Abstract

The anticancer anthracyclines, doxorubicin and daunorubicin, are highly cytotoxic to both cancer and normal cells. In this work, we have investigated the capacity of cellular myeloperoxidase to inactivate these agents. We show that incubation of human leukemia HL-60 cells with the anthracyclines in the presence of hydrogen peroxide and nitrite causes irreversible oxidation of the drugs, suggesting an extensive modification of their chromophores. Methimazole, 4-amino-benzoic acid hydrazide, or azide inhibits the reaction, suggesting that it is mediated by the cellular myeloperoxidase, an enzyme naturally present in large amounts in HL-60 cells. In contrast to the intact drugs, the oxidatively transformed anthracyclines were substantially less cytotoxic for HL-60 (assayed by apoptosis) and PC3 prostate cancer cells and H9c2 rat cardiac myoblasts in vitro (assayed by clonogenic survival), indicating that the oxidative metabolism of these agents leads to their inactivation. Using tandem mass spectrometry, we identified two specific metabolic products of the anthracycline degradation, 3-methoxyphthalic acid and 3-methoxysalicylic acid. These two metabolic products were obtained as authentic compounds and were nontoxic to HL-60 leukemic cells and cardiac myocytes. These findings may have important implications for the cellular pharmacology of anthracyclines and for clinical oncology. (Cancer Res 2005; 65(14): 6346-53)

Introduction

The development of anthracycline resistance during chemotherapy has often been associated with the multidrug resistance pathways in which the net drug accumulation is decreased due to its efficient efflux, resulting in lower intracellular concentrations of the anthracycline and ultimately lower cytotoxicity. These processes are thought to be controlled by specific proteins (1–3).

Another mechanism that may affect efficacy of anthracycline chemotherapy is direct detoxification of the drugs because they can be actively metabolized into products with suppressed anticancer activity. Most mechanisms of anthracycline inactivation/degradation have been associated with reductive mechanisms of drug metabolism (4). For instance, carbonyl reductase (EC 1.1.1.184), aldose reductase (EC 1.1.1.21), and dihydrodiol dehydrogenase 2 (EC 1.5.1.20), which may increase in anthracycline-resistant cell lines, convert daunorubicin to a less tumor active metabolite, daunorubicinol (13-hydroxydaunorubicin), by reduction of the keto group at carbon-13 (see chemical formula below, R: -H, daunorubicin; R: -OH, doxorubicin; ref. 5).

Sublethal concentrations of daunorubicin can induce carbonyl reductases in pancreatic carcinoma cells (6) and may underlie a resistant phenotype. 13-Hydroxydaunorubicin, although a less potent antineoplastic agent, seems to be more cardiotoxic than its parent compound (6, 7) and readily accumulates in the heart.

An alternative reductive metabolism of anthracyclines, which engages NADPH-cytochrome P450 reductase, mitochondrial NADH dehydrogenase, or the reductase domain of endothelial nitric oxide synthase, converts the quinone moiety of the drugs to semiquinones and gives rise to reactive oxygen species via aerobic redox cycling (8–11). It is considered to be less important in killing cancer cells, although it is believed to play a role in the oxidative damage to the heart. This metabolism does not degrade the drugs so their redox active moiety remains unchanged, which renders them capable of generating continuous fluxes of reactive oxygen species for as long as the drugs remain in the body.

The only metabolic pathway that leads to degradation and permanent inactivation of anthracyclines known today is (per)oxidative metabolism (12–17). Many peroxidases such as horseradish peroxidase and lactoperoxidase (12, 17–20), as well as compounds with pseudoperoxidase activity such as microperoxidase (14), ferrylmyoglobin, and hemin (16), are capable of metabolizing anthracyclines in the presence of H2O2. This metabolism involves oxidation of the hydroquinone moieties of the drug, which leads to permanent loss of their characteristic absorption and fluorescent properties (13, 15). Nitrite, tyrosine, and acetaminophen markedly stimulate this peroxidative metabolism of anthracyclines (13, 17). Importantly, products of the oxidatively degraded anthracyclines are less toxic than their parent compounds (16, 21, 22). One such product, generated by ferrylmyoglobin oxidation of anthracyclines, has been identified as 3-methoxyphthalic acid (3-MePA; ref. 16). On the other hand, photosensitized oxidation forms 3-methoxysalicylic acid (3-MeSA; ref. 23). Formation of these species points out that oxidation of
the anthracycline hydroquinone moiety causes a profound modification of the chromophore as both 3-MePA and 3-MeSA represent a residual D-ring species of the anthracycline skeleton. Importantly, 3-MePA has been detected not only in in vitro systems but also in the heart of mice treated with doxorubicin and in human myocardial biopsies exposed to the drug (16). Because purified peroxidases catalyze oxidation of anthracyclines in the presence of H2O2 and nitrite (12–14, 17, 18), we also sought to determine if cellular-derived peroxidase(s) can support these reactions. In addition, we identified two major metabolites and examined their toxicity in cancer cell lines (HL-60 and PC3) and a normal cell line (H9c2). The characterization of these reactions may explain cellular, tissue, or organ sensitivity/resistance patterns to these particular anticancer antibiotics. A better understanding of these relationships and reactions have therapeutic implications as it may serve for manipulating drug toxicity in cancerous as well as normal tissues.

### Materials and Methods

**Chemicals.** Doxorubicin, daunorubicin, sodium nitrite (99%+), methimazole, 4-aminobenzoic acid hydrazide, sodium azide, hydrogen peroxide (8.8 mol/L), catalase, and lactoperoxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Myeloperoxidase (1 mg/mL stock solution), isolated from human neutrophils, was a generous gift from Dr. Jerrold Weiss (University of Iowa, Iowa City, IA). All other chemicals were of the highest purity available.

**Cell culture.** HL-60 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), and supplemented with 1.5 mmol/L L-glutamine, 76 units/mL penicillin, and 76 μg/mL streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were grown in a humidified incubator at 37 °C with 5% CO2. Cells in log growth phase were harvested by centrifugation and washed twice with fresh media or specified buffers. Cells were counted using a Coulter Model Zf cell counter (Coulter, Inc., Hialeah, FL) and the cell density adjusted to 1 × 10^6 cells/mL unless otherwise indicated.

The rat myoblast cell line H9c2 (2-1) was obtained from ATCC. The cells were grown in MEM supplemented with 1.5 mmol/L L-glutamine, 76 units/mL penicillin, and 76 μg/mL streptomycin with 10% FBS. Cells were passed before reaching confluence to maintain the myoblast phenotype and grown in a humidified incubator at 37 °C with 5% CO2.

**PC3 human prostate cancer cells** were obtained from ATCC, and maintained in RPMI media supplemented with 10% FBS. Two days before experimentation, cells were plated into 60 mm Petri dishes and grown exponentially to 50% confluence. The Petri dishes containing cells were kept at 37 °C in a humidified incubator with 5% CO2.

**Oxidation of anthracyclines for the cytotoxicity assays in HL-60 and PC3 cells.** To daunorubicin (50 μmol/L) in 50 mmol/L PBS (pH 7.0) was added 0.75 unit/mL lactoperoxidase followed by the addition of 1 mmol/L nitrite and 1 mmol/L H2O2. The reaction was initiated by the addition of a small aliquot of H2O2 (5 or 10 μL) as the last component. Time course measurements were carried out following changes in absorbance at 480 nm (νmax for daunorubicin and doxorubicin). Data were collected at 2- or 5-second intervals during continuous stirring of the sample in a spectrophotometric cuvette (1 cm light path). The initial rate of oxidation, V0, was determined from the initial linear portion of the A480 versus time traces using the method of linear regression. The initial concentration of drugs was kept low, ~10 μmol/L, to avoid their dimerization. All measurements were carried out at ambient room temperature of 22 °C, and the experiments were repeated at least twice.

**Metabolism of anthracyclines by HL-60 cells.** Metabolism of anthracyclines by cellular myeloperoxidase was carried out by incubating the drug with HL-60 cells either in PBS (pH 7.0), HBSS, RPMI 1640, or NaCl solution (0.9%), in the presence of H2O2 and nitrite (1 mmol/L each) at 37 °C for 1 hour. At specific time points, aliquots of samples were centrifuged at 16,000 × g for 2 minutes and their absorption spectra were measured. Absorbance at 480 nm was then used to calculate the amount of drug remaining in the supernatants using ε_{480} = 11,500 (mol/L)^{-1} cm^{-1} (27).

**Synthesis of authentic 3-methoxyphthalic acid.** 3-MePA was synthesized by the oxidation of 2,3-dimethylanedialdehyde with refluxing with excess KMnO4 in 70% water and 30% t-butyl alcohol (28). The synthesized material precipitated as white crystals with UV-Vis λ_{max} (H2O) near 300 nm, and produced a single TLC spot, which under UV visualization appeared blue. Nuclear magnetic resonance analysis in d6-DMSO showed a strong peak at 3.7 ppm attributed to OCH3, a doublet H1 at 7.4 ppm, a broad singlet H2 at 4.7 ppm, and a triplet H3 at 7.35 ppm, and a doublet H4 at 7.15 ppm. Additional mass spectrometric (MS) analysis confirmed the product as being 3-MePA (as below). Authentic 3-MeSA and 6-MeSA were obtained from Sigma-Aldrich (St. Louis, MO).

**Preparation of samples for mass spectrometry.** To HL-60 cells at 1 × 10^6/mL in PBS (pH 7.0) was added 1 mmol/L NaN3 and 50 to 100 μmol/L doxorubicin. Immediately, an aliquot was centrifuged free of cells and the supernatant frozen at −20 °C. To the remaining sample was added 1 mmol/L H2O2 and incubated at 37 °C. After the first hour of incubation, 1 × 10^6 HL-60 cells/mL, 1 mmol/L H2O2, and NaN3 were added and allowed to incubate for 1 hour. Then the cells were removed by centrifugation and the supernatant frozen at −20 °C. The samples were then selected for mass spectrometry.
were thawed and then filtered through 3,000 MW centrifugal filters (Millipore Corp., Bedford, MA).

**Tandem mass spectrometry analysis of doxorubicin oxidation products.** A Thermo Electron LCQ Deca ion trap mass spectrometer interfaced with a Surveyor liquid chromatograph and a photodiode array UV detector was used to analyze the samples by liquid chromatography (LC)-MS and tandem mass spectrometry (MS/MS). Samples were injected and separated on a 2.1 × 250 mm Vydac C-18 (5 µm) column (Grace Vydac218TP52) using a solvent system consisting of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile). The LC gradient started at 10% Buffer B, then increased over 15 minutes to 70% Buffer B at a flow rate of 200 µL/min. The photodiode array UV detector was set to monitor both 315 and 480 nm for oxidized doxorubicin metabolites and for doxorubicin, respectively. Both positive and negative ion electrospray ionizations were used in the MS analysis. The (M + H)+ and (M − H)− ions of authentic doxorubicin and 3-MePA, respectively, were used to optimize the positive and negative MS variables. The mass spectrometer was set to alternate between collecting full scan MS and MS/MS data. The full scan MS analysis, the spectra were collected over the range m/z 100 to 600. For the MS/MS analysis, the collision-induced decomposition (CID) energy was adjusted to 35% and the CID spectra were collected for each ion of interest based on its retention time determined during the LC-MS/MS analysis of the authentic compounds.

**Results**

Oxidation of anthracyclines by isolated myeloperoxidase. Here we show that metabolism of two anthracyclines by a myeloperoxidase system can be stimulated by nitrite, which is commonly found in micromolar quantities in serum. Figure 1A shows that when daunorubicin is exposed to myeloperoxidase, H2O2, and nitrite, its characteristic absorption band at 480 nm decreases, indicating that the drug has been oxidized. No such changes were observed when myeloperoxidase, H2O2, or nitrite was omitted. Figure 1A (top inset) shows the time course of A480 at various concentrations of nitrite and the effect of the sequential addition of H2O2, myeloperoxidase, and nitrite. It is apparent that the process depends on the simultaneous presence of all these reactants because no significant oxidation was observed when nitrite was omitted. In the studied nitrite concentration range, the initial rate, V_i, of the drug oxidation is linearly dependent on

![Figure 1. Oxidation of doxorubicin by myeloperoxidase/H2O2/nitrite systems in PBS (pH 7.0). A, absorption spectra recorded during daunorubicin (10.5 µmol/L) oxidation by isolated myeloperoxidase (93 nmol/L), H2O2 (265 µmol/L), and nitrite (500 µmol/L). Traces a to k were obtained at time 0, 20, 40, 60, 80, 100, 120, 140, 160, 220, and 260 seconds, respectively, after the start of the reaction (H2O2 addition) to a sample containing all other components. Top inset, time course of oxidation of daunorubicin followed by changes of absorption at 480 nm. Traces a to d were obtained at [NaNO2] = 100, 250, 500, and 1,000 µmol/L, respectively. [H2O2] and [myeloperoxidase] the same as above. It is shown that in the absence of nitrite (NO2−), H2O2 and myeloperoxidase do not oxidize daunorubicin. Only when NO2− was added did the oxidation of daunorubicin become apparent. Bottom inset, initial rate of daunorubicin oxidation, V_i (dA480/dt) plotted versus [NaNO2]. B, absorption spectra resulting from oxidation of doxorubicin (16.5 µmol/L) by HL-60 cells (10^6 cells/mL) in the presence of H2O2 (1 mM/L) and NO2− (1 mM/L). Spectra shown are those measured after 0, 15, 30, and 60 minutes of incubation at 37°C. Inset, time course of A480 in the presence and absence of nitrite. Representative of two separate experiments.]
[nitrite] (Fig. 1A, bottom inset). This additionally shows the dependence of the drug metabolism by myeloperoxidase/H2O2 on the presence of nitrite. The reported concentrations of nitrite in biological fluids range up to 200 μmol/L (29, 30), which covers the initial portion of [nitrite] used in this study (Fig. 1A, bottom inset). Based on the data in Fig. 1A, the loss of daunorubicin has been determined to be 45.4, 72.8, 87.4, and 94.0% at [nitrite] = 100, 250, 500, and 1,000 μmol/L, respectively. When [nitrite] and [H2O2] were ~50 μmol/L, the maximal loss of the drug was 16%, although when nitrite was omitted the loss was only ~2.6% under otherwise identical conditions. The loss of drug absorption is irreversible, indicating profound changes in the drug chromophore. Similar results were obtained when daunorubicin was replaced by doxorubicin (not shown).

**Oxidation of anthracyclines by cellular peroxidase.** The next experiment was designed to find out whether cellular myeloperoxidase is also capable of supporting anthracycline oxidation in the presence of H2O2 and nitrite. For this purpose, we used HL-60 cells, which naturally contain high concentrations of myeloperoxidase (24, 31). When HL-60 cells (1 x 10^6 cells/mL) were incubated in the presence of 16.6 μmol/L doxorubicin, 1 mmol/L nitrite, and 1 mmol/L H2O2 in PBS (pH 7.0), there was a gradual loss of absorption at 480 nm over 1 hour, although the biggest loss occurred during the first 30 minutes of the reaction (Fig. 1B). These changes are similar to those observed during the incubation of the drug with isolated myeloperoxidase (Fig. 1A).

Fig. 1B inset illustrates the time course of A_{480} changes in the presence and absence of nitrite. Much less drug was lost when nitrite was omitted. When cells or H2O2 was omitted, none or very little drug has been lost (not shown). All these observations confirm that oxidation of the drug can also be accomplished by cellular myeloperoxidase. Also, loss of extracellular drug due to intracellular accumulation during 1-hour incubation was determined to be insignificant compared with extracellular drug loss in the presence of nitrite and H2O2 (data not shown).

The efficacy of the oxidation depends on the number of cells used (Fig. 2A). As little as 10^5 cells/mL sufficed to decrease daunorubicin concentration by 8.6 μmol/L or 43% during 1-hour incubation at 37°C in 0.9% NaCl solution. Doxorubicin was oxidized under the same conditions (results not shown).

To confirm the involvement of cellular myeloperoxidase, in the next set of experiments we determined the effect of peroxidase inhibitors on doxorubicin oxidation. Cells treated with methimazole, 4-aminobenzoic acid hydrazide, or azide, all known inhibitors of peroxidases (32, 33), were unable to oxidize doxorubicin in the presence of H2O2 and nitrite (Fig. 2B). Similar inhibitory effects were observed for daunorubicin (17). This confirms that oxidation of anthracyclines by HL-60 cells involves their endogenous peroxidase.

**Cytotoxicity of intact and oxidized anthracyclines in HL-60 cells.** The human promyelocytic leukemia cell line HL-60 is sensitive to anthracyclines, which induce programmed cell death through apoptosis (24, 25, 34). To access whether oxidation of anthracyclines changes their toxicity profile, we did apoptosis assays in HL-60 cells exposed to the intact and oxidatively modified drug. Apoptosis was assessed by endonuclease DNA fragmentation. For these studies, daunorubicin was oxidized using purified lactoperoxidase in the presence of H2O2 and nitrite, as described earlier (13). It should be noted that following the drug oxidation, catalase (500 units/mL) was added and incubation was continued for 1 hour. This was to decompose unreacted H2O2 because our earlier study revealed that even low concentrations of the peroxide can induce apoptosis in this cell line (24). Following this, various aliquots of this reaction mixture and corresponding aliquots of the intact drug were added to HL-60 cells, which were then incubated for 24 hours at 37°C. As is shown in Fig. 3A, at concentrations of 10 to 100 μmol/L, intact daunorubicin induced apoptosis (lanes 3-5). At a higher concentration (250 μmol/L), the drug induced necrotic cell death (lane 6). In contrast, oxidized daunorubicin was unable to induce apoptosis when applied at 10 to 250 μmol/L (lanes 7-10). Also at 500 μmol/L, oxidized daunorubicin did not induce apoptosis or necrosis (not shown).

These results indicate that the oxidized daunorubicin is markedly less cytotoxic than the intact drug. Control experiments showed that lactoperoxidase + H2O2 + nitrite without drug was nontoxic to HL-60 cells (Fig. 3A, lane 2). The results in Fig. 3A clearly establish that there is a loss in the apoptosis-inducing ability of oxidized daunorubicin.
Cytotoxicity in PC3 human prostate cancer cell line. Because anthracyclines are frequently used in the chemotherapy of prostate cancer, we examined the toxicity of oxidized daunorubicin in the PC3 human prostate adenocarcinoma cells. The PC3 cells were treated with intact and oxidized daunorubicin (0, 2, 10, and 50 μmol/L) for 1 hour and cell viability assayed by clonogenic survival using the method described (26). Results obtained show that oxidized daunorubicin is significantly less potent in killing the tumor cells than the intact drug. For example, following exposure to 10 μmol/L daunorubicin, the surviving fraction of cells was \(5.7 \times 10^{-3}\), whereas at the same concentration of oxidized drug, surviving fraction was \(9.2 \times 10^{-2}\) or more than three orders of magnitude higher. This indicates that oxidation of daunorubicin dramatically suppresses its cytotoxic properties in this cell line.

Cytotoxicity in cardiac myocyte H9c2 cell line. In the experiments described above, we compared cytotoxicity of intact and oxidized daunorubicin in two types of cancer cells. It was of interest to find out whether oxidation of daunorubicin decreases its toxicity for normal cells. For this purpose, we chose the rat heart myoblast cell line H9c2, which is known to be sensitive to anthracyclines (16, 35–37). Because cardiotoxicity is the major side effect of anthracycline chemotherapy, investigation of the oxidation of the drugs on their toxicity in these cells is relevant. We assessed changes in clonogenic survival of H9c2 cells treated with intact and oxidized doxorubicin.

In this set of experiments, we used doxorubicin oxidized by HL-60 cells (as the peroxidase source), \(\text{H}_2\text{O}_2\), and nitrite (1 mmol/L each), as described above. As Fig. 4 shows, doxorubicin at concentrations less than 10 mmol/L caused a substantial decrease in clonogenic survival of H9c2 cells; in this case, less than 6% at 10 mmol/L, with 50% of the cells dead between 2.5 and 5 mmol/L. In contrast, the oxidized drug in the same concentration range showed minimal toxicity. It took at least 500 nmol/L of the oxidized drug to cause a 5% survival (not shown). Given these data, the approximate dose modification factor is \(\approx 50\), indicating that the oxidatively modified doxorubicin has significantly reduced toxicity also in this cell line. This change in toxicity is probably a conservative estimate because some residual unreacted doxorubicin could be still present in the oxidized samples and probably contributed to the cell death observed.
Identification of inactivation metabolites. LC-MS and MS/MS studies were done to identify the degradation products of doxorubicin. In these experiments, parent drug decreased to 5% to 20% of the starting concentration of ~100 μmol/L. The UV chromatograms of the untreated and inactivated drug were monitored at both 315 nm (Fig. 5) and 480 nm (not shown). Figure 5 shows that with treatment, a peak at 11.7 minutes (corresponding to authentic doxorubicin) remarkably decreases, and a new peak at 6.8 minutes appears.

The (M + H)+ ion for doxorubicin (m/z 544) is observed in the mass spectrum at 11.7 minutes in the untreated sample, along with fragment ions of m/z 397 (aglycone), 379 (aglycone–H₂O), 361 (aglycone–2 H₂O), and 321 (aglycone-side chain–H₂O). This is in agreement with earlier reports (38, 39). The CID of the (M + H)+ ion of authentic doxorubicin produces two major ions, m/z 397 and 379 (not shown).

In the chromatogram of the inactivated sample, the peak at 6.8 minutes, not present in the untreated sample, is consistent with the retention time of authentic 3-MePA. The negative ion LC-MS results were also consistent with a previous identification of this compound (16). The (M − H)− ion of m/z 195 was observed for the authentic 3-MePA and in the mass spectrum at 6.8 minutes in the inactivated sample (Fig. 6, left). The fragment ion of m/z 151 due to the loss of CO₂ is also observed in both LC-MS and CID spectra. The CID spectra of both the 3-MePA standard and the oxidized sample are similar in that the only product ion observed is m/z 151. Therefore, we believe this degradation product can be identified as 3-MePA based on the LC-MS and MS-MS data presented.

The authentic 3-MeSA was detected on our LC-MS system as a peak at 9.6 minutes when monitored at 315 nm. When the HL-60–mediated oxidized samples were analyzed by LC, there was no detectable UV peak at 9 to 10 minutes that would correspond with 3-MeSA. However, when selectively looking for ions of m/z 167 in the MS data, a signal at 9.6 to 9.8 minutes was revealed. The CID spectrum of m/z 167 at this retention time shows product ion of m/z 152 attributable to 3-MeSA after loss of methyl group. Other minor ions, of m/z 123 (loss of CO₂) and m/z 108 (loss of methyl and CO₂), were also detected (Fig. 6, right). The similar retention times as well as the similar fragment ions, when compared with authentic 3-MeSA, are consistent with small amounts of 3-MeSA being present in oxidized doxorubicin samples.

Lack of toxicity of authentic 3-methoxysalicylic acid and 3-methoxysalicylic acid. We determined the toxicity of authentic 3-MePA and 3-MeSA toward HL-60 cells and H9c2 cardiac myocytes. No evidence of toxicity measured by apoptosis in HL-60 cells treated with 3-MePA and 3-MeSA, up to 10 μmol/L, was found. Figure 3B shows the results with 3-MePA at 100, 500, 1,000, or 10,000 nmol/L and distinct banding with 250 nmol/L doxorubicin.

In addition, we studied the toxicity of these metabolites in H9c2 cells using clonogenic assay. They seem to be nontoxic. At the highest concentration of authentic 3-MePA or 3-MeSA (10 μmol/L), the clonogenic survival was 101.7 ± 4.7% for 3-MePA and 114.7 ± 9.8% for 3-MeSA.

Discussion

Our experimental results show that cell-derived myeloperoxidase can oxidize anthracyclines in the presence of H₂O₂ and nitrite. Most importantly, the products of this oxidative modification of daunorubicin and doxorubicin are substantially less cytotoxic compared with their parent drugs. The loss of anthracycline toxicity was shown by the oxidized drugs possessing markedly suppressed capacity to induce apoptosis (HL-60) and causing higher clonogenic survival in neoplastic (PC3) and nonneoplastic (H9c2) heart cell lines.

Our earlier study showed that in the presence of nitrite, myeloperoxidase/H₂O₂ oxidizes the hydroquinone moiety of doxorubicin to a semiquinone radical (Eq. A; ref. 13). Although the exact mechanism of the nitrite stimulatory action is not known, we hypothesize that the ‘NO₂ radical, a product of the oxidation of NO₂ by myeloperoxidase/H₂O₂ (18, 40, 41), could be involved. ‘NO₂ is a powerful oxidant and could react with the hydroquinone moiety of the drug, causing its oxidation (13).

\[
\text{Doxo } \cdot \text{Q} + \text{QH}_2 + \text{MPO/H}_2\text{O}_2/\text{NO}_2 \rightarrow \text{Doxo } \cdot \text{Q} - \text{QH}^\cdot (\text{semiquinone})
\]

where Q-QH₂ and Q-QH’ are the quinone-hydroquinone moiety of the drug and the corresponding semiquinone radical, respectively, and MPO is myeloperoxidase.

Another possibility is that NO₂ accelerates the conversion of the less reactive forms of the enzyme (myeloperoxidase compounds II and III) accumulating during the reaction back to native enzyme. Both these actions should result in a more efficient oxidation of the anthracyclines. Nitrite is present in vivo, primarily as a result of dietary intake as well as the end product of oxidation of NO. Small amounts of NO are produced during normal cellular functions but its biosynthesis is markedly intensified in response to infections and during inflammatory conditions usually associated with cancer. Therefore, colocalization of nitrite with peroxidase systems and anthracyclines in the vicinity of cancer is likely. Interestingly, it has been shown that doxorubicin itself stimulates the biosynthesis of nitrite in both normal cells (H9c2 cardiac myocytes) and in cancer cells (EMT-6 breast cancer cell line; refs. 42, 43).

Although the exact molecular mechanism through which anthracyclines undergo oxidation has not been fully elucidated,
it seems certain that it involves the hydroquinone moiety of the drug (ring B, formula in Introduction; refs. 13, 14, 17). In contrast to radicals formed by metabolic reduction (8–11), the radicals formed by oxidation, \( \text{Q-QH} \), do not react with \( \text{O}_2 \), but rather disproportionate to the parent compound and the highly electrophilic diquinone (2 \( \text{Q-QH} \) → \( \text{Q-QH}_2 + \text{Q-Q} \)). The diquinone may undergo further transformation to stable products with modified chromophores. Thus, oxidation of the hydroquinone moiety of the drugs is the first step on the pathway to their degradation.

Using MS, we have identified 3-MePA and 3-MeSA as metabolic products of the leukemic cell peroxidase-induced oxidation of doxorubicin. 3-MePA is the more abundant product and is the same compound that results from the oxidative degradation of doxorubicin by ferrylmyoglobin (16). 3-MeSA was previously identified when doxorubicin was oxidized by photoactivated riboflavin (23). Most importantly, authentic chemical preparations of the two compounds failed to manifest toxicity against leukemic or nonneoplastic heart cells. This lack of toxicity explains the inactivation of the parent drug doxorubicin by oxidation.

Concentrations of doxorubicin, \( \text{H}_2\text{O}_2 \), and nitrite used in some experiments are substantially higher than their physiologic or therapeutic concentrations. This was, however, dictated by the need to obtain metabolites at amounts sufficient for subsequent analysis and to overcome the catalase activity of the cells. At therapeutic doses, the concentration of anthracyclines in the blood is submicromolar, which should require less peroxide and nitrite for their oxidation. Under inflammatory conditions, usually associated with cancer, local concentrations of myeloperoxidase, peroxide, and nitrite may be markedly elevated due to higher activity of neutrophils and macrophages. Due to low concentrations of reactants and the presence of alternative targets, the peroxidative metabolism of anthracyclines in tissues may be significantly slower than that in our \textit{in vitro} systems. We speculate, however, that because the process may occur over a prolonged period of time, it still may be responsible for inactivation of a part of the administered drug.

Although data on biological activities of oxidized anthracyclines are scarce, two recent studies have reported that doxorubicin oxidized by horseradish peroxidase/\( \text{H}_2\text{O}_2 \) inhibits creatine kinase and succinate dehydrogenase, two enzymes important for the proper function of the heart (19, 20). Another study has shown that photodegraded doxorubicin was much less effective in inhibiting growth of \( \text{P}388 \) murine leukemia cells compared with nonirradiated compound (21). Finally, a recent study by Cartoni et al. (16) showed that 3-MePA, the major product of the metabolism of anthracyclines by myoglobin/\( \text{H}_2\text{O}_2 \), is markedly less cytotoxic for the \( \text{H}9\text{c}2 \) rat cardiac myocytes than nonoxidized drug. Results of our cytotoxicity studies using the \( \text{H}9\text{c}2 \) cell line agree with this report.

The use of many chemotherapeutics is limited due to toxicity to organs such as heart, liver, kidneys, as well as bone marrow. Besides poor tumor response, in some instances other complications such as drug resistance may develop during the course of treatment that may cause clonal expansion with cross-resistance to other chemotherapeutic drugs. Therefore, strategies aimed at enhancing target cell sensitivity, preventing drug resistance, as well as suppressing toxicity to nontarget cells, tissues, or organs, become especially important with the current generation of drugs. In this respect, we believe that we have addressed a new and unrecognized pathway by which leukemic cells, as well as other cancers that express peroxidases, may potentially inactivate anthracyclines \textit{in vivo} and decrease the efficacy of the anthracycline therapy.

We conclude that peroxidase contained in human leukemia cells is capable of inactivating anthracycline anticancer drugs especially...
in the presence of an additional substrate such as nitrile. It is important to emphasize that nitrile is not the only factor capable of stimulating the process, as many phenolic compounds, including tyrosine and ascorbatin, exert a similar prooxidant activity (17, 44). In addition, given that cellular antioxidants ascorbic acid and reduced glutathione, as well as peroxidase blockers, inhibit oxidative degradation of anthracyclines by myeloperoxidase and HL-60 cells (17), it seems that inactivation of the drugs in vivo could be modulated by pharmacologic interventions. This intervention may affect cellular pharmacology of anthracyclines and have implications to clinical oncology.

References
Inactivation of Anthracyclines by Cellular Peroxidase


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