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Lack of Glutathione Conjugation to Adriamycin in Human Breast Cancer MCF-7/DOX Cells

INHIBITION OF GLUTATHIONE *S*-TRANSFERASE P1–1 BY GLUTATHIONE CONJUGATES FROM ANTHRACYCLINES

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ABSTRACT. One of the proposed mechanisms for multidrug resistance relies on the ability of resistant tumor cells to efficiently promote glutathione *S*-transferase (GST)-catalyzed GSH conjugation of the antitumor drug. This type of conjugation, observed in several families of drugs, has never been documented satisfactorily for anthracyclines. Adriamycin-resistant human breast cancer MCF-7/DOX cells, presenting a comparable GSH concentration, but a 14-fold increase of the GST P1–1 activity relative to the sensitive MCF-7 cells, have been treated with adriamycin in the presence of verapamil, an inhibitor of the 170 P-glycoprotein (P-gp) drug transport protein, and scrutinized for any production of GSH–adriamycin conjugates. HPLC analysis of cell content and culture broths have shown unequivocally that no GSH conjugates are present either inside the cell or in the culture broth. The only anthracycline present inside the cells after 24 hr of incubation was > 98% pure adriamycin. Confocal laser scanning microscopic observation showed that in MCF-7/DOX cells adriamycin was localized mostly in the Golgi apparatus rather than in the nucleus, the preferred site of accumulation for sensitive MCF-7 cells. These findings rule out GSH conjugation or any other significant biochemical transformation as the basis for resistance to adriamycin and as a ground for the anomalous localization of the drug in the cell. Adriamycin, daunomycin, and menogaril did not undergo meaningful conjugation to GSH in the presence of GST P1–1 at pH 7.2. Indeed, their synthetic C(7)-aglycon–GSH conjugates exerted a strong inhibitory effect on GST P1–1, with K_i at 25° in the 1–2 μM range, scarcely dependent on their stereochemistry at C(7). BIOCHEM PHARMACOL **60**;12:1915–1923, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. adriamycin; GSH conjugation; GST inhibition; multidrug resistance; MCF-7; DOX

The resistance of cancer cells acquired upon exposure to a single antitumor drug, such as ADR** (**1**) or DAUN (**2**) (Fig. 1), often extends over a range of other drugs even of a completely different chemical nature. Such ability of tumor cells to develop cross-resistance to many drugs (MDR) poses a major obstacle to successful chemotherapy [1, 2]. In spite of intense studies worldwide, the precise mechanisms of the insurgence and functioning of MDR are far from being understood clearly [1, 3–6].

The insurgence of MDR is commonly associated with the overexpression of P-gp. P-gp, encoded by the human *mdr1* gene, is an ATP-dependent membrane protein and plays a key role in drug efflux by acting as an extrusion pump that lowers the cellular drug concentration below toxic levels [1, 2, 7–9]. However, in many cases, MDR can be acquired through mechanisms other than the overexpression of P-gp or other similar membrane proteins [2, 4–6]. The hypothesis of a different mechanism for MDR first came from the observation that, independent of any overexpression of P-gp, MDR is oftentimes associated with an increase of the cellular GSH concentration and/or GST activity and expression [5, 6, 10–12]. The GSTs (EC 2.5.1.18) are a family of dimeric enzymes that conjugate a wide variety of carcinogenic, mutagenic, toxic, and pharmacologically active electrophiles to the cellular nucleophile glutathione (GSH, γ-Glu-Cys-Gly) [13]. The possible role of GSTs in MDR

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** *Abbreviations*: ADR (DOX), adriamycin (doxorubicin); ADRIGLU, adriamycin–GSH conjugate; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CDNB, 1-chloro-2,4-dinitrobenzene; DAUN, daunomycin (daunorubicin); DAUNOGLU, daunomycin–GSH conjugate; DHM-3, 3,5-dimethyl-5-(hydroxymethyl)-2-oxomorpholin-3-yl; DTT, dithiothreitol; GST, glutathione *S*-transferase; MDR, multidrug resistance or resistant; MENOGLU, menogaril–GSH conjugate; MRP, multidrug resistance protein; PBS, phosphate-buffered saline (pH 7.4, 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl); and P-gp, 170 P-glycoprotein.

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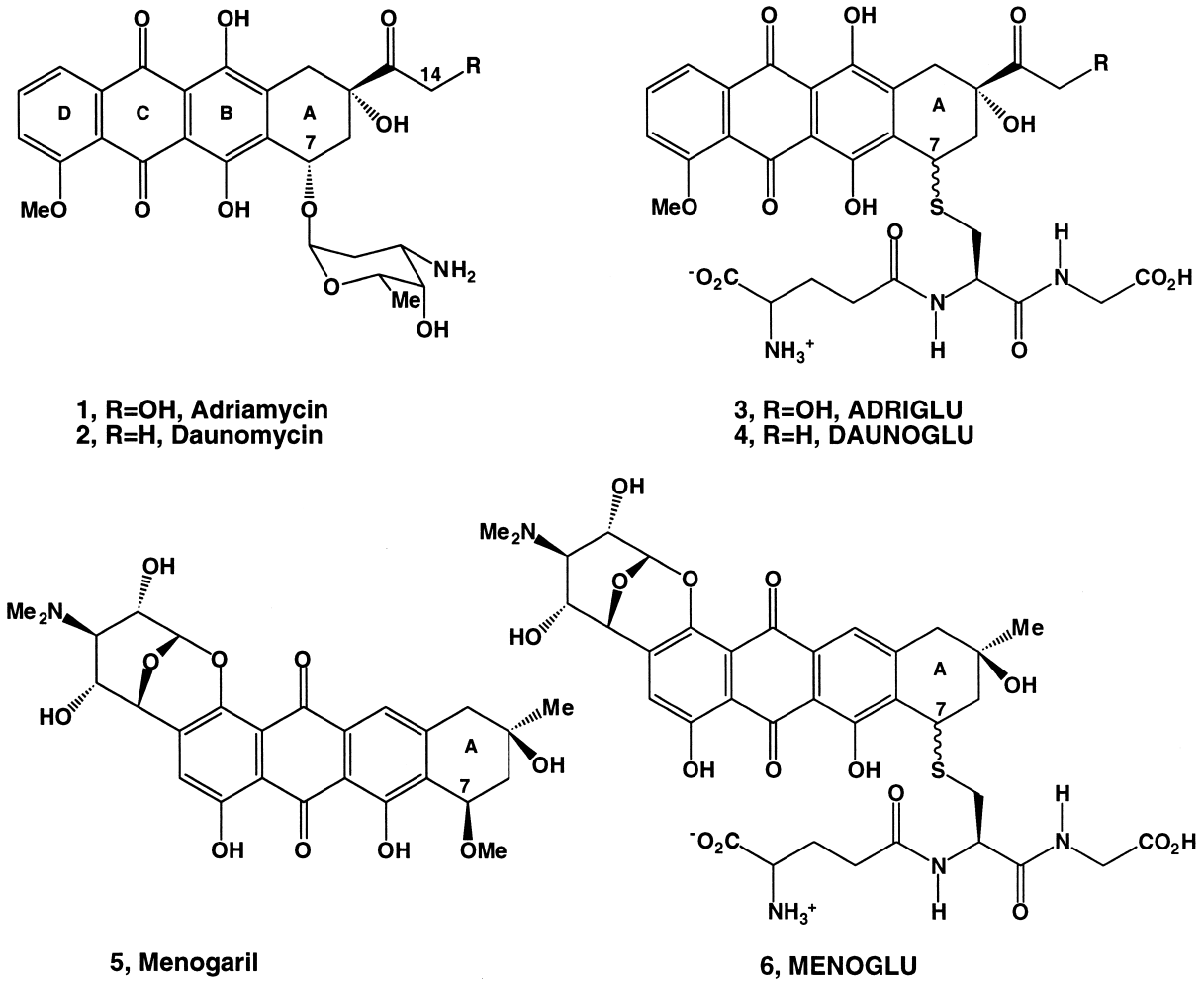

FIG. 1. Structures of the anthracycline antitumor drugs ADR (DOX), DAUN, and menogaril and their respective glutathione conjugates ADRIGLU, DAUNOGLU, and MENOGLU.

would indeed be an example of a widespread defense process in living organisms. The products of the GST-catalyzed GSH conjugation of carcinogenic, mutagenic, toxic, and pharmacologically active compounds, including antineoplastic drugs, or their metabolites, can be extruded and disposed of either as such or after enzymatic transformation [12, 14, 15]. The possibility of this type of detoxifying process being responsible for many cases of acquired MDR during chemotherapy has been investigated and often debated through the last two decades [10, 12, 14, 16–19] and critically reviewed [5, 6, 11]. Relevant to this proposed defense mechanism in MDR cells are the reports on the presence of an ATP-dependent extrusion pump (MRP) for xenobiotics and GSH conjugates, different from P-gp [15, 20–24]. However, beside the oftentimes observed increase in GSH concentration and GST activity and the reports on the presence of MRP, true evidence for a GST/GSH conjugation of anticancer drugs has been offered only for the strongly electrophilic mustard-type (chlorambucil, mephalan, cyclophosphamide, BCNU) or aziridine-type (thiotepa) drugs, with the identification of their GSH conjugates. Mitomycin C, of completely different chemical structure and properties, gives *in vitro* GSH conjugates upon enzymatic or chemical reductive activation [25, 26]. However, no clear evidence of a resistance/GSH-conjugation relationship has been offered. Concerning the anthracyclines, a family of relatively poor electrophilic drugs [27], GSH conjugates have been synthesized only upon chemical reduction of the anthracyclines **1, 2,** and menogaril (**5**) (Fig. 1) to the corresponding more electrophilic quinone methides followed by reaction with GSH [28] or by reaction with GSH in trifluoroacetic acid [29]. So far much speculation has occurred on their possible GSH conjugation in living systems, like MDR cells, but no compelling evidence that might either prove or disprove the formation of GSH conjugates of anthracyclines has been provided by either *in vivo* or *in vitro* experiments. Indeed, beside the above-mentioned reports on the effects of variations of GSH concentration, or GST activity/expression, or MRP overexpression, on MDR, and the successful synthesis of GSH conjugates directly from GSH and anthracyclines, the only other experimental observation that might comfortably be in line with a GSH conjugation of anthracyclines has been offered by our recent report on cytoplasmic localization of

anthracyclines [30]. We have observed that in ADR-resistant MCF-7/DOX human breast cancer cells, displaying a very high GST activity, **1** and **2** seem to be localized, as suggested by fluorescence, in the same Golgi region where their synthetic GSH conjugates **3** and **4** appear to be localized. Instead, in sensitive MCF-7 cells, of relatively low GST activity, **1** and **2** localize in the nucleus, while again their GSH conjugates localize in the Golgi region. The conjugates proved to be less cytotoxic than their parent anthracyclines.

In this paper, we report clear evidence that, in spite of the previous considerations, no GSH conjugation of ADR occurs in human breast cancer MCF-7/DOX cell lines, even in the presence of the P-gp inhibitor verapamil. We also offer our *in vitro* experiments on the possible GSH conjugation of anthracyclines catalyzed by the human placenta GST, a class Pi (46 kDa) homodimeric protein identified as GST P1–1 [31]. This enzyme has been studied extensively in different laboratories because of its potential use as a marker during chemical carcinogenesis [32, 33] and its possible role in the MDR of a number of antineoplastic agents [14, 34–37]. We report that GST P1–1 did not effectively catalyze the conjugation of the antitumor anthracyclines **1, 2,** and **5** with GSH under physiological conditions, either in the presence or in the absence of dioxygen from air. Negative results were obtained even if a preliminary reduction of **1** to the corresponding, more electrophilic quinone methide [38] had been performed. Indeed, the GSH conjugates **3, 4,** and **6** [28] behaved as strong inhibitors of the enzyme.

MATERIALS AND METHODS

General

Solvents and chemicals were purchased from Janssen with the exception of 2,4-dinitrofluorobenzene and DMSO, obtained from Sigma-Aldrich. DHM-3 dimer was prepared as described earlier [39]. The uv-vis spectra were obtained with a Hewlett-Packard 8452A diode array spectrometer.

Anthracyclines and Their GSH Conjugates

ADR (**1**) and DAUN (**2**) were gifts of Pharmacia-Farmitalia. Menogaril (**5**) was a gift from Pharmacia-Upjohn. ADRIGLU (**3**) (Fig. 1) was obtained as a mixture of the epimers **3-I** and **3-II** as reported in the literature [28]. Epimers **3-I** and **3-II** then were separated and purified by submitting the mother liquors from the crude reaction mixture to preparative reverse phase HPLC. Before chromatography, the liquors were concentrated down to a 12 mM total anthracycline concentration. HPLC analysis showed 50% **1,** 25% **3-II,** and 10% **3-I** (% area monitoring at 480 nm). Preparative HPLC was performed on a Rainin semipreparative chromatograph equipped with a Dynamax model UV-1 detector and a Rainin 3 μm C18 Microsorb column, 10 mm i.d. x 50 mm. The eluting solvent was a 45/55 (v/v) mixture of methanol and a 0.3% (pH 4.0) ammonium formate-formic acid buffer, at a flow rate of 3 mL/min, isocratic. Injections were 200 μL each. Under these conditions, the retention times were 8, 11, and 14 min for **3-I, 3-II,** and **1,** respectively. The fractions containing **3-I** and **3-II** were separately combined and evaporated down to 1/20 of the original volume and again passed through the same column using a 50/50 (v/v) methanol–water mixture. The solvent from the anthracycline-containing fractions was removed under vacuum at ambient temperature to leave 99% pure **3-I** and **3-II** as dark-red powders. The two diastereoisomers displayed almost identical uv-vis spectra.

DAUNOGLU (**4-II**) (Fig. 1) was obtained through its DAUNOGLU salt (i.e. its salt with unreacted DAUN) in much the same way as **3-II** was obtained from its ADRIGLU salt ($\mathbf{3}^{-}\cdot\mathbf{1}^{+}$), the only noteworthy difference being the 2-fold higher concentration of the starting reactants **2** and GSH and the longer reaction time (3 days). The sample of **4-II** obtained after chloroform extraction of unreacted **2** from the basic solution of the salt showed less than 1% of unextracted **2.**

MENOGLU **I** and **II** (**6-I** and **6-II**) (Fig. 1) were used as a 1:1 mixture as obtained directly from menogaril (**5**) and GSH as reported [28]. The amount of the excess of GSH, calculated by stoichiometric relationship and confirmed by HPLC analysis, was taken into account for the calculation of the GST inhibition constant, as reported below.

Qualitative and quantitative assays of anthracyclines and their derivatives obtained by synthesis or from biological and GST inhibition tests were performed by HPLC analysis using (method A): a Waters chromatograph equipped with a model 510 two pump solvent delivery system and a uv-vis model 996 diode array detector, monitoring at 480 nm. A Supelcosil RP C18, 2.1 × 150 mm, 5 μm column was used, eluting isocratically with a 50/50 mixture of methanol and a 0.3% (pH 4.0) ammonium formate-formic acid buffer. Alternatively (method B), the HPLC analyses were performed on a Kontron chromatograph provided with a model 420 two pump solvent delivery system and a uv-vis model 430 detector, using a Supelcosil RP C18 4.6 mm × 150 mm, 5 μm column with a flow rate of 0.60 mL/min. Whenever possible, samples were analyzed containing between 1 and 20 nmol of anthracyclines. Lower amounts of anthracyclines, between 0.5 and 1 nmol, could be measured with 90% accuracy. Quantitation of the results from HPLC was done using as reference the linear Lambert-Beer region of a concentration versus peak area plot obtained from pure samples of anthracyclines and derivatives. Proof of identity in critical experiments was established by co-injection and comparison of spectra with authentic samples.

Cell Lines and Drug Treatment

The drug-sensitive human breast cancer cell line MCF-7 and its derivative MDR variant, MCF-7/DOX, were grown as a monolayer in RPMI 1640 medium (Flow) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37° in a humidified atmo-

sphere of 5% CO_2. Cells were passaged serially twice weekly after detachment from culture flasks with 0.05% trypsin and 0.02% EDTA in PBS. The MDR cells were grown in the presence of 10 μM ADR (**1**) and cultured for 4 weeks in drug-free medium prior to use. To analyze the anthracycline content, exponentially growing cells were cultured in 175 cm^2 flasks in RPMI 1640 medium (50 mL) without phenol red for 24 hr before exposure to **1** (1 μg/mL) or **3-II** (6 μg/mL). After exposure to drugs for 2 or 24 hr, the medium was collected, and the cells were rinsed with PBS without Ca^{2+} and Mg^{2+}. The cells were detached with a rubber spatula and collected by centrifugation. The pellet was resuspended in 500 μL of distilled water, and the cells were lysed by three freeze–thaw cycles. The cell lysate was clarified by centrifugation at 13,600 *g* for 10 min, and both pellet and supernatant were stored at −20°.

To study the intracellular distribution of drugs, cells were cultured at 37° for 24 hr before exposure to ADR, ADRIGLU, DAUN, and DAUNOGLU at concentrations ranging from 0.1 to 6 μg/mL for different periods of time. After treatment, cells were washed three times with PBS free of Ca^{2+} and Mg^{2+}, and processed for confocal microscopy. For MDR reversal, MCF-7/DOX cells were pretreated for 1.5 min at 37° in regular medium containing verapamil (Sigma) at a concentration of 10 μg/mL.

Analysis of Anthracyclines from Cell Cultures

Qualitative and quantitative assays for anthracyclines after incubation with MCF-7 and MCF-7/DOX cells were performed by HPLC using method A or B (see above). The values reported for quantitative analyses are averages from analyses performed in duplicate or triplicate, with observed variations being below 15%. The samples to be analyzed were kept in the freezer at −20° throughout the time necessary for the analyses to be completed. The samples tested were (see previous subsection): (i) the supernatant culture media recovered after centrifugation at the end of the incubation, filtered through sterile Acrodisc 0.2 μm Gelman filters; (ii) the clear supernatants from centrifugation of cell lysates; and (iii) the DMSO extract (1 mL/10^6 cells) of the pellet of the cell-fragments obtained by centrifugation of lysates. To improve the accuracy of the HPLC analysis of anthracyclines in the culture media, prior to the analysis the filtered supernatant was passed through reverse phase cartridges (C18 SEP-PAK, Waters). The anthracyclines were retained completely but could be eluted promptly with a 1:1 mixture of methanol–water to obtain an eluant showing a 10-fold increased concentration. With these conditions, using 1-mL HPLC injections, amounts of conjugate as low as 0.5 μmol, corresponding to 5% of conversion, could be detected easily if present.

Confocal Laser Microscopy

Analysis of the intracellular distribution of ADR and ADRIGLU was carried out on cells grown on coverslips and observed after fixing with 2% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, coverslips were mounted on glass microscope slides, in the presence of 4:1 glycerol/PBS and observed on a Leica TCS 4D confocal laser scanning microscope supplemented with an Argon/Krypton laser and equipped with 40 × 1.00–0.5 and 100 × 1.3–0.6 oil immersion lenses. The excitation and emission wavelengths were 488 and 510 nm, respectively. Acquisitions were recorded employing the pseudocolor representation.

GST P1–1 Production and Purification

Human native enzyme GST P1–1 was produced in *Escherichia coli* cells as previously described [36, 37]. The purification of this recombinant enzyme was accomplished by affinity chromatography on immobilized glutathione [40]. After affinity purification, the enzyme was homogeneous as judged by SDS–PAGE [41]. The protein concentration was determined by the method of Lowry *et al.* [42].

GST Activity Measurement

The enzymatic activity of GST was assayed spectrophotometrically at 25° with CDNB as co-substrate under the conditions reported below. Spectrophotometric measurements were performed in a double beam Uvicon 940 spectrophotometer (Kontron Instruments) equipped with a thermostatted cuvette compartment. Initial rates were measured at 0.1-sec intervals for a total period of 12 sec after a lag time of 5 sec. Enzymatic rates were corrected for spontaneous reaction. A suitable amount of GST P1–1 sample (typically 1 μg of protein) was added into a cuvette containing 0.1 M phosphate buffer (pH 6.5), plus 0.1 mM EDTA, in the presence of 1 mM GSH (1 mL final volume). The reaction was started by adding 1 mM CDNB and monitored at 340 nm (ϵ = 9600 M^{-1} cm^{-1}) [43].

Attempts at GSH Conjugation of Anthracyclines Catalyzed by GST

A 100 mM (pH 7.2) Tris buffer containing 1 mM of either compounds **1** or **2,** 1 mM GSH, and 0.1 mM EDTA was kept for 2 hr at 37° in the presence of GST P1–1 (0.1 mg/mL). At the end of this incubation, a suitable aliquot was analyzed by HPLC (method B). In an experiment with compound **5** instead of **1** or **2,** because of the scarce solubility in water, a 5×10^{-3} M solution of **5** was prepared in DMSO and diluted 1:5 with the buffer containing GSH/EDTA solution just before the experiment. During the 2 hr of incubation, a portion of the anthracycline precipitated. HPLC analysis was performed on both the precipitate and the solution.

In another experiment, **1** was reductively activated to its more electrophilic quinone methide by reaction with the one-electron reducing agent DHM-3 [39]. A solution containing **1,** GSH, EDTA, and GST P1–1 at pH 7.2 in 0.1 M

Tris buffer at the above concentrations was deareated with nitrogen. DHM-3 dimer was added to a 5 mM concentration. After 1 hr at ambient temperature under nitrogen, both precipitate and supernatant were analyzed by HPLC for anthracyclines.

Inhibition studies of GST P1–1 with GSH–Anthracycline Conjugates

Inhibition experiments were performed using 1-cm cuvettes with 1 mL (final volume) of 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, GSH (from 0.1 to 3 mM), 1 mM CDNB, suitable amounts of enzyme, and in the presence of fixed inhibitor concentrations ranging from 0 to 40 μM. The enzymatic activities were determined spectrophotometrically at 25° as reported above. Kinetic parameters were determined by fitting the collected data to the Michaelis–Menten equation by non-linear regression analysis using the Graph PAD Prism computer program (Graph PAD Software). The apparent K_m values thus obtained were replotted against the corresponding inhibitor concentration (where K_i is the intercept on the x-axis of the latter plot).

Time course of GST P1–1 inactivation in the presence of ADR

In a typical experiment, GST P1–1 (0.1 mg) was incubated in 1 mL (final volume) of 10 mM Tris–HCl buffer, pH 7.2, in the presence of 0.1 mM ADR (**1**) at 37° for 2 hr. At fixed times, aliquots of sample (10 μL) were withdrawn from the mixture and assayed for GST activity. After a 120-min incubation, 10 mM DTT was added to the mixture, and aliquots of this sample were assayed for GST activity. The same experiment was run in parallel in the presence of 1 mM DTT or 10 mM GSH.

Glutathione Analysis

Glutathione in cells was assayed by a slight modification and adaptation of the method of Reed *et al.* [44]: cells collected by centrifugation from the culture medium were treated with 5% perchloric acid and centrifuged. The supernatant, about 1 mL/10^7 cells, was analyzed for GSH and GSSG by HPLC with a Waters chromatograph (see above), using a Waters μ-Bondapack-NH_2 3.9 × 300 mm column and a 1:4 A/B isocratic mixture as eluant [44], at a flow rate of 1 mL/min. Retention times were 5.5 min (GSH) and 6.8 min (GSSG). Peak areas from HPLC used for calculations were in a linear region of an area/concentration plot obtained from pure GSH.

RESULTS AND DISCUSSION

To address the question, do anthracyclines undergo GSH conjugation in MDR tumor cells, we have chosen the MCF-7/DOX cell line to see if any conjugate with ADR (**1**) forms in significant amounts. The main reason for the choice was the fact that MCF-7/DOX cells were considered to be among the best candidates for GSH conjugation both because of the very high GST activity and the above-mentioned observations on cellular localization and relative toxicity of anthracyclines. Although **1** does not show any center so clearly electrophilic to easily predict the site for a possible GST-catalyzed GSH conjugation, the benzylic C(7) and possibly the C(14) site in the side chain are the favorite candidates for such a reaction to occur on the anthracycline itself. The alternative possibility of reductive activation of **1,** well supported by both *in vivo* and *in vitro* experiments, suggests again the position C(7) as the favorite site for reaction with GSH. Indeed, both epimers **3-I** and **3-II** have been easily obtained by direct reaction of **1** with GSH under anaerobic conditions, through the anthracycline quinone methide, the product of reductive cleavage of **1** [28]. We felt the available epimer **3-II** to be the right compound to assist the search for any GSH conjugate produced in the cells treated with **1.** Easily predictable polar characteristics and chromatographic behavior, along with our personal experience with this family of compounds [38, 45], suggested the use of reverse phase HPLC as the method of choice to detect any GSH derivative that could possibly arise from the conjugation of **1.** ADR (**1**), **3-I, 3-II,** adriamycinone and 7-deoxyadriamycinone all appear in the range of 3–16 min under the eluting conditions. Hence, no GSH conjugate of any sort or any anthracycline-like metabolite thereof should escape from HPLC observation.

Anthracycline in MCF-7 and MCF-7/DOX Cells

Both sensitive and resistant human breast cell lines were treated with either **1** or **3-II** for 2 or 24 hr. The location of the fluorophores of both compounds in cells was established by laser scanning confocal microscopy, taking advantage of the inherent fluorescence of the drugs. ADR appeared predominantly in the nucleus of MCF-7 cells and in the Golgi apparatus of MCF-7/DOX cells, as shown in Fig. 2, panels A and C, respectively, and ADRIGLU, predominantly in the Golgi apparatus of both cell lines as shown in Fig. 2, panels B and D, respectively. In a parallel experiment (same time and same drug concentration), cells were collected and lysed, and the cell debris was collected as a pellet by centrifugation and extracted with DMSO. The recovered culture media, cell lysate supernatant, and DMSO extract of the pellet were all analyzed for **1, 3-II,** and possible anthracycline metabolites by reverse phase HPLC, and the results are reported in Table 1. The HPLC method would detect any anthracycline and/or derivative, from compounds of even higher polarity than ADRIGLU down to low-polarity compounds like 7-deoxyadriamycinone. Inside the cells, only **1** (98% pure) or **3-II** was found from cells treated with **1** or **3-II,** respectively. Most of the anthracycline was found in the DMSO extract of the cell-fragment pellets obtained by centrifugation of the cell

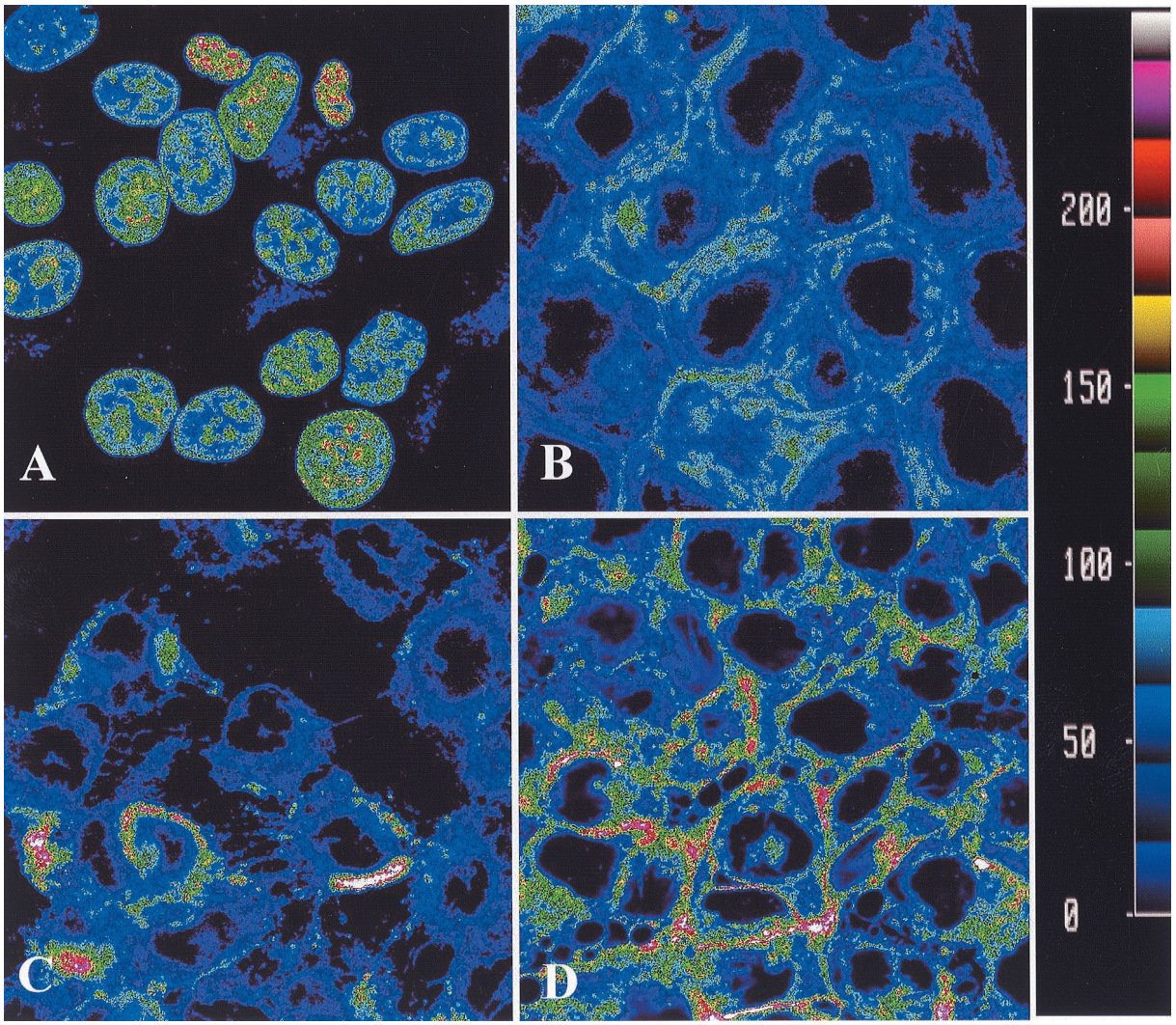

FIG. 2. Confocal fluorescence micrographs of MCF-7 (A and B) and MCF-7/DOX (C and D) cells after a 24-hr treatment with 4 μg/mL of ADR (A and C) or ADRIGLU (B and D). ADR- and ADRIGLU-exposed cells were pretreated with 10 μg/mL of verapamil. Original magnification = 100X. The color bar on the right is relative fluorescence intensity.

lysate. Only a small amount of anthracycline was found in the supernatant from centrifugation of the cell lysate. Analysis of the supernatant culture media with the anthracyclines concentrated using Sep-PAK cartridges showed mostly unchanged **1** (or, respectively, **3-II**). No traces of **3-II** or other polar anthracycline-like derivatives with retention time similar to **3-II** were found in the culture media from MCF-7/DOX cells treated with **1.** Peaks could be detected of < 1% size relative to the peak for **1.** As expected, the cellular uptake of **1** was higher in MCF-7 than in the variant MCF-7/DOX cell line, and the cellular uptake of **3-II** was lower than that of **1** for both cell lines. Basically, no ADRIGLU or any other possible anthracycline metabolite was observed in any of the components of the experiments with ADR-treated MCF-7 or MCF-7/DOX cells. Similarly, no metabolism of ADRIGLU was observed in any of the analytes from the experiments with **3-II**-treated cells.

The lack of ADRIGLU, especially in ADR-treated MCF-7/DOX cells, might have resulted from low levels of GSH or low activity of GST. Analysis for GSH showed 54 nmol/10^6 MCF-7 cells and 6.2 nmol/10^6 MCF-7/DOX cells, and as mentioned earlier, analysis for GST activity showed 14-fold higher activity in MCF-7/DOX cells than in MCF-7 cells. The higher activity of GST should have more than compensated for the lower GSH concentration in MCF-7/DOX cells. If ADR or ADRIGLU were substrates for GST, metabolites should have been observed in MCF-7/DOX and/or MCF-7 cells.

Attempts to Achieve GST-Catalyzed in vitro *GSH Conjugation*

Reaction of **1** with GSH in the presence of GST P1–1 at pH 7.2 at 37° for 2 hr gave only 4% of conjugate **3** (HPLC retention time, 5.7 min) with 95% of **1** (retention time, 7.5 min) accounted for as unreacted ADR. Identical results were obtained in a control reaction missing GST P1–1. Hence, the 4% product resulted from the previously described direct reaction of **1** with GSH [28]. Similar reac-

TABLE 1. HPLC analysis of ADR (1) and ADRIGLU II (3-II) from MCF-7 and MCF-7/DOX cells treated with 1 (1 μg/mL) and 3-II (6 μg/mL) for 2 or 24 hr*

Source	Culture medium supernatant† Area % of anthracycline peaks‡ (*ca.* area % of all peaks§)	Cell lysate supernatant‖	DMSO extract of pellet¶ Area % of anthracyclines peaks‡ (*ca.* area % of all peaks§)
MCF-7 + **1,** 2 hr		Traces of **1**	> 95% **1****, 1.0 μg
MCF-7 + **1,** 24 hr	> 98% **1**** (65%)	Traces of **1**	> 95% **1****, 3.2 μg
MCF-7 + **3-II,** 2 hr		0	Traces of **3-II**
MCF-7 + **3-II,** 24 hr	> 98% **3-II,** (75%)	0	> 70% **1****, 0.5 μg
MCF-7/DOX + **1,** 2 hr	> 99% **1**** (85%††)	Traces of **1****	95% **1****, 0.7 μg
MCF-7/DOX + **1,** 24 hr	> 98% **1**** (85%††)	Traces of **1****	> 95% **1**** (85%††), 0.3 μg‡‡
MCF-7/DOX + **3-II,** 2 h	>98% **3-II** (85%††)	0	0
MCF-7/DOX + **3-II,** 24 hr	*ca.* 90% (80%††)	Traces of **3-II**	Traces of **3-II**

*Percent peak area of **1** or **3-II** is given for samples obtained from cells treated with **1** or **3-II**, respectively. HPLC profiles were obtained as described in Materials and Methods. Retention times for **3-I, 3-II,** and **1** were as follows: (method A) 4.0, 4.6, and 8.5 min, respectively, and (method B) 5.0, 5.7, and 7.5 min. The percent area values reported are the averages from analyses performed in duplicate or triplicate, with observed variations being below 15%. Samples injected contained 0.5 to 20 nmol of anthracyclines.

†Cell culture medium supernatants at the end of the incubation, after filtration through sterile 0.2-μm filters, were concentrated by percolation through reverse phase C18 cartridges followed by elution with a 1:1 methanol-water mixture.

‡Anthracyclines were distinguished by their characteristic absorption in the region λ_{max} 450-550 nm.

§Percent area relative to all peaks absorbing at 480 nm.

‖Clear supernatants from centrifugation of cell lysates were 0.5 mL starting with one flask of cells.

¶DMSO extracts of cell debris pellets were *ca.* 0.5 mL starting with one flask of cells.

No peaks corrsponding to **3-I or **3-II** were detected.

††The major non-anthracycline compounds had retention times of 2.5 and 3.4 min.

‡‡In a similar experiment where verapamil had been added to the culture medium at 10 μg/mL, twice as much **1** was found in the DMSO extract of the pellet.

tions of **2** and **5** with GSH in the presence of GST P1–1 gave 100% recovery of starting anthracycline. The activity of GST P1–1 in an 8/2 (v/v) water–DMSO mixture (the mixture used for the experiment with **5**) showed only a 10% decrease in comparison with pure water. When the chemical reducing agent DHM-3 was added to the reaction mixture of **1,** GSH, and GST P1–1 under anaerobic conditions, the only product observed was 7-deoxyadriamycinone (retention time 12 min) [39]. 7-Deoxyadriamycinone is the major product of direct anaerobic reduction of ADR with DHM-3 and results from reduction to the hydroquinone followed by glycosidic cleavage to the electrophilic quinone methide and tautomerization of the quinone methide to the 7-deoxyaglycon [39]. Even with assistance from a chemical reducing agent, GST P1–1 conjugation of ADR to GSH does not occur.

Effect of ADR on GST P1–1 Activity

When GST P1–1 was incubated at pH 7.2 in the presence of 0.1 mM **1,** a time-dependent inactivation of the enzyme was observed, as shown in Fig. 3. After 2 hr of incubation, the residual activity of GST P1–1 was about 29%. Further, the addition of DTT induced only a partial recovery of the enzymatic activity. On the contrary, when GST P1–1 was incubated in the presence of 1 mM DTT or 10 mM GSH from time zero, only a slight decrease of activity was observed (Fig. 3). As a control, we incubated GST P1–1 alone for the same time and observed at the end of the experiment a partial inactivation (11%). The time-dependent inactivation of GST P1–1 in the presence of **1** and other anthracyclines may be explained as follows: **1** and other anthracyclines produce free radical species that could interact with the side chain of some amino acid residues of proteins, affecting their biological activity. In the case of GST P1–1, potential targets are Cys 47 (amongst cysteine residues) and some tyrosyl residues such as Tyr 7, Tyr 49, and Tyr 108. Among them, Cys 47 is the most reactive species and may undergo oxidation with an intrachain disulfide bond formation with Cys 101. In fact, the inclusion of DTT after a 120-min incubation yielded only a partial recovery of GST activity, indicating that Cys 47 may have been strongly oxidized to other species that do not react with DTT or that the inactivation proceeded also via other residues. The protection afforded by GSH is easily

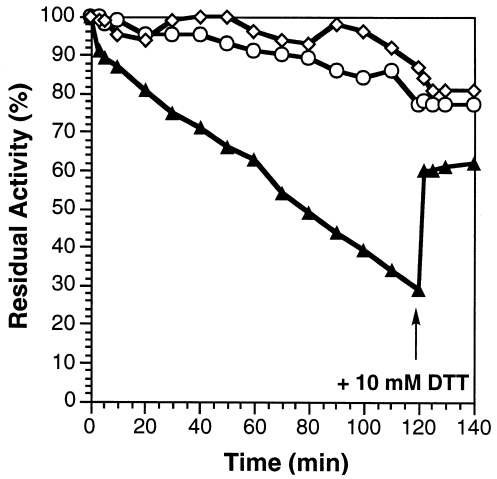

FIG. 3. Time course of GST P1–1 inactivation in the presence of ADR. The experimental conditions are reported in Materials and Methods. Key: (▲) GST P1–1 + 0.1 mM ADR; (○) GST P1–1 + 0.1 mM ADR + 1 mM DTT; and ◇ GST P1–1 + 0.1 mM ADR + 10 mM GSH.

explained by the same reason: the binding of GSH to the active site is accompanied by a conformational change that partially buries Cys 47 into a hydrophobic pocket and significantly increases the distances between Cys 47 and Cys 101 (about 18 Å).

Inhibition of GST-P1–1 by Anthracycline–GSH Conjugates

All of the anthracycline conjugates, **3-I, 3-II, 4-II,** and **6** (1:1 mixture of the two epimers **I** and **II**), were strong, competitive-type inhibitors of GST P1–1. Their K_i values, calculated under conditions described in Materials and Methods, were 2.16 ± 0.09 μM (**3-I**), 1.27 ± 0.03 μM (**3-II**), 1.36 ± 0.10 μM (**4-II**), and 1.01 ± 0.09 μM (**6**). Inhibition was not very sensitive to relatively large changes in structure (compare **4-II** with **6**), indicating primary recognition of the gluthathione group and possibly the A-ring of the anthracycline at the enzyme binding site.

In conclusion, under our conditions no GSH conjugation occurred in MCF-7/DOX cells treated with ADR. Further, no other biochemical change of ADR occurred in resistant cells because of MDR. This is consistent with the parallel unsuccessful attempts of direct GST-catalyzed conjugation of **1, 2,** and **5** and the strong competitive inhibition of GST P1–1 by ADRIGLU, DAUNOGLU, and MENOGLU. We cannot rule out the possibility that other GSTs might yield different results. Our conclusions are reinforced by the recent report that synthetic ADRIGLU and DAUNOGLU as well as the anthracyclines conjugated with GSH at the 14-position are very strong inhibitors of the *MRP/GS-X* efflux pump [29]. Hence, if GSH conjugates were produced by GST in cells, they would not be pumped out of the cells by the efflux pump and should have been observed either in the cell lysate supernatant or in the cell debris pellets. Co-transport of vincristine with GSH not covalently attached by MRP was recently reported; however, the phenomenon was not observed with DOX or DAUN [46]. From a general point of view, lack of evidence of any direct GSH conjugation mediated by GST P1–1 is reasonable by considering that even in the case of strongly electrophilic mustard drugs, as GST substrates, low k_{cat} values have been reported [47]. The overexpression of GST P1–1 in MCF-7/DOX cells might be explained in light of recent findings, suggesting that GST P1–1 is a component of the jun kinase stress system [48]. Therefore, the increased GST activity, as outlined by Tew and co-workers [5, 6], could be a defense against oxidative stress induced by anthracyclines, which is an important component of tumor cell cytotoxicity [49].

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