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Induction of Daunorubicin Carbonyl Reducing Enzymes by Daunorubicin in Sensitive and Resistant Pancreas Carcinoma Cells

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ABSTRACT. Daunorubicin (DRC) and other anthracyclines are valuable cytotoxic agents in the clinical treatment of certain malignancies. However, as is the case with virtually all anticancer drugs, tumor cell resistance to these agents is one of the major obstacles to successful chemotherapy. In addition to an increased energy-dependent efflux of chemotherapeutic agents, enzymatic drug-inactivating mechanisms also contribute to multidrug resistance of tumor cells. In the case of DRC, carbonyl reduction leads to 13-hydroxydaunorubicinol (DRCOL), the major metabolite of DRC with a significantly lower antineoplastic potency compared to the parent drug. In the present study, we compared two pancreas carcinoma cell lines (a DRC-sensitive parental line and its DRC-resistant subline) with respect to their capacity of DRC inactivation *via* carbonyl reduction. In addition, we cultured the two cell lines in the presence of increasing sublethal concentrations of DRC. Evidence is presented that DRC treatment itself leads to a concentration-dependent induction of DRC carbonyl reduction in subcellular fractions of both the sensitive and resistant pancreas carcinoma cells, resulting, surprisingly, in different susceptibilities to DRC. The principal difference between the two cell lines becomes most apparent at high-dose DRC supplementation (1 μg/mL), at which DRC resistant cells exhibited highest inducibility of DRC-inactivating enzymes, whereas respective sensitive cells already showed an impairment of cellular viability. The use of the diagnostic model substrates metyrapone and p-nitrobenzaldehyde reveals that this adaptive enhancement of DRC inactivation can be attributed to the induction of DRC carbonyl reductases different from known aldehyde and carbonyl reductases. In conclusion, these findings suggest that inactivation of anthracyclines by carbonyl reduction is inducible by the substrate itself, a fact that might be considered as one of the enzymatic mechanisms that contribute to the acquired resistance to these drugs. BIOCHEM PHARMACOL 51;2:117–123, 1996.

KEY WORDS. daunorubicin metabolism; anthracycline resistance; carbonyl reduction; enzyme induction; multidrug resistance

A major problem in cytostatic treatment of malignant tumors is the development of chemoresistant cell clones. The phenomenon of multidrug resistance in these tumor cells is usually related to the overexpression of a plasma membrane glycoprotein termed P-170†, which mediates enhanced drug efflux and prevents intracellular drug accumulation. However, it is known that P-170 cannot fully account for the chemoresistance in multidrug resistant cells [1–7].

There are now known mechanisms of multidrug resistance that are not mediated by P-170 overexpression and are not modulated by P-170 inhibitors [8–10]. Two P-170-like proteins, the MRP [11] and the LRP [12], which also act as putative transmembrane pumps, have recently been related to a decreased accumulation and increased energy-dependent efflux of chemotherapeutic agents. Other potential mechanisms include elevated levels of drug-inactivating enzymes, such as glucuronyl transferase, glutathione transferase, and glutathione peroxidase, and alterations in levels and/or activity of topoisomerase II, which also contribute to the development of the multidrug resistance phenomenon in tumor cells [13–18]. Cellular resistance towards anticancer drugs has also been reported to be mediated by elevated levels of cytosolic class-3 aldehyde dehydrogenase, which is capable of catalyzing the detoxification of the oxazaphorines mafosfamide and cyclophosphamide [19].

Anthracyclines, such as DRC, are the most valuable cytostatic agents in clinical chemotherapy, but their usefulness is limited by intrinsic or acquired resistance towards these drugs. Several lines of evidence suggest that anthracycline resistance cannot be accounted for merely by differences in drug uptake and retention [6, 15, 20, 21]. One of the yet to be defined mechanisms of anthracycline resistance may be an enzymatic drug detoxification that is upregulated either intrinsically or on exposure to these noxious agents.

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† *Abbreviations:* DAPI, 4,6-diamine-2-phenylindole-dihydrochloride; DRC, daunorubicin; DRCOL, daunorubicinol; MDR, multidrug resistance; MPON, metyrapone; MPOL, metyrapol; NBA, 4-nitrobenzaldehyde; NBOL, 4-nitrobenzalcohol; P-170, glycoprotein P-170; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; MRP, multidrug resistance associated protein; LRP, lung resistance related protein.

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The metabolism of 13-ketone anthracyclines occurs primarily *via* carbonyl reduction to 13-hydroxy metabolites *in vitro* and *in vivo*, which can then be conjugated to glucuronic acid or glutathione and excreted [22–26]. A DRC reductase was purified to homogeneity from rat liver and identified as aldehyde reductase with a pH 8.5 optimum for DRC [27, 28]. A second enzyme was described that reduced DRC at a pH optimum of 6.0 [29, 30]. In further studies, the two cytosolic enzymes were characterized as being aldehyde reductase (EC 1.1.1.2), which belongs to the aldo-keto reductase protein superfamily, and carbonyl reductase (EC 1.1.1.184), which belongs to the short chain dehydrogenases [31–33]. Recently, in human liver, one isoform of dihydrodiol dehydrogenase has also been demonstrated to catalyze the carbonyl reduction of DRC to DRCOL [34].

Interestingly, 13-hydroxy metabolites of anthracyclines, such as doxorubicinol and DRCOL, are significantly less potent than the parent drug, in terms of inhibiting tumor cell growth *in vitro* [35–40], suggesting that carbonyl reduction is an important biochemical mechanism in the detoxification of carbonyl group-bearing anthracyclines. Hence, elevated levels of anthracycline carbonyl reducing enzymes may constitute an additional mechanism in the development of tumor cell resistance to these cytotoxic agents, whose active form requires a keto group. Moreover, the alcohol metabolites formed are subject to an increased glucuronidation [15] as well as renal elimination *in vivo*.

In the present study, we compared two human cell lines, the DRC-sensitive pancreas carcinoma cell line EPP 85-181 and its DRC-resistant descendant EPP 85-181 RDB with respect to their constitutive capacity to mediate DRC carbonyl reduction in different subcellular fractions. To clarify whether DRC supplementation to the culture medium itself leads to an induction of DRC carbonyl reducing enzymes, we cultured both cell lines in the presence of increasing sublethal concentrations of DRC. Further, to get information on the enzymes involved in DRC inactivation we used MPON and NBA in parallel as diagnostic model substrates.

Our results demonstrate that sublethal doses of DRC, supplemented to the culture media of human pancreas carcinoma cells, indeed, lead to induction of DRC carbonyl reducing enzymes. The elevated levels of these DRC detoxifying enzymes in tumor cells might contribute to the acquired resistance towards 13-ketone anthracyclines during chemotherapy.

MATERIALS AND METHODS

Chemicals

DRC was supplied by Rhône-Poulenc Pharma GmbH (Köln, Germany) and DRCOL was donated by Farmitalia Carlo Erba GmbH (Milano, Italy). MPON, NBA, and NBOL were purchased from Fluka AG (Buchs, Switzerland). MPOL was kindly provided by Prof. Dr. G. F. Kahl (Göttingen, Germany). Leibovits L-15 medium and fetal calf serum were obtained from Gibco BRL (Eggenstein, Germany). All other chemicals were of the highest commercially available grade.

Cell Lines

Two human pancreas carcinoma cell lines were used in this study: the parent daunorubicin-sensitive cell line EPP 85-181 and its daunorubicin-resistant descendant EPP 85-181 RDB. Both were kindly provided by Prof. Dr. M. Dietel (Kiel, Germany). The resistant pancreas cells exhibited a 20-fold higher resistance towards DRC compared to the parent cells, as determined by the MTT-test (unpublished observations).

Cell Culture

Both cell lines were grown in Leibovits L-15 medium completed with 10% fetal calf 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°C 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 DAPI.

For induction of reductive DRC-carbonyl metabolism, DRC-resistant and sensitive pancreas carcinoma cells were grown for 72 hr in the presence of increasing concentrations (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 μg/mL) of DRC. Supplementation of DRC to the culture medium was started after the monolayers had reached approximately 70% cell density in the dishes (when the cells were suspected to be in the logarithmic growth phase). Control values were obtained from cells cultured without DRC supplementation.

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 (Braun Melsungen Braunsonic 300 S) twice for 10 sec on ice. The resulting homogenate was centrifuged 10 min at 600 × g, 10 min at 12,000 × g and 60 min at 170,000 × g. The remaining supernatant represents the cytosolic and the sediment the microsomal fraction, which was resuspended in Tris-HCl buffer, pH 7.4. The nucleic fraction obtained after the first centrifugation step was washed once and resuspended in the same buffer. All steps were carried out at 4°C.

Incubations with Subcellular Fractions

Enzymatic activity was determined in standard assays by incubating 25 μL of biological material and 10 μL of 50 mM sodium phosphate buffer, pH 7.4, in a final volume of 50 μL at 37°C. After addition of 10 μL of NADPH (final concentration 2 mM) the reaction was started by adding 5 μL of substrate (final concentrations: DRC, 0.09 mM; MPON, 1.0 mM; NBA, 1.0 mM).

The reactions were stopped after 30 min by adding 150 μL of ice-cold acetonitril into the incubation mixture and transfer-

ring 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 Reduced Alcohol Metabolites

After enzymatic conversion, the carbonyl substrates DRC, MPON, and NBA as well as their reduced alcohol metabolites DRCOL, MPOL, and NBOL, respectively, were detected on a BioRad (Munich, Germany) reversed 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; and DRC, 10.5 min. Substances were monitored with a spectrofluorimeter (Waters 470, Waters-Millipore) at excitation wavelength 470 nm and emission wavelength 550 nm.

Using a mobile phase of 30% acetonitrile in 0.1% acetic acid, pH 7.4, and a flow rate of 1 mL/min, the following retention times were achieved for the carbonylic model substrates: MPOL, 6.4 min; MPON, 10.3 min; NBOL, 8.2 min; and NBA. 14.8 min. Substances were determined with a UV monitor (BioRad, Munich, Germany) at 254 nm.

Metabolite quantification was performed with the aid of calibration curves generated by using known concentrations of the authentic compounds. Number of experiments performed was 4–12, and relative standard deviations were less than 15%.

Determination of Protein

Protein determination was carried out according to Lowry *et al.* [41].

Statistical Analysis

The results of DRCOL formation in sensitive and resistant pancreas carcinoma cells were analyzed by the Student's *t*-test. The criterion of significance of the differences between the means (± SD) was $P < 0.05$.

RESULTS

As shown in Figs. 1 and 2, constitutively expressed carbonyl reduction of DRC (without DRC induction) was detectable in the cytosolic as well as nucleic fraction of both cell lines, but the cytosol had by far the highest activity (30-fold compared to the nuclei). Without DRC supplementation, microsomal fractions were completely devoid of reductive DRC metabolism (Fig. 3). In addition, no differences in DRC reduction between the two cell lines were observed when cells were grown in the absence of DRC (Figs. 1–3).

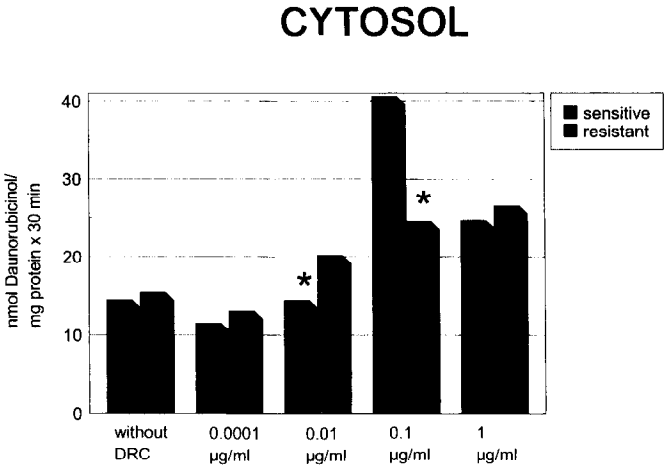

FIG. 1. Effect of DRC pretreatment on DRC carbonyl reduction in the cytosolic fraction of pancreas carcinoma cells. Sensitive and resistant cells were cultured in the absence or presence of increasing concentrations of DRC. Specific activities are expressed as nmol/mg protein DRCOL formed in 30 min. Each bar represents the mean of 4–12 determinations. Standard deviations were less than 12%. *$P < 0.05$.

Interestingly, supplementation of DRC to the culture media led to a strong and concentration-dependent induction of DRC carbonyl reduction in all subcellular fractions of both cell lines, suggesting the occurrence of an adaptive anthracycline detoxifying mechanism (Figs. 1–3). Even in the microsomal fractions, DRC reduction could be detected after culturing the cells in the presence of DRC. Considerable values of microsomal DRC reduction in resistant cells could already be

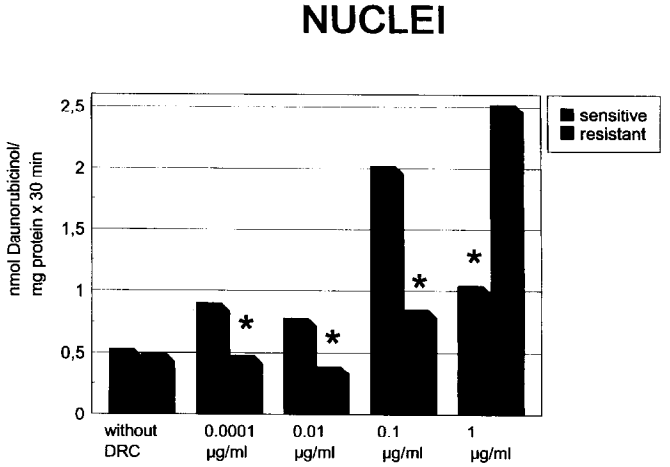

FIG. 2. Effect of DRC pretreatment on DRC carbonyl reduction in the nucleic fraction of pancreas carcinoma cells. Sensitive and resistant cells were cultured in the absence or presence of increasing concentrations of DRC. Specific activities are expressed as nmol/mg protein DRCOL formed in 30 min. Each bar represents the mean of 4–12 determinations. Standard deviations were less than 12%. *$P < 0.05$.

induced by DRC concentrations of 10^{-4} μg/mL, the same reduction in the sensitive cells required 100-fold higher amounts of DRC (10^{-2} μg/mL). Combined, these results demonstrate that DRC carbonyl reduction is inducible by DRC itself.

Comparing the profiles of the concentration-dependent enzyme induction between the two cell lines, it is clear that the nonresistant cells are more sensitive to DRC induction as long as the DRC concentration does not exceed 0.1 μg/mL. A possible explanation for this phenomenon is that, up to this concentration, the resistant cells utilize other mechanisms (e.g. P-170) to eliminate intracellular DRC, whereas the sensitive cells (lacking other detoxifying mechanisms) seem to be dependent on an enhanced DRC inactivation *via* carbonyl reduction.

At 1 μg/mL DRC, the principal difference between the two cell lines becomes apparent. At this concentration the sensitive cells showed an impaired viability as judged by the MTT test, as well as morphologic alterations observed by light microscopy (data not shown), whereas the resistant cells exhibited their maximum of anthracycline inactivation. This difference is true in all subcellular fractions, but becomes most distinct in the particulate fractions. It might be speculated that at 1 μg/mL DRC, other protective mechanisms (e.g. P-170) are overstressed even in the resistant cells, such that here as well, DRC carbonyl reduction serves as supplementary means against DRC toxicity.

DRC carbonyl reduction has been shown to be mediated by carbonyl reductase (EC 1.1.1.184) as well as by aldehyde reductase (EC 1.1.1.2) [31–33]. To obtain information on the enzymes being induced in our pancreas cell lines by DRC culture media supplementation, we tested the diagnostic model substrates MPON and NBA in the same subcellular fractions. Surprisingly, DRC supplementation caused a strong decrease in MPON and NBA carbonyl reduction (down to one fifth compared to the control) in all subcellular fractions of the

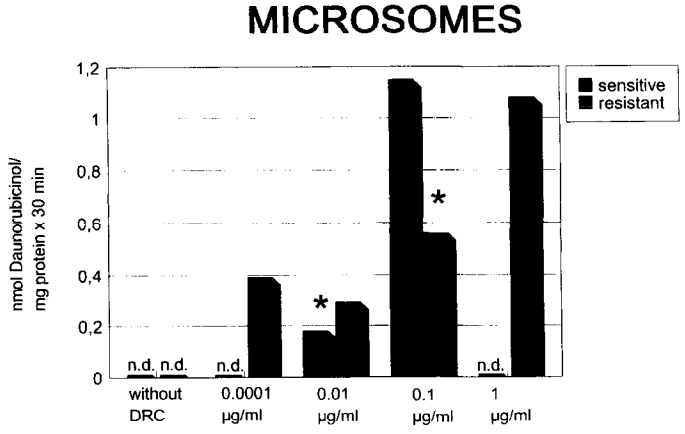

FIG. 3. Effect of DRC pretreatment on DRC carbonyl reduction in the microsomal fraction of pancreas carcinoma cells. Sensitive and resistant cells were cultured in the absence or presence of increasing concentrations of DRC. Specific activities are expressed as nmol/mg protein DRCOL formed in 30 min. Each bar represents the mean of 4–12 determinations. Standard deviations were less than 12%. n.d. = not detectable; activity was below the limit of detection of the assay method. *P < 0.05.

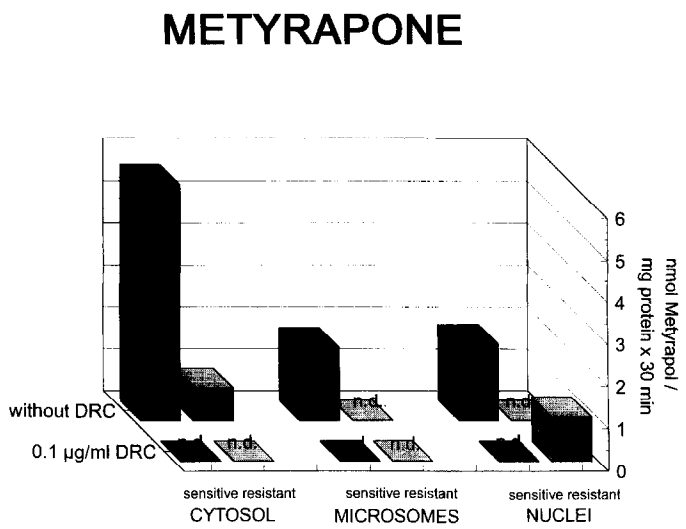

FIG. 4. Effect of DRC pretreatment on MPON carbonyl reduction in subcellular fractions of pancreas carcinoma cells. Sensitive and resistant cells were cultured in the absence or presence of 0.1 μg/mL DRC. Specific activities are expressed as nmol/mg protein MPOL formed in 30 min. Each bar represents the mean of 4–12 determinations. Standard deviations were less than 15%. n.d. = not detectable; activity had fallen below the limit of detection of the assay method.

DRC-sensitive cell line, as well as in the cytosol and microsomes of the resistant cell line (Figs. 4 and 5). However, in the nucleic fraction of the resistant cells, a counteracting increase in reductive metabolism was observed for both model compounds.

Because cytosolic carbonyl reduction of MPON and NBA is mediated *via* carbonyl reductase (EC 1.1.1.184) and/or aldehyde reductase (EC 1.1.1.2) [42, 43], the negative correlation between increases in DRCOL formation and decreases in MPOL and NBOL formation are possibly due to a DRC-derived selective induction of DRC carbonyl reductase activity different from known aldehyde and carbonyl reductases.

DISCUSSION

Resistant tumor cells can still proliferate with intracellular anthracycline concentrations that are lethal to sensitive cells [6, 35], a fact that raises the question of their mechanism of resistance. The widely accepted theory of drug extrusion through a glycoprotein pump as primary mechanism for resistance cannot explain this observation, and it must be admitted that other supplementary mechanisms may be developed during selection of cellular variants resistant to anthracyclines. Moreover, multidrug resistance appears so complex that the likelihood that only one mechanism of resistance is expressed in a malignant cell line is small. Rather, increasing evidence suggests that cellular drug resistance is a multifactorial event where multiple resistance factors occur within tumor cells simultaneously [5, 44].

Several experimental models have clearly shown that the degree of resistance exceeds that attributable to enhanced anthracycline efflux [20, 21], and that elevated levels of anthra-

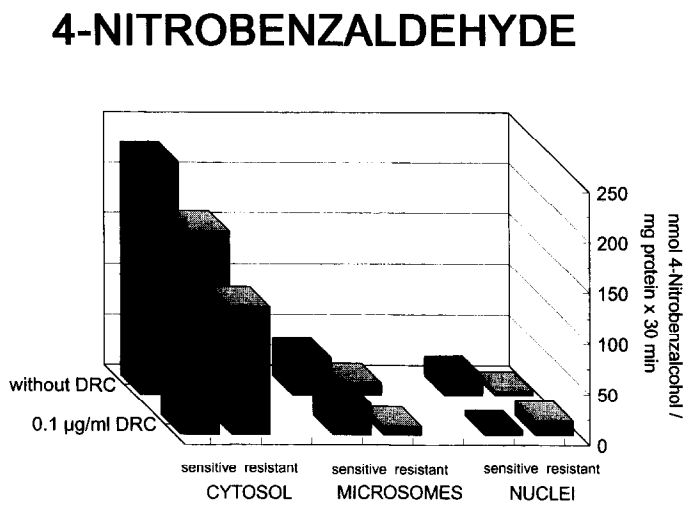

FIG. 5. Effect of DRC pretreatment on NBA carbonyl reduction in subcellular fractions of pancreas carcinoma cells. Sensitive and resistant cells were cultured in the absence or presence of 0.1 µg/mL DRC. Specific activities are expressed as nmol/mg protein NBOL formed in 30 min. Each bar represents the mean of 4–12 determinations. Standard deviations were less than 15%.

cycline detoxifying enzymes might account for this disproportion between intracellular drug accumulation and cytotoxicity [6, 15].

The metabolism of 13-ketone anthracyclines occurs primarily *via* carbonyl reduction to 13-hydroxy metabolites, which are significantly less potent in terms of cytotoxicity compared to the parent drug [35]. Elevated levels of anthracycline carbonyl reducing enzymes might, therefore, constitute a general mechanism for the acquired resistance towards these toxic agents. DRC, which serves as a model compound for the study of anthracycline carbonyl reduction, has recently been shown to be reduced at its carbon 13-ketone moiety to DRCOL in human liver by aldehyde reductase (EC 1.1.1.2), carbonyl reductase (EC 1.1.1.184), and one isoform of dihydrodiol dehydrogenase (EC 1.3.1.20) [34].

Overexpression of an aldo-keto reductase in Chinese hamster ovary cells has recently been related to the inactivation of a cytotoxic synthetic tripeptide, by catalyzing the carbonyl reduction of its active aldehyde group to the corresponding alcohol [45]. The authors postulated that elevated levels of aldo-keto reductases may constitute a general mechanism for the acquired resistance to toxic agents whose active form requires an aldehyde or a keto group.

In the present study, we demonstrate that sublethal concentrations of DRC, supplemented to the culture media, lead to an enhanced expression of DRC carbonyl reduction in both a parent DRC-sensitive pancreas carcinoma cell line and in its DRC-resistant descendant. As carbonyl reduction of DRC to DRCOL signifies loss of activity of this antitumor agent [35–40], induction of respective enzymes may result in a decreased antineoplastic potency of DRC during therapy. This phenomenon might contribute to the development of resistance against cytotoxic agents, the mechanism of inactivation of which involves reduction of a carbonyl group.

Studies of Schott and Robert [35] have shown that when cells are exposed to the same extracellular dose of 13-hydroxy anthracyclines, the incorporation of these metabolites into the cells is reduced by a factor of 2–80 when compared to the parent drug, both in sensitive and resistant cells. Moreover, for some anthracyclines the incorporation of 13-hydroxy metabolites is reduced by a factor of 5–10 in resistant cells compared to the wild cell line.

This raises the question of whether there exists a synergism between metabolic drug inactivation and drug efflux or intracellular drug sequestration. It might be possible that, once these 13-hydroxy metabolites have been formed through carbonyl reduction, they are effluxed out of the cell or sequestered into subcellular organelles by transmembrane pumps such as P-170, MRP, or LRP. Due to their decreased lipophilicity, these 13-hydroxy anthracyclines can not easily traverse membranes, which hinder their re-entry into the cells, so that the concerted action of metabolism and transmembrane transport might be very effective in preventing these drugs from reaching their intracellular targets.

In addition, efflux of DRC may be aided by further intracellular metabolism to glucuronides, which are readily excretable and eliminated from cells. DRCOL, the major metabolite of DRC, has been shown to be metabolized to DRCOL glucuronide in P388 cells and eliminated into the surrounding medium [15]. These studies have shown that P388 DRC-resistant cells produced as much DRCOL glucuronide as the wild-type P388 cells, in spite of taking up to 5 times less DRC. This strongly suggests that both elevated DRC carbonyl reduction and DRCOL glucuronidation may significantly augment the metabolism of anthracyclines in resistant cells.

In our studies the concentration-dependent induction of DRC carbonyl reducing enzyme(s) results in different susceptibilities to DRC in sensitive and resistant pancreas carcinoma cells. In particular, at high DRC concentrations (1 µg/mL), the sensitive cells showed a strong regression of cellular activity, whereas the resistant cells generated mechanisms to reduce even clearly higher amounts of DRC.

Because carbonyl reducing enzymes from the aldo-keto reductase and short-chain dehydrogenase superfamilies have a broad substrate specificity and their respective primary structures are highly homologous [46], we do not know which member(s) of these reductase groups is (are) induced in our pancreas carcinoma cells. Also, we have no information concerning the mechanism of induction of this DRC carbonyl reductase(s). Decreases in carbonyl reduction of the diagnostic model substrates MPON and NBA suggest that this adaptive enhancement of DRC inactivation may be attributed to the induction of DRC carbonyl reductases different from known aldehyde and carbonyl reductases. A similar anthracycline-derived "negative induction" has already been observed in the case of decreased levels of DT-diaphorase, NADH cytochrome b_5 reductase, and NADPH cytochrome P450 reductase after pretreatment of tumors with doxorubicin [47].

Little is known about carbonyl reducing enzymes of the particulate fractions within the cell [48, 49], so that an interpretation of the increasing activity of MPON and NBA car-

bonyl reduction in the nucleic fraction of the resistant cell line remains uncertain. Considering the fact that DNA intercalation is one of the effective mechanisms discussed in terms of DRC cytotoxicity, it is likely that several DRC-inactivating enzymes are grouped particularly in the nuclear envelope to prevent active anthracyclines from reaching the nucleus and to protect the genetic material. This hypothesis is supported by the finding that reductive DRC metabolism is strongly induced in the nucleic fraction (Fig. 2).

The fact that expression of DRC carbonyl reducing enzymes is increased by DRC treatment itself is particularly noteworthy, because until now there has been controversy as to whether or not these enzymes are inducible by xenobiotics such as the cytochrome P-450 monooxygenase system. Although some reports have proposed the noninducibility of carbonyl reducing enzymes [50 and references therein, 51], few studies have considered these enzymes to be weakly inducible by xenobiotics, such as ethoxyquin [52], chlordecone [53], pyrazine [54], and ethacrynic acid [55], as well as by 2,(3)-t-butyl-hydroxyanisole, β-naphthoflavone, and Sudan I [56], and phenobarbital and 3-methylcholanthrene [57].

However, many of these studies have used a diverse spectrum of substances to modify the levels of detoxification enzymes and it is, therefore, unclear as to which particular inducers are of functional importance in the enzymic inactivation of anti-neoplastic chemotherapeutics. In the case of DRC, this question gains even more importance, considering that the incidence of cardiotoxicity caused by DRC therapy is attributed to the occurrence of its alcohol metabolite DRCOL [38], which is produced *via* carbonyl reduction of the parent drug. Hence, inhibition of DRC carbonyl reduction to DRCOL could have two-fold beneficial consequences during DRC anti-tumor therapy, both preservation of the antineoplastic potency of the parent drug and prevention of cardiomyopathy caused by its reduced alcohol metabolite DRCOL.

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