 or HCT-8ox cells were allowed to attach in 6-well plates for 12 to 14 h. When the effect of buthionine sulfoximine (BSO) was investigated, cells were incubated with 100 μM BSO directly after being seeded out. Cells were then incubated with 100 μM oxaliplatin. Cells were harvested using trypsin–EDTA after washing with ice-cold PBS and resuspended in medium. The suspension was centrifuged and the cell pellet washed with 1 mL PBS twice. The cell pellet was lysed in 65% nitric acid (1 h, 80 °C) and platinum was quantified using flameless atomic absorption spectrometry (SpectrAA® Zeeman 220; Varian, Darmstadt, Germany) (Kloft et al. 1999). The platinum concentration was related to the protein content determined by the bicinchonic acid (BCA) assay (Novagen® BCA Protein Assay Kit, EMD Chemicals Inc., Gibbstown, USA).

4.6. DNA platination

10^6 HCT-8 or HCT-8ox cells were seeded out in 6-well plates and allowed to attach for 12 to14 h. Cells were then incubated with 100 μM of oxaliplatin with or without 200 μM Gü83 co-incubation. After 3 h cells were harvested as for platinum accumulation described above. The DNA was isolated from the pellet with solid-phase extraction (QIAmp®, Qiagen, Hilden, Germany) and DNA concentration was determined by UV photometry. Afterwards the samples where lysed using 1% nitric acid (24 h, 70 °C) and platinum quantified using inductively coupled plasma mass spectrometry (ICP-MS) (Varian 820®; Varian, Darmstadt, Germany). Platinum isotope Pt^{195} was used for analysis. Samples were measured in five replicates each resulting from 20 single scans. Precision and accuracy were checked during measurements. Finally, the platinum-nucleotide ratio was calculated.

4.7. Statistical analysis

Experiments were performed in triplicate. Arithmetic mean values were calculated from the dependent experiments. Usually the means of at least three independent experiments were calculated and the results were presented as mean and standard deviation (SD). Since experiments were performed using a small sample size, data could not be tested for normal distribution. Assuming that data were normally distributed, the two-sided Student's t-test was used to analyze differences. In case of dependent experiments the paired t-test and in case of independent experiments the unpaired t-test was used. In order to analyze the influence of two independent variables, two-way ANOVA with the *post-hoc* test of Bonferroni was applied. In case p values were ≤ 0.05 a difference between results was considered to be statistically significant; p values > 0.05 were considered not significant (n. s.). EC_{50} values describing cytotoxicity were assumed to be log-normally distributed. Thus, the negative decimal logarithms (pEC_{50}) were calculated and means were calculated from pEC_{50} values. For those experiments the standard error of the mean (SEM) was calculated to describe the accuracy of the determination of the mean.

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