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Development of Daunorubicin Resistance in Tumour Cells by Induction of Carbonyl Reduction

*Wolfram Ax, Michael Soldan, Lutz Koch and Edmund Maser**

Department of Pharmacology and Toxicology, School of Medicine, Philipps-University of Marburg, D-35033 Marburg, Germany

ABSTRACT. A resistant descendant of the human stomach carcinoma cell line EPG85-257 was selected in the presence of increasing concentrations of daunorubicin (DRC). To avoid the expression and activity of P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP), cells were cultured in the presence of verapamil. The resulting cells were used to evaluate an induced carbonyl reduction as a new determinant in DRC resistance. The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) toxicity assay was performed to estimate sensitivity to DRC in both cell lines. IC_{50} values of DRC increased almost 8-fold in the resistant descendants compared to the parental cell line. P-gp transcripts were detectable in both cell lines at only very low levels, and no significant alterations between sensitive and resistant cells were observed. MRP mRNA expression was markedly higher compared to P-gp mRNA, but, as was the case with P-gp, MRP mRNA levels in sensitive and resistant cells showed no alteration. This was probably due to the effect of the presence of verapamil during cell selection. Another known drug resistance factor, the lung resistance-related protein (LRP), was not at all detectable. Interestingly, resistant cells possessed 6-fold higher levels of DRC carbonyl-reducing activity, leading to the less toxic 13-hydroxy metabolite daunorubicinol (DRCOL). The 6-fold higher DRCOL formation roughly parallels the 8-fold increase in DRC IC_{50} values during cell selection, and therefore may account for DRC resistance in these cells. The determination of specific carbonyl reducing enzymes, known to be involved in DRC detoxification, revealed that mRNA expression of carbonyl reductase (EC 1.1.1.184), aldose reductase (EC 1.1.1.21), and dihydrodiol dehydrogenase 2 (EC 1.3.1.20) increased in the resistant descendant. In contrast, the phase II-conjugating enzyme activities of glutathione *S*-transferases were significantly lower in resistant than in sensitive cells, whereas those of glucuronosyl transferase were not detectable in either cell line. Apparently, conjugating enzymes are not involved in DRC resistance in human stomach carcinoma cells. These studies indicate that DRC resistance in human stomach carcinoma cells may appear as a result of an induction of metabolic DRC inactivation via carbonyl reduction to the less active 13-hydroxy metabolite DRCOL. BIOCHEM PHARMACOL **59**;3:293–300, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. cancer cells; daunorubicin; resistance; carbonyl reduction; induction

Inherent or acquired resistance to multiple anticancer drugs is a problem of major importance in chemotherapy, and numerous mechanisms of resistance have been investigated until now. MDR† is the result of, at least in part, the overexpression of P-gp and MRP, which act by decreasing intracellular drug concentrations through an ATP-dependent, verapamil-sensitive efflux of unmodified drug from the cell [1, 2]. Multidrug-resistant cell lines also frequently overexpress LRP which, at time of diagnosis, provides a strong and independent prognostic factor for response to chemotherapy and outcome in different tumour types [3]. On the other hand, the development of non-classical MDR is generally associated with alterations in levels and/or activity of topoisomerase II and the overexpression of drug-detoxifying enzymes, including glutathione *S*-transferase, glucuronosyl transferase, cytosolic class 3 aldehyde dehydrogenase, and AKR [4–11].

Anthracyclines are the most valuable cytostatic agents in chemotherapy, but their usefulness is limited by intrinsic or acquired resistance towards these drugs. In previous investigations, it was shown that anthracycline resistance is not merely the result of alterations in drug uptake and retention [6, 12–14], but is also mediated by enzymatic anthracycline detoxification that is up-regulated upon exposure to these drugs [15, 16]. Interestingly, 13-hydroxy metabolites of anthracyclines, such as doxorubicinol and DRCOL, are significantly less potent than the parent drug in terms of inhibiting tumour cell growth *in vitro* [17–23], suggesting

* Corresponding author: Dr. Edmund Maser, Department of Pharmacology and Toxicology, School of Medicine, Philipps-University of Marburg, Karl-von-Frisch-Strasse 1, D-35033 Marburg, Germany. Tel. +49-6421-286-5465; FAX +49-6421-286-5600; E-mail: maser@mailer.uni-marburg.de.

† *Abbreviations:* MDR, multidrug resistance; P-gp, P-glycoprotein 170; MRP, multidrug resistance-associated protein; LRP, lung resistance-related protein; DRC, daunorubicin; DRCOL, daunorubicinol; AKR, aldo–keto reductase; DD2, dihydrodiol dehydrogenase 2; CR, carbonyl reductase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; RT–PCR, reverse transcriptase–polymerase chain reaction; CDNB, 1-chloro-2,4-dinitrobenzene; ALR1, aldehyde reductase; ALR2, aldose reductase.

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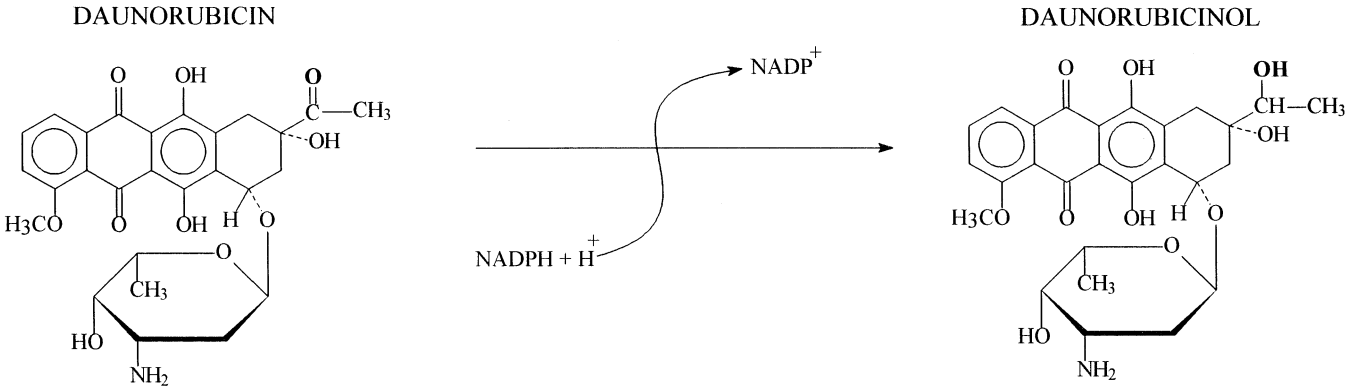

FIG. 1. Scheme of daunorubicin carbonyl reduction.

that carbonyl reduction is an important biochemical mechanism in the detoxification of carbonyl group-bearing anthracyclines (Fig. 1). Therefore, elevated levels of anthracycline carbonyl-reducing enzymes constitute an additional mechanism in the development of resistance towards DRC [15, 16].

Three enzymes capable of catalyzing DRC carbonyl reduction have been identified in human liver: aldehyde reductase (ALR1; AKR1A1), dihydrodiol dehydrogenase 2 (DD2; AKR1C2), and carbonyl reductase (CR) [24]. Moreover, overexpression of an unknown AKR in Chinese hamster ovary cells has recently been related to the inactivation of a cytotoxic synthetic tripeptide, with AKR catalyzing the carbonyl reduction of its active aldehyde group to the corresponding alcohol. This inducible reductase, which has been shown to catalyze DRC carbonyl reduction, exhibits about 70% sequence identity to aldose reductase (ALR2; AKR1B1) [25], which is also able to reduce aldehyde and ketone substrates. Accordingly, it was necessary to elucidate the extent of DRC carbonyl-reducing activity and the levels of mRNA expression of all known DRC carbonyl reductases in sensitive and resistant stomach carcinoma cells.

Acquired resistance to anticancer drugs is a multifactorial event in which several resistance mechanisms are active simultaneously. Accordingly, under experimental conditions as well as in chemotherapy, an induction of drug-inactivating enzymes may be masked by an enhanced drug efflux via MDR and MRP. Therefore, by evaluating levels of DRC carbonyl reduction, we concomitantly blocked the overexpression of MDR and MRP. For the present investigation, we generated a DRC-resistant human stomach carcinoma cell line by continuous culture in the presence of stepwise increasing concentrations of DRC. To circumvent the selection for the P-gp or MRP phenotype, the culture media were co-supplemented with verapamil, a competitive inhibitor of P-gp and MRP activity and mRNA expression [26, 27]. Due to suppression by verapamil, the resulting cell descendants showed no significant increase in P-gp and MRP mRNA expression. LRP was not detectable in either cell line. However, resistance towards DRC increased by a factor of eight, as revealed by the MTT cytotoxicity assay. This increase in DRC resistance was paralleled by a sixfold higher DRC carbonyl reduction and concomitant induction of ALR2, DD2, and CR mRNA expression. Our data demonstrate that, if P-gp and MRP expression was suppressed, long-term exposure to DRC led to a strong induction of DRC carbonyl-reducing activity by DRC itself, a fact which may represent an important determinant in the acquired resistance towards this drug.

MATERIALS AND METHODS

Chemicals

DRC was supplied by Rhône–Poulenc Pharma GmbH and DRCOL was donated by Farmitalia Carlo Erba GmbH. Leibovits L-15 medium and fetal bovine serum were obtained from GIBCO BRL. All other chemicals were of highest commercially available grade.

Cells and Cell Culture

A DRC-sensitive human stomach carcinoma cell line (EPG85-257), isolated from liver metastases, was kindly provided by Prof. M. Dietel. Cells were grown in Leibovits L-15 medium completed with 10% fetal bovine serum, insulin 80 I.E./L, transferrin 2.5 mg/mL, fetuin 6.25 mg/mL, aprotinin 20,000 K.I.U./L, glucose 1 g/L, and gentamicin 5 mg/L. Cells were propagated as monolayer cultures at 37° in flasks, containing the growth medium described above; the atmosphere of 5% CO_2 in air was fully humidified. Mean population doubling times were approximately 24 hr. The cells were free of mycoplasma as judged by staining with 4,6-diamino-2-phenylindole-dihydrochloride.

Generation of DRC-resistant Sublines

DRC-resistant descendants were derived from DRC-sensitive cells over a period of 5 months by passage in increasing sublethal concentrations of DCR and permanent supplementation of 20 μM verapamil to the culture media. DRC concentrations were doubled every two weeks beginning with 0.1 ng/mL, finally resulting in a DRC-resistant subline grown at 12.8 ng/mL DRC.

MTT Assay

The MTT assay involves the conversion of tetrazolium salt to colored formazan by cells, serving as an indirect measurement of cell proliferation and viability [28]. The cells were plated in flat-bottom (Sarstedt) microtiter plates at a cell density of 10,000 cells/well with 6 replicates for each drug concentration. After 24-hr preincubation time, cells were incubated with DRC for another 24 hr and then tested for viability with 5 mg/mL MTT (exposure time 3 hr). The formazan crystals were dissolved in a solution containing 0.1 N HCl in isopropanol. The optical density of the colored product was measured at 570 nm using a BioRad microplate reader. Cytotoxicity (IC_{50}) data were determined by analysis with the GraphPad computer software.

Preparation of Subcellular Fractions

The monolayers were rinsed twice with isotonic (1.15% KCl) Tris–HCl buffer, pH 7.4, scraped off with a cell scraper in buffer and sedimented by centrifugation at 200 × *g*. After centrifugation cells were ruptured by ultrasonication (Braunsonic 300 S) twice for 10 sec on ice. The resulting homogenate was centrifuged for 10 min at 600 × *g*, 10 min at 12,000 × *g*, and 60 min at 170,000 × *g*. The remaining supernatant and the sediment represent the cytosolic and microsomal fraction, respectively, with the latter being resuspended in Tris–HCl buffer, pH 7.4. All steps were carried out at 4°. Microsomes were washed twice before use. Lactate dehydrogenase activities were determined as marker enzyme for cytosol.

DRC Carbonyl Reduction Assay

DRC carbonyl reduction was determined in standard assays by incubating 25 μL of cytosolic fraction and 10 μL of 50 mM sodium phosphate buffer, pH 7.4, in a final volume of 50 μL at 37°. After addition of 10 μL of NADPH (final concentration 2 mM), the reaction was started by adding 5 μL of DRC to a final concentration of 0.44 mM. The reactions were stopped after 30 min by adding 150 μL of ice-cold acetonitril into the incubation mixture and transferring the reaction vessel on ice for 15 min. The samples were centrifuged in an Eppendorf centrifuge at 8000 × *g* for 6 min in the cold to sediment organic material, and 20 μL of the supernatant served for HPLC determination of the substrates and their reduced alcohol metabolites. Preliminary examinations proved linearity of the reaction within the chosen incubation time. Control experiments were performed without biological material.

Determination of DRCOL

After enzymatic conversion, DRC and DRCOL were detected on a BioRad reverse-phase HPLC system with a Nova-Pak C18 cartridge column (4.6 mm × 25 cm; Waters–Millipore). HPLC separation of DRC and DRCOL was achieved using an eluent of 28% acetonitril in 50 mM ammonium formate buffer, pH 4.0, with doxorubicin as internal standard and a flow rate of 1.5 mL/min. Under these conditions, anthracyclines eluted as follows: doxorubicin, 4.3 min; DRCOL, 5.8 min; DRC, 10.5 min. Substances were monitored with a spectrofluorimeter (Waters 470, Waters–Millipore) at excitation wavelength 470 nm and emission wavelength 550 nm. Metabolite quantification was performed with the aid of calibration curves generated by using known concentrations of authentic DRCOL. Six experiments were performed and relative standard deviations were less than 15%.

Glutathione S-Transferase Assay

Enzyme activities of glutathione *S*-transferase were assayed spectrophotometrically according to standard procedures [29] by incubating cytosolic fractions with CDNB as broad specificity substrate. In brief, CDNB and glutathione (both at final concentrations of 1 mM) were preincubated in a cuvette for 2 min at 25° in a total volume of 500 μL. The reaction was started with 100 μg of cytosolic protein and recorded in a Kontron Uvicon 930 spectrophotometer at 340 nm over a period of 5 min.

UDP–Glucuronosyl Transferase Assay

UDP–glucuronosyl transferase (UDP–GT) activity in microsomal fractions was determined with the two model substrates 7-hydroxycoumarin (umbelliferone) and 4-nitrophenol. 7-Hydroxycoumarin conjugation was assayed spectrophotometrically according to [30], and glucuronosylation of 4-nitrophenol was measured by HPLC analysis as described in [31] and [32]. Positive control reactions were run using rat liver microsomes as source of UDP–GT. In brief, 2 mM UDP-glucuronic acid, 0.2 mM 4-nitrophenol, 5 mM $MgCl_2$ (all final concentrations) and 0.2 mg/mL to 1 mg/mL microsomal protein were incubated for 15 min at 37°. The reaction was stopped with ice-cold ethanol, containing 4-nitrophenylsulfate as internal standard. After centrifugation, 20 μL of the supernatant served for UV analysis of the samples at 300 nm on a Merck–Hitachi HPLC.

RT–PCR of P-gp, MRP, LRP and DRC Carbonyl Reductases

All RT–PCR were carried out with an RT–PCR kit (Ready To Go, Pharmacia Biotech). Total cellular RNA was isolated by the RNeasy Kit (Qiagen). An amount of cDNA representing 1 μg of RNA was subjected to PCR for 45 cycles in a final volume of 50 μL using 15 pmol of each primer. Following an initial denaturation of 5 min at 95°, each cycle consisted of 45 sec at 94°, 45 sec at 60°, and 60 sec at 72°. The encoding cDNA-specific primers used were:

P-gp: forward primer, 5′-GCCTGGCAGCTGGAAGA-CAAATACACAAAAAT, reverse primer, 5′-CAGA-CAGCAGCTGACAGTCCAAGAACAGGAC;
MRP: forward primer, 5′-AGAACCTCAGTGTCGGG-CAGCG, reverse primer, 5′-TCGCATCTCTGTCT-CTCCTGGG;
LRP: forward primer, 5′-CCTCGAGATCCATTGTGC-TGG, reverse primer, 5′-CACAGGGTTGGCCACT-GTGCA;
CR: forward primer, 5′-TGCCTCTGGAACACGCTGC-GGGGCTCC, reverse primer, 5′-GACCAGCACGT-CCAGGCCCCCGTACTC;
ALR2: forward primer, 5′-AGCGACCTGAAGCTGG-ACTACCTGG,reverseprimer,5′-GGTCACCACGAT-GCCTTTGGACTGG;
ALR1: forward primer, 5′-CATTGATTGTGCTGCTA-TCTACGG, reverse primer, 5′-GCCTTCCAAGTCT-CCTTGTAGTGG;
DD2: forward primer, 5′-GTTGGTCCGACCAGCCTT-GGAAAGG, reverse primer, 5′-GTAAGGATGACA-TTCCACCTGGTTGC;

Primers of the internal standard β2-microglobulin were:

forward, 5′-GTGGAGCATTCAGACTTGTCTTTCAGC; reverse, 5′-TTCACTCAATCCAAATGCGGCATC-TTC.

Aliquots (20 μL of RT–PCR products) were then subjected to electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide. Positive controls were performed using RNA prepared from sensitive pancreas carcinoma cells (EPG85 P181), known to express P-gp, MRP, and LRP at high levels. Gels were photographed and analyzed by scanning densitometry using the ImageMaster VDS (Pharmacia Biotech) detection and analysis system. Quantification of bands was performed by calculating the area under the curve for each resulting peak. Expression of all measured gene products (density units) was compared in sensitive and resistant cell lines after being normalized against β2-microglobulin signals as the internal standard to account for RT–PCR and DNA loading variations.

Determination of Protein

Protein determination was carried out according to [33].

Statistical Analysis

The results of enzymatic activities in sensitive and resistant stomach carcinoma cells were analyzed by the Student's *t*-test.

RESULTS

Generation of DRC-resistant Stomach Carcinoma Cells

The generation of a DRC-resistant subline was accomplished by culturing human stomach carcinoma cells in the absence (control) or presence of stepwise increasing concentrations of DRC, ranging from 0.1 ng/mL (starting concentration) to 12.8 ng/mL (final concentration) after 5 months. DRC concentrations were doubled every two weeks, finally resulting in a DRC-resistant subline grown at 12.8 ng/mL DRC (EPG Res). To suppress the overexpression of P-gp or MRP, DRC was co-supplemented with 20 μM verapamil to the culture media, thus ensuring high levels of intracellular DRC. The MTT assay was conducted to evaluate the DRC concentration–response characteristics of both cell lines. IC_{50} values of DRC were 0.055 and 0.410 μg/mL for the sensitive and resistant cell lines, respectively, i.e. a nearly 8-fold increase in resistance against DRC compared to the parental cells.

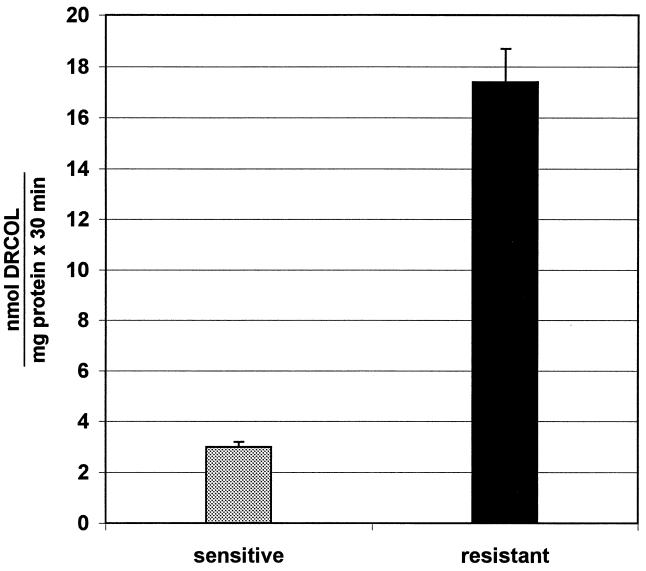

FIG. 2. Specific activity of DRC carbonyl reduction in sensitive and resistant stomach carcinoma cells. Specific activity is expressed as nmol/mg protein DRCOL formed in 30 min. Data represent the means ± SD of N = 6 determinations; standard deviations were less than 10%.

DRC Carbonyl Reduction in Sensitive and Resistant Cells

Constitutively expressed specific activity of DRC carbonyl reduction in the sensitive parental cell line was found to be 2.99 nmol per mg of protein within 30 min (Fig. 2). Interestingly, the development of the DRC-resistant phenotype by DRC treatment was paralleled by an increase in the specific activity of DRCOL formation, resulting in 17.39 nmol per mg of protein within 30 (Fig. 2). Accordingly, resistant stomach carcinoma cells exposed to DRC showed a 6-fold induction of DRC carbonyl reduction compared to their untreated counterparts. This finding confirms previous investigations on pancreas carcinoma cells, which revealed that carbonyl reduction of DRC is inducible by the substrate itself and contributes to the acquired resistance towards DRC [15, 16].

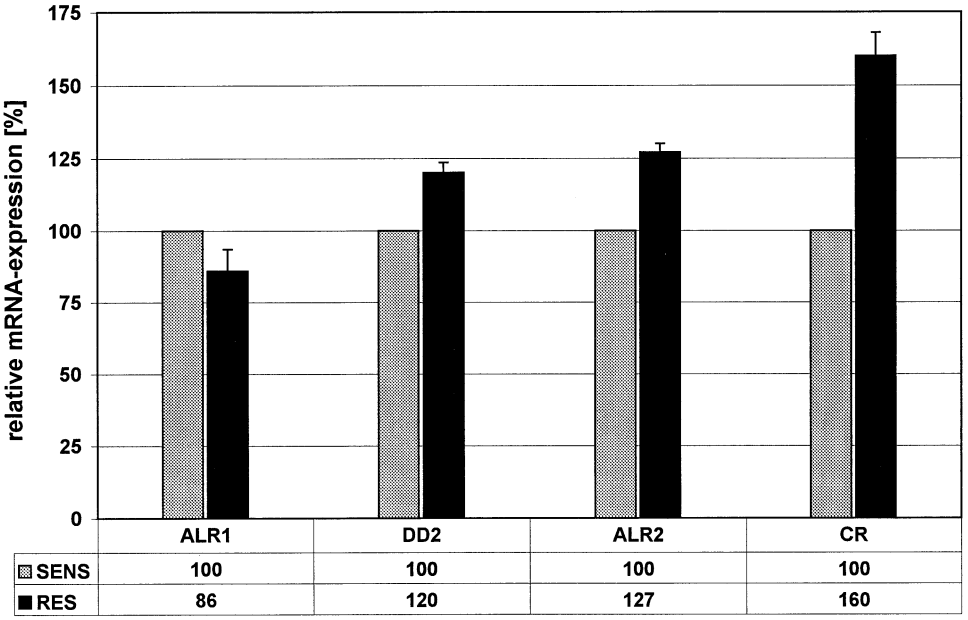

FIG. 3. mRNA expression of DRC carbonyl reductases in sensitive (sens) and resistant (res) stomach carcinoma cells by RT–PCR. Values of sensitive cells were set as 100%. Each bar represents the means ± SD of N = 6 determinations. Standard deviations were less than 5% (less than 10% for ALR1). For ALR2 and CR, $P < 0.05$. ALR1, aldehyde reductase; DD2, dihydrodiol dehydrogenase 2; ALR2, aldose reductase; CR, carbonyl reductase.

mRNA Expression of DRC Carbonyl Reductases

Constitutive mRNA expression of DRC carbonyl reductases in the sensitive tumour cells was set as 100% and compared with that in resistant cells. Higher amounts of mRNA in the resistant tumour cells were determined for CR (160%), ALR2 (127%), and DD2 (120%). In contrast, ALR1 mRNA expression showed a weak decrease to 86% of the value of sensitive cells (Fig. 3). This co-induction of three DRC carbonyl reductases, most prominently CR, indicates that a single DRC carbonyl reductase is not the reason for higher DRC metabolism. Rather, the concerted action of CR, ALR2, and DD2 could protect against DRC toxicity and contribute to DRC resistance. However, it is also possible that hitherto unknown carbonyl reductases are also involved in this reduction.

Activity of Phase II-conjugating Enzymes

To determine if the changes in resistant cells extend to phase II-conjugating enzymes, the activities of GST and UDP–GT were measured. Compared to the sensitive parental cells, resistant cells had markedly decreased GST activities (Table 1). Surprisingly, UDP–GT activities in microsomes were completely absent in both sensitive and resistant human stomach carcinoma cells. It is noteworthy that different methods and two different substrates (umbelliferone and 4-nitrophenol) were employed in these experiments to record possible UDP–GT activities in the cell lines, and that both yielded positive results in control experiments with rat liver microsomes (shown for 4-nitrophenol in Table 1).

P-gp and MRP mRNA Expression

As already indicated above, cells were co-supplemented with 20 μM verapamil to the culture media in order to circumvent the formation of the MDR phenotype and achieve high levels of intracellular DRC. The expression of P-gp and MRP in sensitive and resistant cells was determined on the mRNA level by RT–PCR. Simultaneous supplementation of DRC and verapamil during the culture period of five months led to no significant increase in P-gp, which was barely detectable in both cell lines (Fig. 4). Compared to P-gp, MRP mRNA expression occurred at a higher level, but like P-gp, showed no alteration during the increase in drug resistance. This suggests that the development of DRC resistance in the EPG85-257 stomach carcinoma cells does not necessarily require amplification of P-gp or MRP.

TABLE 1. Specific activities of phase II-conjugating enzymes in stomach carcinoma cells

	rat liver	DRC-sensitive	DRC-resistant	*P*
GST activity	—	23.35 ± 1.65	9.16 ± 0.34	<0.0001
UDP-GT activity	88.60	ND	ND	—

GST activities were determined with CDNB as substrate according to standard procedures [29] and given as nmol CDNB conjugate per mg of protein in 1 hr. Data represent the means ± SD of N = 6 determinations. $P < 0.0001$. UDP–GT activity was assayed using 4-nitrophenol and 7-hydroxycoumarin (not shown) as substrates. Activities are given in percent of 4-nitrophenol glucuronide formation as calculated from the initial amount of 0.2 mM 4-nitrophenol. Conjugates were determined by HPLC analysis. Positive control reactions were run using rat liver microsomes as source of UDP–GT. ND = not detectable.

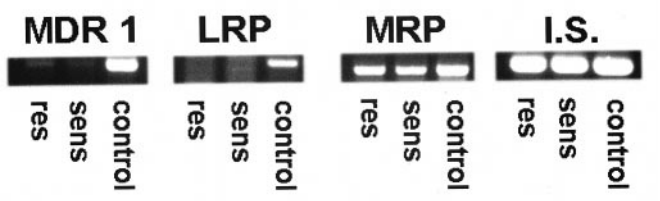

FIG. 4. Determination of resistance factors in DRC-sensitive (sens) and -resistant (res) stomach carcinoma cells with control experiments of DRC-induced pancreas carcinoma cells (control). RT–PCR of MDR1 (P-gp), LRP, MRP, and β_2-microglobulin as internal standard (I.S.) was run as described in the experimental section.

LRP Expression

Sensitive and resistant cells were complete devoid of detectable LRP mRNA (Fig. 4).

DISCUSSION

In general, well-characterized sensitive and resistant cell lines derived from the same origin represent suitable models to study the mechanisms of acquired resistance to selected chemotherapeutics and to trace these mechanisms to classical and/or non-classical MDR. In this study, we developed a DRC-resistant stomach carcinoma cell line by continuous passage in increasing sublethal concentrations of DRC. After eight passages (five months), cells grown at 12.8 ng/mL DRC had IC_{50} values for DRC of 0.410 μg/mL, an 8-fold increase in DRC resistance compared to the IC_{50} values of the parental cells (0.055 μg/mL DRC).

Whether DRCOL is less toxic to tumour cells than DRC has long been a matter of controversy in the literature [17, 23, 34–36]. However, it has now been accepted that conversion of DRC to DRCOL is a significant step in the detoxification [37], as the products of anthracycline carbonyl reduction, the 13-hydroxy derivatives, are significantly less potent in terms of cytotoxicity [17–23]. In pancreas carcinoma cells, we demonstrated previously that DRC carbonyl reduction is inducible by the substrate itself and that elevated carbonyl reduction is a likely contributor to resistance against anthracyclines [15, 16, 38]. The present results clearly indicate that inducible DRCOL formation may constitute an important determinant in the resistance to DRC in human stomach carcinoma cells used in this study. Compared to the sensitive cells, a 6-fold induction of DRC carbonyl-reducing activities occurred in the DRC-resistant subline, which parallels the 8-fold increase in DRC resistance. No correlation between the expression of other known drug resistance mechanisms (P-gp, MRP, LRP, and phase-II enzymes) and DRC resistance was observed. GST activity was not higher in resistant cells even though this activity has been correlated with cellular resistance to alkylating agents [39–41]. In two other studies, tumour cells, as compared to normal tissue specimens, showed decreased levels of GST activity [42, 43]. The high inherent drug sensitivity of these cells was attributed by the authors to the changes in expression and activity of several GST isoforms. Moreover, since UDP–GT activities were not detectable in either stomach carcinoma cell line (although several methods and substrates were applied and positive controls were conducted with rat liver microsomes), it is questionable whether phase II-conjugating enzymes are actually involved in the DRC resistance mechanism in the tested cells. The 8-fold increase in resistance against DRC is also in discordance with the constant levels of MRP or the low levels of P-gp expression which were blocked by verapamil. This indicates that the pattern of resistance does not conform to a classical MDR phenotype and that P-gp and MRP do not contribute to DRC resistance in these cells. Rather, the acquired resistance to DRC is consistent with the 6-fold increase in DRC carbonyl reduction.

It is, at present, unclear whether this mechanism of non-classical MDR also accounts for other cancer cells expressing resistance against anthracyclines bearing a carbonyl group. An additional question arising from our results concerns the nature of the DRC carbonyl-reducing enzymes undergoing induction by treatment with DRC. Carbonyl reduction of endo- and xenobiotics is generally mediated by members of two protein superfamilies, the AKR [44] and the short-chain dehydrogenases/reductases [45]. In the case of DRC, ALR1 and DD2 (AKR enzymes), as well as CR (belonging to the short-chain dehydrogenases/reductases), have been shown to perform this reaction in human liver [24]. Interestingly, non-MDR-resistant Chinese hamster ovary cells resistant to calpain inhibitor (*N*-acetyl-leucyl-leucyl-norleucinal) have been described to overexpress an NADPH-dependent AKR that exhibits about 70% sequence identity with ALR2 [25], the latter also being capable of reducing aldehyde and ketone substrates, including DRC [11]. All four enzymes (ALR1, ALR2, DD2, and CR) were therefore investigated in terms of their levels of expression in sensitive and resistant cells. Compared to all others, CR was induced strongest by DRC. The elevated level of CR mRNA in the resistant cells is in accordance with the finding that transfection of CR in CR-deficient K562 leukemia cells protects against DRC toxicity and contributes to DRC resistance [37]. However, although being less strongly induced, ALR2 and DD2 apparently do account for the higher DRC-reducing activity in resistant cells. Hence, the 6-fold higher levels of DRCOL formation do not result from an induction of one specific enzyme; rather, several DRC reductases seem to be involved. It is quite possible that hitherto unknown reductases additionally participate in DRC clearance. Although several resistance mechanisms were considered in our study, the possibility remains that further factors of resistance (e.g. suppression of apoptosis, alterations in levels and/or activity of topoisomerase II) could also be of significance in the investigated cell lines.

The clinical consequences of our findings remain to be established. It is noteworthy that several flavonoids not only function as scavengers of free radicals [46], but also

serve as potent inhibitors of carbonyl-reducing enzymes [47, 48]. In addition, it has been reported that the flavonoid quercetin strongly inhibits P-gp-mediated drug efflux [49, 50], thus rendering MCF-7 cells, for example, more sensitive to doxorubicin [50]. On the other hand, the anthracycline doxorubicin is also subject to metabolic inactivation via C13 carbonyl reduction and may be intensely detoxified in resistant cells [51]. Co-administration of flavonoids during anthracycline chemotherapy may, therefore, represent an attractive means to simultaneously bypass classical and non-classical drug resistance via inhibition of DRC detoxification by carbonyl reduction and enhanced drug accumulation by P-gp inhibition.

Taken together, our results suggest that an increased enzymatic detoxification of DRC via carbonyl reduction to the less toxic 13-hydroxy metabolite DRCOL may contribute to the resistance of cancer cells towards DRC.

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