Mitochondrial Cytochrome c Oxidase as a Target Site for Daunomycin in K-562 Cells and Heart Tissue

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ABSTRACT

Daunomycin and other structurally related anthracyclines can cause myelosuppression and cardiomyopathy. We explored the possible mechanisms by which daunomycin (DAU) interacts with target sites in neoplastic hematopoietic cells and heart tissue. We observed that [3H(G)]DAU interacts selectively with mitochondrial hemoproteins isolated from K-562 cells and rat and bovine heart and forms relatively stable protein complexes. Isolation, purification, and chromatographic analysis of the mitochondrial components complexed with [3H(G)]DAU revealed that one of the major components involved is cytochrome c oxidase (COX). Both DAU and ADR caused a dose-dependent inhibition of COX activity in vitro, an event prevented by exogenous hemin. The interaction of DAU with COX appears to occur via more than one site, one of which at least appears to be the prosthetic group of heme. Therefore, mitochondrial COX, a pivotal mitochondrial enzyme for cell respiration, may serve as a potential target site for DAU and other related anthracyclines.

INTRODUCTION

DAU and other structurally related anthracyclines like ADR are potent antitumor agents with wide clinical applications in the therapy of a large variety of neoplasms (1). Unfortunately, their long-term clinical use is limited due to a cumulative dose-dependent cardiovascular toxicity (2) and severe bone marrow suppression (3).

Although several different mechanisms have been proposed so far to explain anthracycline-induced cytotoxicity, it is as yet unknown which of these mechanisms is most responsible for cardiovascular toxicity, myelosuppression and antitumor activity of anthracyclines. The original hypothesis that anthracyclines kill cells primarily by interacting directly with the genome, promoting DNA damage and retarding RNA synthesis (4) has been challenged over the years. Evidence now exists to indicate that anthracyclines can also kill neoplastic cells by interacting with cellular components other than DNA. In particular, ADR (a) interacts with the plasma membrane phospholipid bilayer (5); (b) promotes lipid peroxidation (6, 7); (c) stimulates formation of free radical species with destructive capacity (8–11); (d) stabilizes the DNA-topoisomerase cleavage complex (12); (e) forms a quite stable complex with iron (Fe3+) (13, 14); and (f) deteriorates mitochondrial structural and functional integrity (15–19).

Earlier studies from our laboratory have shown that hemin (iron-protoporphyrin IX) (a) selectively counteracts the induced cytotoxicity of ADR in normal and transformed hematopoietic cells (20) and (b) interacts directly with DAU (21). In addition, DAU was found to form relatively stable complexes with mitochondrial proteins enriched in hemoproteins (22). These findings prompted us to further explore the effects of DAU on mitochondria.

In this study, we used [3H(G)]DAU and intact mitochondria prepared from human K-562 cells and bovine and rat heart and demonstrated that (a) hemin counteracts DAU-induced cytotoxicity in K-562 cells like ADR (20); (b) DAU interacts selectively with mitochondrial COX; these interactions appear to be specific in nature and may occur in part via the prosthetic group of heme located in two of the several subunits of this enzyme; (c) DAU and ADR inhibited COX activity in a dose-dependent fashion that was prevented by exogenously added hemin. In light of these observations, we propose that mitochondrial COX may serve as a target site for DAU and presumably other anthracyclines on highly proliferating neoplastic cells and heart tissue.

MATERIALS AND METHODS

Chemicals and Biologicals. Hemin was purchased from Eastman Kodak (Rochester, NY) and dissolved in slightly alkaline solution. ADR-HCl, DAU-HCl, cytochrome c (bovine heart type V), cytochrome c oxidase (ferrocytochrome c: oxygen oxidoreductase, EC 19.3.1 from bovine heart), cytochrome c-agarose (type VIA from horse heart, 6.6 mg/ml gel), DEAE-cellulose, NP-40, and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO). Pyrromycin, kindly donated by Bristol Laboratories (Syracuse, NY), was dissolved in 0.1 M HCl solution. Protoporphyrin IX was purchased from Porphin Products, Inc. (Logan, Utah) and dissolved like hemin. [3H(G)]DAU (specific activity, 5.0 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Bio-Gel P-150 and gel filtration standards were purchased from Bio-Rad Laboratories (Richmond, CA). Sephadex G-25 and Sephadex LH-20 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Protein molecular weight standards were purchased from Gibco, Life Technologies, Inc. (Paisley, Scotland). Collagenase II (150 units/mg protein) was purchased from Worthington Biochemical Corp. (Freehold, NJ), proteinase K (27 mg Anson units/mg protein) from E. Merck (Darmstadt, Germany), and Triton X-100 from Serva (NY). Phosphate-buffered saline (PBS) containing NaCl (8 g/liter), KCl (0.2 g/liter), Na2HPO4·2H2O (1.15 g/liter), and KH2PO4 (0.2 g/liter) was prepared in our laboratory.

Cell Cultures. Human K-562 erythroleukemia cells, originally developed by Loozio and Loozio (23), were seeded in culture at a concentration of 2·3·105 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum, streptomycin (100 ìg/ml), and penicillin (100 units/ml) (Gibco, Life Technologies, Inc.). Cells were kept in exponential growth at 37°C in 5% CO2 humidified atmosphere by replenishing the cultures with fresh medium every 48–72 h. Cell growth was determined at various time intervals by measuring the number of cells with a hemocytometer under a light microscope.

Animals. Adult albino Wistar rats, used throughout this study, were bred in our animal house. Bovine heart was kindly donated by the local slaughterhouse.

Preparation of Mitochondria from K-562 Cells and Heart Tissue. Intact mitochondria were prepared from drug-treated and untreated K-562 cells as well as from heart tissue. In the case of K-562 cells, mitochondria were isolated by homogenizing cells in buffer solution A (10 mM Tris-HCl, 1 mM CaCl2, 0.5 mM PMSF, 7% w/v sucrose, pH 7.0) for 30 min at 4°C. The homogenate was centrifuged at 600·g for 10 min (4°C) to remove nuclei, and the postnuclear supernatant was spun at 9000·g for 30 min (4°C) in order to collect intact mitochondria as previously described (24). Isolated mitochondria were resuspended into buffer solution (0.25 mM sucrose/1 mM PMSF). Mitochondria from bovine and rat heart tissue were prepared according to the method of Vercesi et al. (25). Briefly, 1 g of bovine or rat heart tissue was placed in a Petri dish carrying ice-cold 0.25 mM sucrose solution and dissected in small pieces. The mixture was then treated with 10 ml buffer solution B (0.25 mM sucrose, 0.5 mM EGTA, 3 mM 4-[2-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-HCl, pH 7.25) and 100 µg collagenase for 15 min at 4°C. The dissected and collagenase-treated parts of the heart tissue were then washed twice with 0.25 mM sucrose solution to remove collagenase. Finally, the mixture was resuspended in 0.25 mM sucrose solution containing PMSF (1 mM), homogenized in a Dounce Potter homogenizer (10 strokes), and centrifuged at 600·g.
**Gel Filtration.** Separation of solubilized mitochondrial components was performed by gel filtration using Bio-Gel P-150 (1 x 46 cm) or Sephadex G-25 (fine, 1 x 50 cm) columns.

**Membrane Equilibrium Dialysis Experiment.** Intact mitochondria prepared from K-562 cells were incubated with \([^{3}H(G)]DAU (2 \times 10^{-7} \text{M})\) at 37°C for 60 min. The mixture was then transferred into a membrane dialysis bag and dialyzed for 4 h. A membrane dialysis bag bearing only \([^{3}H(G)]DAU\) (at the same concentration) served as a control experiment.

**Binding of \([^{3}H(G)]DAU\) to Isolated Mitochondria.** Aliquots of intact mitochondria prepared from cultured K-562 cells were incubated with varying concentrations of \([^{3}H(G)]DAU\), in the presence and absence of excess unlabeled DAU (1 \times 10^{-4} \text{M}), for 60 min at 37°C in a final volume of 200 \(\mu\text{L}\) solution (0.25 \text{M sucrose/1 mM PMSF}). By the end of this period, the reaction mixture was cooled to 4°C, overlayered on a 9% ice-cold sucrose solution, and centrifuged at 11,500 \(\times g\) for 5 min. The pellet was collected, washed twice with PBS, centrifuged as above, solubilized in 100 \(\mu\text{L}\) 0.1 \text{M NaOH}, neutralized with 100 \(\mu\text{L}\) 0.1 \text{M HCl}, and counted for radioactivity. Specific binding of \([^{3}H(G)]DAU\) was expressed as the difference between total binding and non-specific binding observed in the excess of unlabeled DAU. The data were evaluated by Scatchard plot analysis (27).

**Assessment of Cytochrome c Oxidase Activity.** COX activity was measured according to the method of Sakai et al. (28) with the use of a UV-Vis spectrophotometer (Hitachi U-2000; Hitachi, Ltd., Tokyo, Japan).

In those occasions where mitochondrial protein fractions were used as source of COX, enzyme activity was measured in the presence of rotenone and antimycin A (final concentrations, 6 and 10 \(\mu\text{g/mL}\), respectively) to inhibit complex I and complex II activities in the electron transfer chain reactions (29).

**SDS-PAGE Analysis of Mitochondrial Protein Fractions.** Purified protein fractions highly enriched in mitochondrial COX were analyzed by SDS-PAGE (30).

**Preparation of Delipidated Cytochrome c Oxidase.** Depletion of cardiolipin, bound tightly to COX, was carried out by elution of the enzyme from a Sephadex LH-20 column in the presence of Triton X-100, according to the method of Fry and Green (31). Briefly, 6 mg of commercially available (lyophilized) COX were solubilized in 1 mL of buffer solution containing 50 mM Tris-HCl (pH 7.4), 5% (v/v) Triton X-100, and 20% (v/v) glycerol and incubated for 1 h at 4°C. The solubilized enzyme was loaded onto a Sephadex LH-20 (4 x 18 cm) and eluted with the 50 mM Tris-HCl (pH 7.0) solution, 5% (v/v) Triton X-100, at a flow rate of 0.25 ml/min. The eluted enzyme was saturated with ammonium sulfate (25%), collected by centrifugation at 600 \(\times g\) for 5 min at 4°C as an oily floating layer, and extracted with toluene. Delipidated COX was further purified by chromatography of the aqueous phase on a Sephadex G-25 (1 x 5 cm) column.

**Spectrophotometric Measurements.** Spectra of hemin, protoporphyrin IX, ADR-HCl, DAU-HCl, and pyrromycin-HCl, dissolved in aqueous solutions, as well as spectra of their mixtures were recorded by using a UV-Vis spectrophotometer (Hitachi U-2000).

**RESULTS.**

**Hemin Reduces DAU-induced Cytotoxicity on K-562 Cells.** To demonstrate if hemin reduces DAU-induced cytotoxicity in K-562 cells, as reported earlier with ADR (20), we exposed cells to varying concentrations of DAU in the presence and absence of 30 \(\mu\text{M}\) hemin and measured their cell growth. As illustrated in Fig. 1, hemin increased cell growth at concentrations of DAU ranging from 0.5 \times 10^{-4} \text{M} to 2 \times 10^{-3} \text{M}. Hemin treatment altered the median inhibiting concentration of DAU (2 \times 10^{-5} \text{M}). The best possible protective effect of hemin was observed at 30 \(\mu\text{M}\) hemin in cells treated with 5 \times 10^{-6} \text{M} DAU. However, this protective effect was less pronounced when higher concentrations (>45 \(\mu\text{M}\)) of hemin (Fig. 1, inset) and DAU (>1 \times 10^{-7} \text{M}) were evaluated. These results indicate that hemin, as in the case of ADR (20), prevented DAU-induced cytotoxicity, suggesting that hemin and DAU may interact at the cellular level in a dose-dependent manner.

**\([^{3}H(G)]DAU\) Interacts Selectively with Mitochondrial Protein Components.** Our observation that intracellular \([^{3}H(G)]DAU\) becomes associated with components of the postnuclear fraction of K-562 cells (22) prompted us to further explore the interaction of \([^{3}H(G)]DAU\) with mitochondrial components.

Intact mitochondria prepared from K-562 cells were incubated with \([^{3}H(G)]DAU\). The mixture was solubilized (0.2% NP-40) and subsequently analyzed by Bio-Gel P-150 column chromatography. A small portion (23%) of \([^{3}H(G)]DAU\) formed high-molecular-weight complex with mitochondrial proteins from K-562 cells while the rest (67%) of \([^{3}H(G)]DAU\) remained unbound (Fig. 2A). Similarly, \([^{3}H(G)]DAU\) formed complexes with mitochondrial proteins prepared from rat or bovine heart (Fig. 2B and C). These results indicate that \([^{3}H(G)]DAU\) interacts with mitochondrial protein components of different origin.

**Equilibrium membrane dialysis studies, with \([^{3}H(G)]DAU\) as the ligand, indicated that intact mitochondria retarded the efflux of \([^{3}H(G)]DAU\) substantially and increased its steady-state level inside the bag (Fig. 3). Additional binding studies have indicated that \([^{3}H(G)]DAU\) interacts selectively with intact mitochondria from K-562 cells (Fig. 4). A relatively linear binding behavior at the lower concentrations (1 \times 10^{-9} to 5 \times 10^{-8} \text{M}) followed by saturation kinetics later on at higher concentrations (>10^{-7} \text{M}), indicated that the specific binding of \([^{3}H(G)]DAU\) in mitochondria is saturated. Scatchard analysis of binding data indicated a cooperative effect in binding (Fig. 4, inset), suggesting that at least two binding sites for \([^{3}H(G)]DAU\) may exist in mitochondria.

**\([^{3}H(G)]DAU\) Interacts with Mitochondrial Cytochrome c Oxidase.** When intact mitochondria prepared from rat heart were incubated with \([^{3}H(G)]DAU\), solubilized, and finally separated by Bio-Gel P-150 column chromatography, COX activity was observed in fractions 5–10, where the majority of proteins associated with \([^{3}H(G)]DAU\) were eluted (Fig. 5). This observation suggests that \([^{3}H(G)]DAU\) may interact with COX, a major multisubunit hemoprotein located within the inner mitochondrial membrane. When commercially available COX (bovine heart) was incubated with \([^{3}H(G)]DAU\) (Fig. 6) and the mixture was separated by Bio-Gel P-150 column...
under the same conditions, we observed that COX was also eluted at the same position complexed with \( ^3\text{H}\text{DAU} \).

To demonstrate whether COX represents a major component in the interaction of \( ^3\text{H}\text{DAU} \) with mitochondrial components prepared from K-562 leukemia cells, intact mitochondria (2.2 mg of protein) prepared from K-562 cells were resuspended in 0.5 ml solution buffer (10 mm Tris-Cl, pH 7.0) and incubated with \( ^3\text{H}\text{DAU} (2 \times 10^{-7} \text{M}) \) at 37°C for 60 min. The mixture was then transferred to a membrane dialysis bag (cutoff, Mr 6000) and dialyzed into 50 ml solution (50 mm Tris-Cl, pH 7.0) at room temperature. At time intervals as indicated, 200-μl aliquots were removed from the dialysis buffer and counted for radioactivity. Efflux was measured for \( ^3\text{H}\text{DAU} \) incubated in the presence (●) and in the absence (○) of intact mitochondria. This experiment is representative of three similar experiments.

Hemin Prevents Anthracycline-induced Inhibition of Mitochondrial Cytochrome c Oxidase Activity in Vitro. To elucidate the biological significance of the interaction of DAU with mitochondrial COX, we determined the effects of varying concentrations of DAU-HCl on COX activity. Indeed, DAU-HCl caused a dose-dependent inhibition of COX activity (Fig. 9A). Exogenous hemin reduced daunomycin-induced inhibition of COX activity at concentrations as high as 100–200 μM. Similar data were obtained with ADR-HCl under the same experimental conditions (Fig. 9B).

\( ^3\text{H}\text{DAU} \) Interacts with Delipidated (Cardiolipin-free) Mitochondrial Cytochrome c Oxidase. To determine whether the interaction of \( ^3\text{H}\text{DAU} \) with COX involves sites other than cardiolipin as claimed earlier for Adriamycin (32), commercially available cytochrome c affinity chromatography confirmed the presence of several peptides similar to those obtained from commercially available COX (Fig. 8).

**Fig. 2.** Association of \( ^3\text{H}\text{DAU} \) with components from mitochondria prepared from K-562 cells (A), rat heart (B), and bovine heart (C). Intact mitochondria prepared from K-562 cells (9.2 mg) as well as from rat (4.4 mg) and bovine heart (4.8 mg) were incubated with \( ^3\text{H}\text{DAU} \) (2 × 10^{-7} M) for 60 min at 37°C. Then, the detergent lysate (0.2% NP-40) was loaded onto a Bio-Gel P-150 column and eluted with 50 mM Tris-HCl buffer solution (pH 7.0) (flow rate, 6 ml/h). Each fraction (2 ml) was assessed for protein content at 280 nm (D), while aliquots of 200 μl were assessed for radioactivity (•) in a liquid scintillation counter. Arrows, elution position of molecular weight standards.

**Fig. 3.** Membrane equilibrium dialysis study of the interaction of \( ^3\text{H}\text{DAU} \) with isolated mitochondria prepared from K-562 cells. Equal amounts of intact mitochondria (22 μg of protein) were incubated with varying concentrations of \( ^3\text{H}\text{DAU} \) in the presence and absence of 1 × 10^{-4} Mcold DAU for 60 min at 37°C. Mitochondria were then overlaid on 9% ice-cold sucrose solution and centrifuged at 11,500 × g for 5 min at 4°C. Radioactivity was measured in the collected pellets, and specific binding was determined by subtracting the nonspecific binding from the total binding of \( ^3\text{H}\text{DAU} \). □, total; ○, nonspecific; ●, specific binding of \( ^3\text{H}\text{DAU} \). Inset, Scatchard analysis of the binding data. Bound/free is expressed as nmol/mg of protein/mg and free \( ^3\text{H}\text{DAU} \) in nM. This experiment is representative of two similar experiments.

**Fig. 4.** Binding of \( ^3\text{H}\text{DAU} \) with isolated mitochondria prepared from K-562 cells. E

**Fig. 5.** Bio-Gel P-150 chromatographic analysis of mitochondrial components prepared from K-562 leukemia cells. Intact mitochondria (1.8 mg) prepared from rat heart were incubated with \( ^3\text{H}\text{DAU} \) (2 × 10^{-7} M) for 60 min at 37°C, loaded onto a Bio-Gel P-150 column, and eluted with 50 mM Tris-HCl buffer solution (pH 7.0). A, each fraction (2 ml) assessed for protein content (□) and aliquots of 200 μl assessed for radioactivity (●), B, 100 μl from each fraction mixed with 800 μl freshly reduced cytochrome c (final concentration, 20 μM) also containing rotenone and antimycin A, the ability of included COX to oxidize the cytochrome c was recorded (A, DA550nm/min).
CYTOCHROME c OXIDASE AS TARGET FOR DAUNOMYCIN

Fig. 6. Bio-Gel P-150 chromatographic analysis of the association of [3H(G)]DAU with commercial cytochrome c oxidase of mitochondrial origin (bovine heart). Solid cytochrome c oxidase (5 mg) was suspended in 200 µl PBS, incubated with 1 × 10^{-6} M [3H(G)]DAU for 60 min at 37°C, and solubilized with 0.2% NP-40. The reaction product was then loaded onto a Bio-Gel P-150 column and eluted with 50 mM Tris-HCl buffer solution (pH 7.0). Each fraction (2 ml) was assessed for protein content (C), while aliquots of 200 µl were assessed for radioactivity (B).

Fig. 7. Affinity chromatography on cytochrome c-agarose column of commercial cytochrome c oxidase (A) and of mitochondrial protein components prepared from K-562 cells (B), rat heart (C), and bovine heart (D). Mitochondria prepared from different tissues were incubated with [3H(G)]DAU (2 × 10^{-3} µM) for 60 min at 37°C and then analyzed by column chromatography (Bio-Gel P-150 and DEAE-cellulose). Such samples of solubilized mitochondria prepared from various sources were separated by agarose-cytochrome c affinity chromatography in columns (0.3 ml) equilibrated with buffer solution containing 10 mM Tris-HCl (pH 7.5) and 0.1% Triton X-100 (42). The COX was selectively eluted with high concentrations (a, 25 mM; b, 100 mM) NaCl in the buffer solution (flow rate, 24 ml/h; fraction size, 2 ml).

COX was delipidated, incubated with [3H(G)]DAU, and then analyzed by Bio-Gel P-150 column chromatography. [3H(G)]DAU was able to interact with delipidated COX (Fig. 10) as well as with the native enzyme (Fig. 6) despite phospholipid depletion. This suggests that there may be more than one sites for the interaction of [3H(G)]DAU with COX, as suggested by the binding studies.

The Association of [3H(G)]DAU with Mitochondrial Hemoproteins May Depend on Molecular Conformation and Occur via the Prosthetic Group of Heme. To demonstrate directly if [3H(G)]DAU interacts with COX via the prosthetic group of heme is not an easy task, due to the complexity of this enzyme. We used cytochrome c, a single peptide hemoprotein (M, 12,200), to explore the interactions between [3H(G)]DAU and hemoproteins like COX.

In this study, we examined the possibility that [3H(G)]DAU interacts via the heme group. Two experiments were carried out. First, cytochrome c was incubated with [3H(G)]DAU, and the reaction product was then loaded onto a Bio-Gel P-150 column and eluted with 50 mM Tris-HCl buffer solution (pH 7.0). Each fraction (2 ml) was assessed for protein content (C), while aliquots of 200 µl were assessed for radioactivity (B).

Fig. 8. SDS-PAGE analysis of commercially available cytochrome c oxidase and of cytochrome c oxidase prepared from mitochondrial extracts of K-562 cells and rat and bovine heart and purified by column chromatography (Bio-Gel P-150, DEAE-cellulose, and cytochrome c-agarose). The gel was silver stained for protein. Lanes A, 50 µg of mitochondrial protein from bovine heart; Lanes B, 50 µg of mitochondrial protein from rat heart; Lanes C, 50 µg of mitochondrial protein from K-562 cells; Lanes D, 90 µg of commercial COX. Molecular weight markers are indicated in kilodaltons (K) on the left ordinate. The subunits of cytochrome c oxidase are designated along the right ordinate according to References 42-47.

Fig. 9. Effect of hemin on the dose-dependent inhibition of cytochrome c oxidase activity induced by DAU-HCI (A) and ADR-HCI (B). Commercially available COX (10 µM) was incubated with varying concentrations of DAU-HCl or ADR-HCl in the absence or presence of hemin (26.6 µM) for 60 min at 37°C. COX, treated as above, was assessed for enzymatic activity in a reaction medium (total volume, 200 µl) containing 770 µl of freshly reduced cytochrome c (final concentration, 12 µM). The rate of substrate oxidation (DAU or ADR, nmol/min) was recorded immediately following the addition of COX. The concentration of hemin was fixed at 26.6 µM. The results indicate that hemin inhibited the enzyme activity in a dose-dependent manner.

Fig. 10. Effect of hemin on the dose-dependent inhibition of cytochrome c oxidase activity induced by DAU-HCl (A) and ADR-HCl (B). Commercially available COX (10 µM) was incubated with varying concentrations of DAU-HCl or ADR-HCl in the absence or presence of hemin (26.6 µM) for 60 min at 37°C. COX, treated as above, was assessed for enzymatic activity in a reaction medium (total volume, 200 µl) containing 770 µl of freshly reduced cytochrome c (final concentration, 12 µM). The rate of substrate oxidation (DAU or ADR, nmol/min) was recorded immediately following the addition of COX. The concentration of hemin was fixed at 26.6 µM. The results indicate that hemin inhibited the enzyme activity in a dose-dependent manner.
Cytochrome c oxidase as target for daunomycin

Cytochrome c. Cytochrome e (3 mg) dissolved in 0.5 ml solution (50 mM Tris-HCl, 1 mM EDTA, pH 7.5) was incubated with [3H(G)]DAU (1 x 10^-6 M) and then analyzed by Bio-Gel P-150 column chromatography as described in Fig. 2. □, protein content at A280; ○, radioactivity.

When pyrromycin (an anthracycline differing both in the aglycone and the sugar moieties from DAU-HCl and ADR-HCl; Fig. 14) was used, differences between the addition spectrum of pyrromycin and iron-free protoporphyrin IX and the spectrum of their mixture were observed (Fig. 13H). Such differences in spectra, however, were not observed when hemin was mixed with pyrromycin (Fig. 13D). These data tend to indicate that (a) the interaction between hemin and DAU or ADR is likely to occur via the porphyrin ring and (b) the structural features of anthracyclines can influence their interaction with hemin.

The interaction of the complex formed between heme and DAU-HCl (Fig. 11). A barely detectable amount of [3H(G)]DAU was found to be associated with cytochrome c. Second, cytochrome c incubated with [3H(G)]DAU and simultaneously digested with proteinase K was analyzed by Sephadex G-25 column chromatography. These experiments indicated that the proteolytic digestion of cytochrome c yielded peptide fragments, two sets of which (fractions 26-32 and 33-42) were enriched in both [3H(G)]DAU and heme (Fig. 12), since radioactivity was eluted bound to peptide fragments bearing heme (absorbance at 361 and 410 nm). Comparatively higher amounts of [3H(G)]DAU were associated with the peptide fragments of cytochrome c rather than intact cytochrome c. This discrepancy may be due to the fact that cytochrome c is a single peptide in globular conformation having the heme group in the center (33), which is not accessible enough for full interaction with [3H(G)]DAU. Considering that the peptide fragments differ in size and heme content, we propose that the heme group may be one of the potential target sites for interaction with [3H(G)]DAU.

Interaction of [3H(G)]DAU with cytochrome c via heme residue implies that [3H(G)]DAU may also interact with other hemoproteins. In a set of studies not shown here, we attempted to demonstrate the association of [3H(G)]DAU with other hemoproteins such as catalase and hemoglobin. From molecular filtration experiments, we observed that iron-free protoporphyrin IX and the spectrum of their mixture were observed (Fig. 13H). These findings indicate that iron does not have a pronounced effect on the interaction of heme with DAU. Similar spectra were obtained with ADR-HCl (Fig. 13, B and F).

In spite of the plethora of information accumulated on how anthracyclines act as antineoplastic agents, there has not been a unified theory to explain the majority of their effects on malignant tissues, bone marrow, and heart. Nevertheless, we must underline the fact that

Address issue from a different point of view, UV-Vis spectra of pure compounds (hemin and DAU-HCl or ADR-HCl) and their addition spectra were compared with those obtained from mixtures of hemin and each anthracycline (34). The aim of this study was to detect spectrophotometric changes that may reveal specific interactions between anthracyclines and heme in vitro. Fig. 13 (A and E) depicts the UV-Vis spectra of hemin and iron-free protoporphyrin IX, respectively, in the range of 250-700 nm. Addition spectra of hemin and DAU-HCl as well as spectra resulting from mixing these agents at the same concentrations are given in Fig. 13C. The differences observed in spectra suggest that bond formation may occur when hemin is mixed with DAU-HCl. The same differences in spectra prevailed when iron-free protoporphyrin IX was mixed with DAU-HCl (Fig. 13G). These findings indicate that iron does not have a pronounced effect on the interaction of heme with DAU. Similar spectra were obtained with ADR-HCl (Fig. 13, B and F).

In a set of studies not shown here, we attempted to demonstrate the association of [3H(G)]DAU with other hemoproteins. In a set of studies not shown here, we attempted to demonstrate the association of [3H(G)]DAU with other hemoproteins. In a set of studies not shown here, we attempted to demonstrate the association of [3H(G)]DAU with other hemoproteins. In a set of studies not shown here, we attempted to demonstrate the association of [3H(G)]DAU with other hemoproteins. In a set of studies not shown here, we attempted to demonstrate the association of [3H(G)]DAU with other hemoproteins. In a set of studies not shown here, we attempted to demonstrate the association of [3H(G)]DAU with other hemoproteins.

**Spectrophotometric Determination of the Complex Formed between Hemin and ADR-HCl or DAU-HCl.** As reported earlier, [3H(G)]DAU interacts directly with heme to form a complex (21). To
each of these tissues undergoes extensive mitochondrial damage, an event that leads to the depletion of energy sources, tissue hypoxia, and cell death (1).

The central objectives of this work have been to demonstrate (a) that hematin treatment counteracts DAU-induced cytotoxicity like ADR; (b) whether DAU interacts with pivotal hemoproteins in mitochondria that may serve as potential target sites for anthracyclines; and (c) whether the interactions of DAU with these sites bear any biological significance. The results presented here indicate that \(^{3}H\)(G)DAU interacts selectively with mitochondrial protein components of various sources (K-562 tumor cells and bovine and rat heart), whose major component is COX.

The data shown in Fig. 1 indicated that hematin (30 \(\mu\)M) prevented DAU-induced cytotoxicity in K-562 cells, as in the case of ADR (20). This finding suggests that the effect of hematin is unique for anthracyclines and depends on a critical concentration ratio of these agents. This dependency in concentration may reflect the direct interaction of both agents intracellularly or modulation of the action of DAU by hematin on cellular target sites like COX.

Studies of \(^{3}H\)(G)DAU binding to mitochondria revealed that \(^{3}H\)(G)DAU may interact via at least two sites differing in affinity but acting cooperatively. It is conceivable that the binding of \(^{3}H\)(G)-DAU to one site facilitates binding to the other. Additional evidence that cardiolipin (a phospholipid tightly attached to peptide backbone of the enzyme) may not be the only site for interactions of anthracyclines with COX, as claimed earlier for ADR (35), stems from the observation that lipid-free COX still binds substantial amounts of \(^{3}H\)(G)DAU.

That the prosthetic group of hematin may be a potential target site on hemoproteins for interaction with \(^{3}H\)(G)DAU is based on the following evidence: (a) peptide fragments derived from cytochrome \(c\) and enriched in hematin \(c\) were associated with higher amounts of \(^{3}H\)(G)DAU as compared to intact cytochrome \(c\); (b) exogenous hematin prevented both DAU- and ADR-induced cytotoxicity on K-562 malignant hemopoietic cells and inhibition of COX activity; and (c) \(^{3}H\)(G)DAU forms complexes with hemoproteins as well as with hematin by direct interaction in vitro (21).

Heme (ferroporphyrin IX) serves as a prosthetic group in hemoproteins. During the oxidation of cytochrome \(c\) by COX both substrate and enzyme interact electrostatically via the positively charged lysines of cytochrome \(c\) and the negatively charged aspartic and glutamic acid of subunit II of COX. These interactions may facilitate electron transfer (36). It is also known that the transfer of electrons from one molecule of heme (of cytochrome \(c\)) to another (on COX) is achieved either via a coupling system of the porphyrin ring or via iron (37).

Our observations that \(^{3}H\)(G)DAU interacts with mitochondrial hemoproteins (like COX) and that exogenous hematin prevents DAU and ADR from suppressing COX activity suggest that anthracyclines may interact directly with the prosthetic group of heme in hemoproteins as well as with free hematin. One could view these interactions from different points since, in the case of hemoproteins, heme is immobilized, while in the case of exogenous hematin, heme is free as a molecule. Direct interactions of hematin with DAU may occur via electrostatic interactions, hydrogen bonding, or van der Waals interactions rather than via iron. Electrostatic interactions can be developed due to the negatively charged carboxyl groups of hematin and positively charged groups of DAU (e.g., the NH\(_2\)- group of daunomycin). Indeed, studies from this laboratory have indicated that considerably less \(^{3}H\)(G)DAU was associated with hematin at pH 3.5, which prevents electrostatic interactions from occurring.

Spectrophotometric analysis of interactions between hematin and DAU or hematin and ADR indicated that the spectrum of hematin was affected by either anthracycline, like that of iron-free protoporphyrin IX (Fig. 13). These data suggest that the interaction of DAU and ADR with hematin may not involve iron, since iron is highly coordinated and perhaps unable to interact with either anthracyline, regardless of its high affinity (\(K_{a} = 10^{33.4}\) M) (14, 38). Spectrophotometric studies with hematin and pyrromycin, on the other hand, suggested that there may be structural features of the anthracyclines that influence their ability to interact with hematin via the porphyrin ring. This molecular interaction, however, needs further investigation.

The finding that both DAU and ADR inhibited COX activity at relatively high concentrations is in agreement with previous work (39). This inhibition could result either from direct interaction of DAU with the prosthetic group of hematin or from interaction with other sites on COX. Therefore, the reduction of DAU- or ADR-induced inhibition of COX activity obtained by hematin could be viewed at least in part as a result of the direct interaction of anthracyclines with exogenous hematin; this would limit the interaction of anthracyclines with COX and preserve the function of the enzyme. Thus, hematin allows COX-mediated oxidation to occur, even in the presence of anthracyclines.

The evidence described thus far refers to the interaction of anthracyclines with mitochondrial COX. It is also possible for anthracyclines to impair the biosynthesis of this enzyme at the level of transcription of mitochondrial and nuclear genes coding for COX.

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* A. S. Tsiftsoglou et al., unpublished observations.
subunits. DAU, like ADR, may break mitochondrial DNA and impair the replication as well as transcription of mitochondrial DNA (40). If so, then it is reasonable to assume that DAU and ADR deteriorate mitochondrial functions by exerting a complementary action; they inactivate COX, on the one hand, by acting directly on the enzyme and, on the other, presumably by inhibiting the biosynthesis of the enzyme at the transcriptional level. The latter possibility is currently under investigation in our laboratory. In the context of this hypothesis, we also could view heme as an agent interacting at the nuclear level to prevent DAU-induced effects on DNA as reported elsewhere (48). Such a dual action of DAU on mitochondrial enzyme COX could explain in part the unique toxicity of anthracyclines on heart (41), a tissue that is extremely rich in mitochondrial hemoproteins and depends highly on energy sources and cell respiration.

ACKNOWLEDGMENTS

We would like to thank Dr. C. Tsiamis, Department of Chemistry, Aristotle University of Thessaloniki, for his constructive comments regarding the spectrophotometric studies. In addition, we would also like to thank Prof. A. C. Sartorelli of Yale University for reading this paper.

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