We show that the pseudoperoxidase activity of ferrylmyoglobin (MbIV) promotes oxidative degradation of doxorubicin (DOX), an anticancer anthracycline known to induce severe cardiotoxicity. MbIV, formed in vitro by reacting horse heart MbIII with H2O2, caused disappearance of the spectrum of DOX at 477 nm and appearance of UV-absorbing chromophores that indicated opening and degradation of its tetracyclic ring. Electron spray ionization mass spectrometry analyses of DOX/MbIV ultratratisses showed that DOX degradation resulted in formation of 3-methoxyphthalic acid, the product of oxidative modifications of its methoxy-substituted ring D. Other methoxy-substituted anthracyclines similarly released 3-methoxyphthalic acid after oxidation by MbIV, whereas demethoxy analogs released simple phthalic acid. Kinetic and stoichiometric analyses of reactions between DOX and MbIII/H2O2 or hemin/H2O2 showed that the porphyrin radical of MbIV-compound I and the iron-oxo moiety of MbIV-compound II were sequentially involved in oxidizing DOX; however, oxidation by compound I formed more 3-methoxyphthalic acid than oxidation by compound II. Sizeable amounts of 3-methoxyphthalic acid were formed in the heart of mice treated with DOX, in human myocardial biopsies exposed to DOX in vitro, and in human cardiac cytosol that oxidized DOX after activation of its endogenous myoglobin by H2O2. Importantly, H9c2 cardiomyocytes were damaged by low concentrations of DOX but could tolerate concentrations of 3-methoxyphthalic acid higher than those measured in murine or human myocardium. These results unravel a novel function for MbIV in the oxidative degradation of anthracyclines to phthalic acids and suggest that this may serve a salvage pathway against cardiotoxicity.

Myoglobin (Mb) has been implicated as a potential catalyst of cardiac damage induced by increased formation of hydrogen peroxide (H2O2). Under normal conditions the majority of Mb is found in its oxygenated form (MbO2), which interacts slowly with H2O2 (k = 20.8 s⁻¹ M⁻¹); however, both deoxy-MbI and metmyoglobin (MbIII) react rapidly with H2O2 (k = 3.6 × 10⁸ and 3.4 × 10⁸ s⁻¹ M⁻¹, respectively) (1, 2). An ideal setting for reactions between Mb and H2O2 has therefore been identified in cardiac ischemia-reperfusion, a condition characterized by conversion of MbIII to MbIV/deoxy-MbIV during ischemia and by formation of H2O2 during blood reflow (3, 4). Hydrogen peroxide causes two-equivalent oxidation of MbIII to ferrylmyoglobin (MbIV), a hypervalent species that oxidizes polysaturated fatty acids and several other biomolecules in a fashion similar to that described for the compound I or II of peroxidases. The reaction sequence through which MbIV is generated from MbIII has been considered as shown in Reactions 1 and 2.

\[
\text{Por-MbIII-globin} + \text{H}_2\text{O}_2 \rightarrow \text{Por-MbIV-globin} + \text{H}_2\text{O} \\
\text{REACTION 1}
\]

\[
\text{Por-MbIV-globin} \rightarrow \text{Por-MbIV-globin} \\
\text{REACTION 2}
\]

In Reaction 1, H2O2 converts MbIII to a compound I-like species in which both oxidizing equivalents are retained in the heme pocket, one in the form of a long lived iron-oxo moiety (FeIV=O) and the other in the form of a transient porphyrin σ-cation radical (Por⁺) (5). In Reaction 2, the porphyrin radical dissipates in the globin, causing formation of amino acid radicals while leaving the heme moiety in a FeIV=O form similar to compound II (5–8).

We developed an interest in possible reactions between Mb and doxorubicin (DOX), an anticancer anthracycline which exhibits activity against several tumors but also causes severe cardiotoxicity. The rationale for investigating DOX-Mb interactions was offered by several considerations. On the one hand, cyclic reduction-oxidation of a quinone moiety in the tetracyclic ring of DOX (Fig. 1) generates H2O2 in excess of the detoxifying capacity of cardiomyocytes (9–11). On the other hand, DOX causes a 4-fold stimulation of the autoxidation of MbII to MbIII (12) and inhibits MbIII reductases that would regenerate

MbIII, metmyoglobin; MbIV, ferrylmyoglobin; H2O2, hydrogen peroxide; HRP, horseradish peroxidase; LPO, lactoperoxidase; DNR, daunorubicin; IDA, idarubicin; ABTS, 2,2'-diazinobis(3-ethylbenzothiazoline-6-sulfonic acid); ESI-MS, electron spray ionization-mass spectrometry; TIC, total ion count; C.V., cone voltage; M, molecular ion; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; HPLC, high pressure liquid chromatography.
MbIVO2 (13). Thus, several factors seem to enable DOX to promote reactions between MbIII and H2O2, possibly exposing cardiomyocytes to lipid peroxidation or other forms of oxidative injury induced by MbIV. In contrast to these premises, however, we found that DOX inhibited lipid peroxidation induced by MbIV in reconstituted chemical models (14). We demonstrated that the “antioxidant” effect of DOX was due to its ability to reduce MbIV back to MbIII, a process mediated by a hydroquinone-induced injury induced by MbIV. In contrast to these premises, however, logs used in this study.

The lack of a methoxy residue in ring D). Primary alcohol; IDA, same as DNR and a side chain methyl terminus in place of a lack of a methoxy residue in ring D).

We designed experiments to elucidate how Mb IV oxidizes DOX and to identify the product(s) of DOX degradation. Moreover, we characterized the possible role of degradation product(s) in anthracycline-induced cardiotoxicity.

Attempts to identify the product(s) of Mb IV-dependent DOX degradation were unsuccessful (14), and similar problems were encountered by other investigators when DOX was oxidized with horseradish peroxidase, microperoxidase 11 (product of the proteolytic digestion of cytochrome c), or NO2 radicals generated through lactoperoxidase/NO2 or myeloperoxidase/NO2 (15–18). Lack of information about the nature of degradation products precludes an appraisal of the mechanisms and consequences of Mb IV-anthracycline interactions. Therefore, we designed experiments to elucidate how Mb IV oxidizes DOX and to identify the product(s) of DOX degradation. Moreover, we characterized the possible role of degradation product(s) in anthracycline cardiotoxicity.

EXPERIMENTAL PROCEDURES

Chemicals—Doxorubicin, daunorubicin (DNR), and 4-demethoxy-daunorubicin (idarubicin, IDA) were obtained through the courtesy of Pharmacia-Upjohn, Milan, Italy. Thymol-free bovine liver catalase, horse heart MbIII, hemin, type VI-A horseradish peroxidase (HRP), bovine milk lactoperoxidase (LPO), 2,2'-diazinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), naphthazarin (5,8-dihydroxy-1,4-naphthoquinone), and all other chemicals were from Sigma. 3-Methoxyphthalic acid was synthesized by oxidation of 2,3-dimethylanisole with a large excess of potassium permanganate (19) and purified by crystallization from water.

MbIV Formation—MbIV was formed by reacting MbIII with a 2-fold excess of H2O2. As shown previously (14), this H2O2:MbIII ratio gives complete oxidation of MbIII to MbIV while also avoiding confounding factors due to the possible release of iron from the heme pocket. MbIII was quantitated by assuming e600 nm = 3.5 mm-1 cm-1; MbIV was quantitated according to the formula: MbIV (mM) = (249 × A550 nm) / (367 × A600 nm)) (20). Unless otherwise indicated, the experiments were carried out at 37 °C in 0.3 mM NaCl, carefully adjusted to pH 7.0. This was done because we noticed that common buffers like phosphate or Tris or Heps accelerated a spontaneous decay of MbIV to MbIII; buffers are also known to participate in anthracycline redox reactions (21). Although unbuffered, the pH of incubations did not vary throughout the experiment.

Spectrophotometric Assays for MbIV-dependent DOX Degradation—After correction of MbIII absorbance, known amounts of DOX were added, and the spectrum of the anthracycline at 477 nm was recorded immediately. Next, H2O2 was added, and the disappearance of the spectrum of DOX was monitored at regular times. Similar settings were adopted when hemin was used in place of MbIII; in the latter case 10 mM stock solutions of hemin were prepared in 100 mM NaOH and diluted appropriately with 50 mM phosphate buffer to obtain 1 mM working solutions.

Spectrophotometric Assays for DOX-dependent MbIV Reduction—In the experiments described in the preceding section, involving sequential additions of MbIII and DOX and H2O2, stepwise H2O2-dependent oxidation of MbIII to MbIV/FeIV–O and DOX-dependent reduction of MbIV/FeIV–O to MbIII were monitored at 426 or 410 nm, respectively (6). In other experiments, MbIII was added, and its spectrum (peaks at 502 and 630 nm) was recorded. Next, H2O2 was added, and the spectrum of MbIV/FeIV–O (peaks at 546 and 586 nm) was taken at regular times until DOX was included. Doxorubicin-dependent MbIV/FeIV–O reduction and MbIII regeneration were eventually monitored as disappearance of peaks at 546 and 586 nm or reappearance of peaks at 502 and 630 nm, respectively.

HPLC-UV-ESI(+)/MS Analysis of DOX Degradation—Varying amounts of DOX were incubated with equimolar MbIII and a 2-fold excess of H2O2. After 10 min the reaction mixtures were added with catalase (2690 units) to decompose any residual H2O2 and ultrafiltered through YM 10 Centricon® (Millipore Corp., Bedford, MA). Myoglobin-free ultrafiltrates were injected without further manipulations into a BioSys 510 Liquid Chromatography System (Beckman Instruments, Fullerton, CA) equipped with a (1 × 20 cm) Ultragel A34 column.
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(BioSeps SA, Villeneuve la Garenne Cedex, France) equilibrated with 2.5 mM NaCl, 2.5 mM CH₃COONH₄. Samples were eluted with the same medium at the flow rate of 0.5 mL/min. UV-absorbing fractions were pooled, passed through Anotop 250 filters (Merck), and concentrated by evaporation. After suspension in a minimum volume of CH₃OH, the sample was laid into a Waters 2790 separation module (alliance HT) equipped with a Waters 996 photodiode array and a ZMD single quadrupole mass spectrometer (Micromass, UK) as detectors in series. Reversed phase HPLC was performed using a LiChrospher (Merck) analytical column (100 RP-18 (5 μm), 150 × 4 mm) and a mobile phase composed of (H₂O + 0.1% trifluoroacetic acid):(CH₃CN + 0.1% trifluoroacetic acid) at an initial ratio of 80:20, which was decreased to 30:70 in 15 min and returned to initial conditions in 20 min, at the flow rate of 0.5 mL/min. The chromatography was performed with a Micromass Quattro Ultima triple quadrupole mass spectrometer (Micromass, UK) equipped with a Z-Spray ESI source, operated in negative ion mode. Standard ESI ion source, operated in positive ion mode, was used with capillary voltage of 3.25 kV and cone voltage (C.V.) of 20 or 50 V to obtain mass spectra under low or high energy conditions. The source and desolvation temperatures were 100 and 250 °C, respectively. Nitrogen was used as the nebulizer gas at a flow rate of 59 liters/h and as the desolvation gas at a flow rate of 430 liters/h.

Liquid Chromatography-Tandem Mass Spectrometry—Because HPLC-UV-ESI+ MS identified 3-methoxyphthalic acid (molecular mass = 196 daltons) as a possible product of MB³⁻-dependent DOX degradation, we used liquid chromatography-mass spectrometry to further characterize DOX/Mb³⁻ ultratransferrin in comparison with authentic 3-methoxyphthalic acid. Reversed phase HPLC was carried out using a Waters Alliance 2795 separation module and an Agilent HyperSil ODS² column (2.1 × 50 mm; C₁₈, 5 μm, 120 Å). Ten microliter samples were eluted at a flow rate of 200 μL/min with (H₂O + 0.2% HCOOH) - (10% CH₃CN + 0.2% HCOOH). After 2 min CH₃CN was increased linearly to reach 70% in 6 min and was then maintained isocratically for 4 more min. The HPLC system was directly coupled with a Micromass Quattro Ultima™ triple quadrupole mass spectrometer equipped with a Z-Spray ESI source, operated in negative ion mode to improve sensitivity. Analytical conditions were optimized with direct infusion of standard solutions of 4.5 μL/min. Mass spectra were acquired at low (unity) resolution in the mass range of 50–350 m/z. The product ion scan mode for tandem MS was used. The mass spectrometer monitored the deprotonated molecule [M – H]⁻ of 3-methoxyphthalic acid at m/z 195 via the first quadrupole filter, and collision-induced dissociation was performed at the second quadrupole (collision gas, argon, at 2.4–3 mбар, and collision energy at 8 eV). The daughter ions of m/z 195 were monitored via the third quadrupole in the mass range of 50–300 atomic mass units. Spectra were acquired in the continuous mode. The capillary and cone voltages were set at −3 kV and −40 V, respectively. The source and desolvation temperatures were set at 110 and 400 °C, respectively, and the desolvation gas (N₂) flow was 800 L/h. Cone gas was not used, whereas the nebulizer gas (N₂) was left at its maximum flow.

Other HPLC Assays for Anthracyclines and Phthalates—Anthracene/Mb³⁻-H₂O₂ incubations were extracted with a 4-fold excess of CHCl₃/CH₃OH (1:1) and subjected to 10 min of low speed centrifugation. Next, 20 μL of the methanolic phase were analyzed for 3-methoxyphthalic acid or simple phthalic acid by reversed phase HPLC using an HP 1100 system (Hewlett-Packard Co., Palo Alto, CA). Chromatography was performed using a Hewlett-Packard Zorbax CN column (250 × 4.6 mm, 5 μm) operated at 25 °C. Samples were eluted at the flow rate of 1.5 mL/min by using a 15-min linear gradient from 50 μM NaH₂PO₄ to CH₃CN, 25 μM NaH₂PO₄, all adjusted to pH 4.0 with orthophosphoric acid, and filtered through a 0.22-μm membrane (Millipore Corp.). Phthalates were detected at 300 nm by on-line diode array spectrometer. Identification was obtained by co-elution with authentic standards and ESI (+)-MS analysis of peak fractions; quantification was obtained against standard curves prepared with known amounts of phthalates subjected to the same extraction and chromatographic procedures. Quantification was performed with 3-methoxyphthalic acid or simply phthalic acid were 4.1 and 4.7 min, respectively. Anthracenes were detected by diode array and fluorescence spectroscopy (excitation at 470 nm, emission at 550 nm); retention times of DOX, DNR, IDA, and naphthazarin were 12.1, 13.8, 14.3, or 14.5 min, respectively. Quantification was obtained against appropriate standard curves of each anthracene.

DOX (10 mg/kg, intravenously) and sacrificed after 4 or 24 h. Heart, liver, and kidneys were removed, and 0.2-g aliquots were homogenized in ice-cold 0.3 M NaCl. Next, the homogenates were added with 5 ml of ice-cold acetone, incubated at −20 °C for 30 min, and centrifuged to precipitate proteins. The supernatants were vacuum-dried, dissolved of gross particulates by low speed centrifugation in CH₃OH, and assayed for DOX and 3-methoxyphthalic acid by HPLC.

Fluorescent anthracycline metabolites, like the C-13 secondary alcohol metabolite and hydroxy- or deoxyglycrones, were also measured using appropriate standards (retention times: 10.9, 12.5, and 13.8 min, respectively).

3-Methoxyphthalic Acid (Micromass Formation in Human Myocardium)—Small myocardial samples were taken from the lateral aspect of excluded right atrium of patients undergoing aorto-coronary bypass grafting. All specimens were routinely disposed of by the surgeons during cannulation procedures; therefore, patients were not subjected to any unjustified or ethically unacceptable loss of tissue (22). Thin myocardial strips (< 0.1 g) were dissected and placed in 5 mL of Krebs bicarbonate buffer in the presence of 1–10 μM DOX. After 4 h at 37 °C the strips were removed, washed extensively in ice-cold 0.3 M NaCl, and extracted for HPLC analysis of DOX and fluorescent metabolites or 3-methoxyphthalic acid as described in the preceding section. In other experiments, pools of 10–15 anonymous biopsies were processed by sequential homogenization, 20-min centrifugations at 16,000 and 25,000 × g, and 90-min ultracentrifugation at 105,000 × g. The supernatant (cytosol) was used immediately or after precipitation with 65% ammonium sulfate, a procedure known to remove Mb by “salting out” (4, 14). Mb-containing or Mb-depleted cytosol (referred to as Mb³⁻ or Mb⁻ cytosol, respectively) was eventually dialyzed against two 1-liter changes of 0.3 M NaCl, 1 mM EDTA to remove chromatinous iron and other low molecular weight contaminants, and then against two 1-liter changes of 0.3 M NaCl to remove EDTA-iron complexes (23). Based on nephelometric and spectral assays by us and others (24), the myoglobin content of right atrium homogenates and Mb³⁻ or Mb⁻ cytosol was determined to be 1.3 and 2.9 or 0.6 nmol/mg of protein, respectively. Whole homogenates and Mb³⁻ or Mb⁻ cytosol were eventually reconstituted with known amounts of DOX and H₂O₂ and assayed for anthracycline degradation and 3-methoxyphthalic acid formation by HPLC.

Experiments with Isolated Cardiomyocytes—The embryonic rat heart-derived cell line H9c2 (American Type Culture Collection (CRL 1446)) was grown in Dulbecco’s modified Eagle’s medium as described previously (25). Subconfluent cardiomyocytes were plated in quadruplicate in 24-well plates (10⁶ cells/well) and incubated overnight in the absence or presence of increasing amounts of DOX or 3-methoxyphthalic acid. At the end of treatment, cell viability was measured by 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) conversion assay as an indicator of mitochondrial function (Sigma kit) (26).

Other Conditions and Assays—Proteins were assayed by the bicinchoninic acid method (27). The peroxidative activity of HRP, LPO, Mb³⁻ and hemin was determined by monitoring oxidation of ABTS to its cation radical ABTS⁺ (ε₆₅₀₉₅ = 12 × 10³ M⁻¹ cm⁻¹) (16). All values are given as means ± S.E.; where indicated, data were analyzed by unpaired Student’s t test, and differences were considered significant when p < 0.05. Other conditions are indicated in the legends to figures and tables.

RESULTS

Mb³⁻-dependent DOX Degradation, Role of Compound I and II—Incubation of DOX with H₂O₂ and Mb³⁻ resulted in a time-dependent decay of the spectrum of the anthracycline and concomitant development of absorbance at 350 nm (Fig. 2A). These spectral changes were similar to those observed by others when monitoring reactions of DOX with H₂O₂ and HRP or lactoperoxidase (16); under those conditions the loss of absorbance at 477 nm was attributed to degradation of the anthraquinone chromophore, whereas the increase of absorbance at 350 nm was attributed to accumulation of degradation product(s). In our study putative product(s) of anthracycline degradation were found to exhibit more complex characteristics. In fact, difference spectra obtained by subtracting the initial scan of DOX/Mb³⁻ mixtures to the scans obtained at regular times after H₂O₂ addition revealed that the increase in absorbance at 350 nm was accompanied by additional peaks at −210, 242, and 280 nm, possibly reflecting the formation and spectral overlapping of a complex mixture of degradation products (Fig. 2B).

Doxorubicin degradation increased with the ratio of Mb³⁻ to DOX, near-to-complete degradation occurring at Mb³⁻:DOX ratios around unity (Fig. 2C). Mb⁰ was less effective than HRP.
Fig. 2. MbIV-dependent DOX degradation. A, incubations (1 ml final volume) contained 3 μM MbIII in 0.3 M NaCl, pH 7.0, 37 °C. After correction for MbIII absorbance, 5 μM DOX was added, and its spectrum was recorded immediately. Next, 6 μM H2O2 was added, and spectra were recorded every minute. All samples were corrected for background absorbance of reference cuvettes lacking DOX. B, difference spectra obtained by subtracting the spectrum of the unchanged DOX to the scans recorded at different times after H2O2 addition; the boldface trace is the difference spectrum recorded 1 min after H2O2 addition. C, increasing MbIV:DOX ratios were obtained by incubating 5 μM DOX with 0.1–10 μM MbIII, always reacted with a 2-fold excess of H2O2. Values are means ± S.E. of three determinations; values without vertical bars have S.E. within the symbols.

or LPO at oxidizing a typical peroxidatic substrate like ABTS, its kcat/km for formation of ABTS+ being ~2 orders of magnitude lower than that determined for either peroxidase (1.8 ± 0.2 × 108 for MbIV versus 7 ± 0.4 × 108 or 1.1 ± 0.3 × 108 M⁻¹ s⁻¹, respectively; n = 3). However, MbIV was more effective than HRP or LPO at degrading DOX, its kcat/km for DOX disappearance being ~1 order of magnitude higher than that determined for either peroxidase (1.1 ± 0.2 × 108 versus 0.9 ± 0.1 × 108 or 3 ± 0.2 × 108 M⁻¹ s⁻¹, respectively; n = 3). Thus, a pseudoperoxidase like MbIV was more effective than authentic peroxidases at inducing oxidative degradation of DOX.

Incubation of DOX with H2O2 and MbIII was also accompanied by appearance of peaks at 426 and 410 nm. The peak at 426 nm developed immediately after the addition of H2O2; it reflected oxidation of MbIII with H2O2 and consequent formation of FeIV=O (6). The peak at 410 nm developed later during the course of the reaction and eventually replaced the peak at 426 nm; it reflected reduction of FeIV=O to MbIII (6). These results suggested that DOX degraded by oxidizing with FeIV=O/compound II, but the kinetics of DOX degradation did not match the kinetics of FeIV=O reduction/MbIII regeneration. In fact, the absorbance of DOX at 477 nm decreased immediately after mixing MbIII with H2O2, and dropped to ~60% its initial level in ~4 min, i.e. the time when FeIV=O was at its maximum detectable level (Fig. 3, A and B). Based upon these findings, we characterized whether DOX degradation was initiated by a compound I involving FeIV=O and a porphyrin radical. To obtain this information, we incubated DOX with H2O2/hemin, an established source of FeIV=O/porphyrin radical (28). Preliminary experiments showed that hemin was less effective than equimolar MbIII at catalyzing ABTS oxidation in the presence of H2O2, even when H2O2:hemin ratios were much higher than H2O2:MbIII ratios (units of peroxidative activities (nmol ABTS⁻/nmol heme/min): 0.02 for 20:1 H2O2:hemin versus 0.4 for 2:1 H2O2:MbIII). Despite its reduced peroxidative activity, hemin/H2O2 was quite effective at degrading DOX, giving the same spectral changes obtained with MbIV/H2O2 (loss of absorbance at 477 and formation of multiple peaks at ~210, 242, 280, and 350 nm; Fig. 4A and inset). Doxorubicin degradation actually occurred much faster with hemin-H2O2 than with MbIII-H2O2. This was observed when DOX was incubated with equimolar MbIII or hemin, and anthracycline degradation was normalized to peroxidative activities (Fig. 4B). It was also observed when the concentrations of H2O2 and MbIII or hemin were adjusted to achieve similar peroxidative activities in the incubations (Fig. 4C). The anthracycline-degrading activity of hemin/H2O2 could not be attributed to a denaturation of the heme pocket and consequent release of redox-active iron due to the high H2O2:hemin ratios used in this study; in fact, DOX underwent degradation also in the presence of EDTA (cf. Fig. 4, B and C). Thus, DOX oxidative degradation was initiated by the porphyrin radical of compound I, followed by the action of FeIV=O/compound II.

HPLC-UV-ESI(+)-MS Characterizations of DOX Degradation Products—Previous ESI and MALDI mass spectral analyses of organic extracts derived from peroxidase-anthracycline incubations did not reveal anything but reduced intensity or disappearance of molecular ions attributable to intact anthracyclines or fragments generated under high energy conditions; unambiguous evidence for newly formed degradation products was not obtained (16). In attempting characterization of products of MbIV-dependent DOX degradation, we therefore considered that it was necessary to avoid organic extractions or other extensive manipulations that might have caused a loss of products with unknown polarity and partitioning in laboratory solvents. We developed a flow chart in which MbIV/DOX incubations were subjected to ultrafiltration in place of organic extractions. Next, the ultrafiltrates were subjected to gel permeation under low ionic strength conditions (2.5 mM NaCl, 2.5 mM CH3COONH4), a procedure needed to reduce the concentration of NaCl in the samples and to avoid interferences of Na+ clusters with ESI-MS analyses (cf. “Experimental Procedures”). This procedure enabled us to isolate a gross fraction that eluted before DOX, possibly due to micelle formation and/or gel electrostatic repulsions, and exhibited the same retention time when the eluant was monitored at 210, 242, 280, or 350 nm (i.e. the UV peaks of MbIV or H2O2/hemin-degraded

2 In preparing for analyses of DOX degradation product(s), we acquired ESI(+)-MS spectra of DOX standards subjected to the same ultrafiltration/gel permeation procedure and dissolved in a (1:1) H2O:CH3CN mixture containing 2 mM CH3COONH4. Low energy mass spectrum showed a quasi-molecular ion at m/z 544 [M + H]+, whereas in the high energy mass spectrum several fragments were observed: m/z 415 (aglycone), 397 (aglycone without one water molecule), 379 (aglycone with aromatized ring A), 361 (probably aromatized aglycone without –OH in the side chain), 321 (aromatized aglycone without side chain), 148 (protonated sugar), and 130 (sugar without –OH in anomeric position) (see also Refs. 16, 29, and 30).
Different times after H2O2 addition; the changed DOX to the scans recorded by subtracting the spectrum of un-

derived from MbIV-degraded DOX (Fig. 5A). Reversed phase HPLC resolved this material into several peaks, as

shown in the chromatogram obtained at λ210–600 nm (Fig. 5B). Among these peaks only that corresponding to a retention time of 5.02 min gave also a significant total ion count (TIC), which was detected at a retention time of 5.21 min in the ESI-positive

mass spectra acquisition (Fig. 5C). The slight peak-to-peak delay (from 5.02 to 5.21 min) was due to the dead volume between photodiode array and mass spectrometer.

Because mass spectra acquisition is fundamental for characterizing molecules, these conditions gave us an opportunity to identify only one component of the complex material analyzed. As also shown in Fig. 5, the UV spectrum of the unknown compound was characterized by a single maximum at 299 nm and no absorbance over 350 nm. Under low energy conditions (C.V. = 20 V) the mass spectrum displayed a quasi-molecular ion [M + H]+ at m/z 197, indicating a possible molecular mass of 196 daltons for the molecule; under high energy conditions (C.V. = 50 V) a main fragment at m/z 179 was recorded, possibly indicating the loss of water [M + H – 18]⁺ from the quasi-molecular ion. Based upon these mass spectra, and taking the structure of DOX into account, we tentatively assigned the unknown molecule to 3-methoxyphthalic acid, product of oxidative modifications of ring D of DOX. Indirect support to such conclusion was also offered by the striking similarity between the UV spectrum of the unknown molecule and that reported for 3-methoxyphthalic acid in ethanol (λmax = 298 nm) (31). Moreover, (i) authentic 3-methoxyphthalic acid co-chromatographed with the ultrafiltrates of MbIV/DOX in gel permeation while also giving the same retention time in HPLC/ESI(+)-MS; (ii) the UV spectrum of HPLC-eluted 3-methoxyphthalic acid exhibited the same λmax = 299 nm of the spectrum of the unknown compound; (iii) the low energy mass spectrum of 3-methoxyphthalic acid gave the expected quasi-molecular ion [M + H]+ at m/z 197, and the high energy mass spectrum gave a fragment at m/z 179 (Fig. 6). This fragment ion, similar to that observed when recording a mass spectrum of the unknown compound under high energy, was therefore consistent with H2O elimination from protonated 3-methoxyphthalic acid and with the possible formation of its protonated anhydride (see also Fig. 6).

**LC-ESI(−) MS/MS of 3-Methoxyphthalic Acid and MbIV-degraded DOX**—To strengthen identification of 3-methoxyphthalic acid as a product of DOX degradation, we characterized a more detailed fragmentation pattern of DOX/MbIV ultrafiltrates vis à vis authentic 3-methoxyphthalic acid. For this purpose we used LC-ESI MS/MS in product ion scan mode. Under the analytical conditions adopted in our study (cf. “Experimental Procedures”), the negative ion mode proved to be

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**Fig. 3. Time courses of FeIV=O formation, MbIII regeneration, and DOX degradation.** Incubations and spectral analyses were as described in Fig. 2A, and absorbances at 410, 426 (A), and 477 nm (B) were monitored simultaneously.

**Fig. 4. Hemin-dependent DOX degradation.** A, incubations (1 ml final volume) contained DOX and hemin (both 50 μM); reactions were started by adding H2O2 (1 mM), and spectra were taken every 90 s. The *inset* shows spectra obtained by subtracting the spectrum of unchanged DOX to the scans recorded at different times after H2O2 addition; the *boldface trace* is the difference spectrum recorded 90 s after H2O2 addition. B, incubations contained 25 μM DOX and equimolar MbIII or hemin; reactions were started by adding H2O2 at 2 or 20:1 ratios to MbIII or hemin, respectively, and rates of DOX degradation were normalized to peroxidative activities. C, incubations contained 50 μM DOX and either 2.5 μM MbIII or 50 μM hemin, corresponding to 1 unit of peroxidative activity/ml; reactions were started by adding H2O2 at 2 or 20:1 ratios to MbIII or hemin, respectively. Where indicated incubations contained 0.1 mM EDTA.
more sensitive and convenient than the positive mode for performing these experiments. Fig. 7A shows gel permeation of ultrafiltrates derived from MbIV-degraded DOX (100 μM (line a), 200 μM (line b), or 500 μM (line c)); the upper arrows indicate the retention times of MbIII or DOX standards chromatographed under comparable conditions. B shows HPLC of gel-filtered fraction c in A, and C shows the corresponding HPLC/ESI (+)-MS chromatogram. ESI (+)-MS of the HPLC fraction eluted at 5.21 min was carried out at C.V. = 20 or 50 V; the inset in the upper panel shows the UV spectrum associated with the HPLC fraction eluted at 5.02 min.

Fig. 5. Gel permeation, HPLC, and HPLC/ESI (+)-MS of DOX/MbIV ultrafiltrates. Incubations (1 ml final volume) contained DOX (100–500 μM) and equimolar MbIII, always reacted with a 2-fold excess of H2O2. After 10 min, reactions mixtures were added with catalase (2600 units) and ultrafiltered. A shows gel permeation of ultrafiltrates derived from MbIV-degraded DOX (100 μM (line a), 200 μM (line b), or 500 μM (line c)); the upper arrows indicate the retention times of MbIII or DOX standards chromatographed under comparable conditions. B shows HPLC of gel-filtered fraction c in A, and C shows the corresponding HPLC/ESI (+)-MS chromatogram. ESI (+)-MS of the HPLC fraction eluted at 5.21 min was carried out at C.V. = 20 or 50 V; the inset in the upper panel shows the UV spectrum associated with the HPLC fraction eluted at 5.02 min.

Stoichiometries and Time Courses—HPLC analyses of DOX/MbIII/H2O2 incubations showed that 3-methoxyphthalic acid formation closely paralleled DOX degradation induced by increasing levels of MbIV (Fig. 8A).

Doxorubicin degradation and 3-methoxyphthalic acid formation occurred in a time-dependent manner (Fig. 8B), but the stoichiometry of 3-methoxyphthalic acid formation versus DOX degradation decreased from ~0.5 to ~0.12 in 3–4 min (Fig. 8C). This was not attributable to DOX depletion or secondary metabolization of 3-methoxyphthalic acid; in fact, ~60–70% of DOX was still available for further degradation after 3–4 min reaction between MbIII and H2O2, and 3-methoxyphthalic acid was stable to prolonged incubation with MbIII or H2O2 or MbIII/H2O2 (cf. Fig. 8, B and C, and inset). Data analysis showed that the stoichiometry decreased due to a 4-fold increase of DOX degradation that was not accompanied by an increased formation of 3-methoxyphthalic acid. Because these changes occurred 3–4 min after mixing MbIII with H2O2, which is the time when the anthracycline began reacting with FeIV=O, we considered that compound II was highly effective at oxidizing DOX but generated several product(s) other than 3-methoxyphthalic acid, eventually decreasing the stoichiometry of 3-methoxyphthalic acid formation versus DOX degradation. This possibility was anticipated by the fact that hemin/H2O2 (a surrogate of compound I) allowed DOX oxidation and 3-methoxyphthalic acid formation to proceed stoichiometrically coupled for several more minutes than did MbIII/H2O2 (a source of both compound I and II) (not shown). Further evidence was obtained by adding DOX 1 h after mixing MbIII with H2O2, a time when both porphyrin and globin radicals had decayed (2, 32, 33), whereas the long lived FeIV=O was still present at ~50% its initial absorbance (Fig. 9A). Under these defined conditions, the ad-
dition of DOX resulted in a very rapid (~1 min) and complete disappearance of residual FeIV O, coupled with regeneration of the spectrum of MbIII and extensive degradation of the anthracycline; however, the yield of 3-methoxyphthalic acid was very low, according to a stoichiometry of 3-methoxyphthalic acid formation versus DOX degradation of less than 0.02 (Fig. 9, A and B). Finally, the apparent kcat/km value of H2O2/MbIII for formation of 3-methoxyphthalic acid was 2 orders of magnitude lower than that determined for DOX degradation (1.5 ± 0.2 × 10^3 versus 1.8 ± 0.2 × 10^5 M^-1 s^-1; n = 3), which was consistent with the fact that both compound I and compound II were involved in oxidizing DOX but compound II uncoupled anthracycline degradation from formation of 3-methoxyphthalic acid.

Studies of Other Anthracyclines—Doxorubicin was compared with other approved or investigational anthracyclines like DNR and IDA or naphthazarin. The only difference between DOX and DNR is that the side chain of DOX terminates with a primary alcohol, whereas that of DNR terminates with a methyl (cf. Fig. 1). This difference did not alter the usual pattern of MbIV-anthracycline reactions, as evidenced by DNR degradation and formation of essentially the same amounts of 3-methoxyphthalic acid observed with DOX at low or high MbIV-anthracycline ratios (Table I). Idarubicin (4-demethoxy-DNR) was similarly susceptible to degradation but released phthalic acid in place of 3-methoxyphthalic acid due to the absence of a methoxy residue in ring D. Naphthazarin, an investigational model compound reproducing only the quinone-hydroquinone rings of DOX, underwent degradation but obviously released no phthalate due to the absence of ring D (see also Fig. 1 and Table I); attempts to identify the products of naphthazarin degradation by ESI-MS were unsuccessful. Comparisons between DOX and DNR or IDA therefore showed that 3-methoxyphthalic acid and simple phthalic acid were common products of MbIV-dependent degradation of anthracyclines with methoxy or demethoxy ring D. On the other hand, results obtained with naphthazarin showed that anthracycline degradation occurred in the central quinone-hydroquinone portion of these molecules.

3-Methoxyphthalic Acid Formation in Vivo—Mice were treated with DOX intravenously, and their organs were analyzed after 4 or 24 h (Table II). At 4 h both the heart and liver and kidneys contained sizeable amounts of DOX or
metabolites (like the C-13 secondary alcohol metabolite and hydroxy- or deoxyaglycones), which retained the same fluorescence of unchanged DOX due to the presence of an intact tetracyclic ring in their molecule. The values of DOX and/or fluorescent metabolites decreased, to a variable extent, at 24 h due to a tissue clearance of both parent drug and its metabolites. Different results were obtained in regard to 3-methoxyphthalic acid. As also shown in Table II, 3-methoxyphthalic acid was found in the heart at 4 h and increased further at 24 h, reaching levels that were higher than those of DOX and fluorescent metabolites. The liver contained 3-methoxyphthalic acid only at 24 h, but its levels were only $\sim 30\%$ of those found in the heart at the same post-treatment time. Kidneys did not contain 3-methoxyphthalic acid at either 4 or 24 h. These results showed that (i) 3-methoxyphthalic acid was formed in vivo after DOX administration; (ii) the heart was more active than other organs in degrading DOX to 3-methoxyphthalic acid; and (iii) there were conditions when the cardiac levels of 3-methoxyphthalic acid exceeded those of unchanged DOX or its metabolites.

3-Methoxyphthalic Acid Formation in Human Myocardium—

Human myocardial strips were exposed to 1 or 10 $\mu$M DOX, concentrations found in the plasma of patients after slow or bolus infusions of the drug (34). At the end of the experiments the strips contained very low amounts of residual unchanged DOX but contained sizeable amounts of fluorescent metabolites and 3-methoxyphthalic acid. At 1 $\mu$M DOX intramyocardial levels of 3-methoxyphthalic acid were higher than those of fluorescent metabolites; at 10 $\mu$M DOX the levels of fluorescent metabolites increased and exceeded those of 3-methoxyphthalic acid, but the latter remained several times higher than unchanged DOX (Table III). By having demonstrated that 3-methoxyphthalic was an important component of the metabolic fate of DOX in human myocardium, we performed experiments to confirm that it was formed by H$_2$O$_2$-activated myoglobin. Three lines of evidence showed that this was the case. First, incubation of a whole homogenate of human myocardium with DOX and H$_2$O$_2$ resulted in anthracycline degradation and 3-methoxyphthalic acid formation, but these processes became more evident when H$_2$O$_2$ was added to cytosol, i.e. the subcellular fraction containing myoglobin. Second, treatment of cytosol with 65% ammonium sulfate removed $\sim 80\%$ of myoglobin by salting out and gave an Mb$^-$ cytosol that was essentially inactive at degrading DOX or generating 3-methoxyphthalic acid upon incubation with H$_2$O$_2$. Finally, Mb$^-$ cytosol regained activity in DOX degradation and 3-methoxyphthalic acid formation after reconstitution with its salted out myoglobin (see Fig. 10, A–D, and legend).

Studies with Isolated Cardiomyocytes—We have shown previously (25) that the embryonic rat heart-derived cell line H9c2...
offers a convenient and reproducible model for evaluating cardiotoxicity induced by DOX or related compounds in vitro. Therefore, H9c2 cardiomyocytes were exposed to 0.01–10 μM 3-methoxyphthalic acid to see whether it caused increased or decreased toxicity compared with equimolar DOX. Inasmuch as cardiac tissue has a density very similar to that of water (1 g/ml) (14), the range of concentrations of 3-methoxyphthalic acid in the incubation medium was broad enough to include and exceed those detected in mouse heart (1–3 nmol/g, cf. Table II) or in human myocardial strips (0.37–0.44 nmol/g, cf. Table III). As shown in Fig. 11, 3-methoxyphthalic acid never reduced cardiomyocyte viability in the MTT assay; in contrast, a significant loss of viability occurred if cardiomyocytes were exposed to ≥1 μM DOX. Thus, 3-methoxyphthalic acid was essentially non-toxic compared with DOX.

**DISCUSSION**

We have shown that MbIV, a pseudoperoxidase, is more effective than authentic peroxidases at promoting the oxidative degradation of DOX, exhibiting a $k_{cat}/k_m$ order of magnitude higher than that of HRP or LPO. Time course analyses of FeIII=O formation and decay versus DOX degradation, and experiments conducted with hemin/H2O2 as a source of porphyrin radical/FeIII=O, indicate that DOX is oxidized by the porphyrin radical of a compound I-like species and then by the FeIII=O moiety of a compound II-like species (cf. Figs. 2–4).
The ease with which the porphyrin radical of compound I dissipates in the globin clearly calls attention also on a possible involvement of amino acid radicals centered at Tyr<sub>103</sub> or Trp<sub>14</sub>, for example (4–7). In this regard we have data showing that tyrosophan radicals, generated by oxidizing tryptophan with H<sub>2</sub>O<sub>2</sub>, lack reactivity toward DOX; tyrosine radicals, generated through the same procedure, did degrade DOX but acetylation of myoglobin tyrosine residues did not decrease the k<sub><sup>cat</sup>/k<sub>m</sub></k> of Mb<sup>III</sup>/H<sub>2</sub>O<sub>2</sub> for degradation of DOX (not shown). These reasonings do not completely rule out a possible role for globin-centered reactive species in oxidizing DOX. Free radicals are known to transfer from one site of the globin to another, and chemical modifications or site-directed mutagenesis of the primary sites of free radical formation may not prevent the formation of other radicals on nearby residues (32). Moreover, Mb<sup>III</sup> may display an additional radical signal that is not centered at Tyr or Trp and has been tentatively assigned to oxidation of an unidentified aromatic amino acid in close proximity to the heme pocket (32). The possible role of globin radicals

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*Fig. 9.* DOX degradation and 3-methoxyphthalic acid formation. Results were obtained by adding DOX 1 h after mixing Mb<sup>III</sup> with H<sub>2</sub>O<sub>2</sub>. A, Mb<sup>III</sup> (50 μM) was reacted with H<sub>2</sub>O<sub>2</sub> (100 μM) to form Mb<sup>IV</sup>/Fe<sup>IV</sup>=O (peaks at 546 and 586 nm). Spectra were taken at 4 and 10 min and then every 10 min until 60 min (solid lines). At 60 min DOX (50 μM) was added, and spectra were taken every min for 10 consecutive min (dotted lines). The boldface trace is the spectrum of unreacted Mb<sup>III</sup>. B, experimental conditions were as in A, except that Mb<sup>III</sup> and H<sub>2</sub>O<sub>2</sub> were 100 and 200 μM, respectively. After 60 min, DOX (100 μM) was added, and aliquots were taken every min and assayed for DOX degradation (initial DOX – residual DOX) or 3-methoxyphthalic acid formation. Values were means ± S.E. of three determinations.

| Drug | Mb<sup>IV</sup>:drug | Degradation | 3-Methoxyphthalic acid | Phthalic acid |
|------|----------------------|-------------|------------------------|--------------|
| DOX  | 0.5:1 | 53 ± 7 | 3.8 ± 0.8 | — |
|      | 2:1  | 98 ± 2 | 16 ± 1  | — |
| DNR  | 0.5:1 | 40 ± 4 | 3.6 ± 0.7 | — |
|      | 2:1  | 98 ± 1 | 13 ± 1.5  | — |
| IDA  | 0.5:1 | 62 ± 9 | — | 14 ± 1.5 |
|      | 2:1  | 99 ± 1 | — | 25 ± 2 |
| Naphthazarin | 0.5:1 | 83 ± 20 | — | — |
|      | 2:1  | 97 ± 2 | — | — |

*Table I* Levels of DOX, fluorescent metabolites, and 3-methoxyphthalic acid in organs of mice treated with DOX

Other conditions are as described under “Experimental Procedures.” –, not detectable.

| DOX | Fluorescent metabolites<sup>a</sup> | 3-Methoxyphthalic acid | nmol/g |
|-----|---------------------------------|------------------------|-------|
| Heart | 4 h | 8.1 ± 0.5 | 0.3 ± 0.05 | 1.7 ± 0.1 |
|      | 24 h | 1.9 ± 0.2<sup>b</sup> | 0.2 ± 0.1 | 3.0 ± 0.3* |
| Liver | 4 h | 6.1 ± 0.8 | 0.4 ± 0.1 | — |
|      | 24 h | 1.3 ± 0.2<sup>e</sup> | 0.1 ± 0.02<sup>g</sup> | 1.0 ± 0.2 |
| Kidney | 4 h | 12.6 ± 1 | 1.9 ± 0.3 | — |
|      | 24 h | 9.1 ± 0.8<sup>f</sup> | 0.2 ± 0.1<sup>f</sup> | — |

<sup>a</sup> Sum of C-13 secondary alcohol metabolite and hydroxy- or deoxyaglycones. Values were means ± S.E. (n = 9).

<sup>b</sup> p < 0.01 or < 0.05 versus 4 h.

<sup>c</sup> p < 0.025 versus 4 h and p < 0.05 versus DOX 24 h.

<sup>d</sup> p < 0.01 versus 4 h.

<sup>e</sup> p < 0.05 versus 4 h.

<sup>f</sup> p < 0.01 versus 4 h.

| DOX | Unchanged DOX | Fluorescent metabolites<sup>a</sup> | 3-Methoxyphthalic acid |
|-----|----------------|---------------------------------|------------------------|
|      | μM             | nmol/g                          |                        |
| 1    | 0.02 ± 0.01    | 0.27 ± 0.04                     | 0.44 ± 0.06            |
| 10   | 0.04 ± 0.01    | 1.2 ± 0.2<sup>g</sup>           | 0.37 ± 0.05            |

<sup>a</sup> Sum of C-13 secondary alcohol metabolite and hydroxy- or deoxyaglycones. Other conditions were as described under “Experimental Procedures.”

<sup>g</sup> Values were means of ± S.E. (n = 4), p < 0.01 versus 1 μM.

as additional determinants of DOX oxidation therefore remains a matter of consideration.

Chromatographic, UV, and mass spectrometry analyses provide novel evidence that a biologic oxidant like Mb<sup>IV</sup> degrades DOX to 3-methoxyphthalic acid, product of oxidative modifications of the methoxy-substituted ring D (cf. Figs. 1 and 5–7). Biochemical evidence for the formation of oxidized ring D products was also offered by the fact that methoxy or demethoxy analogs, like DNR or IDA, similarly released 3-methoxyphthalic acid or simple phthalic acid after exposure to Mb<sup>IV</sup> (cf. Table I). Previously, the formation of 3-methoxyphthalic acid or other products of ring D oxidation (like e.g. 3-methoxyxalyl acid) was only observed under artificial conditions such as permanganate oxidation of investigational anthraquinones (35) or riboflavin-mediated photooxidation of DOX (36).
The complete sequence of events leading to the formation of 3-methoxyphthalic acid cannot be envisaged at this time as MbIV-DOX interactions released other products that were detected by HPLC but could not be characterized by ESI-(2.9 nmol of MbIII/mg of protein), or Mb−cytosol (0.6 nmol of MbIII/mg of protein). Reactions were started by adding increasing amounts of H2O2, as indicated. After 10 min incubations were assayed for DOX (A) or 3-methoxyphthalic acid (B). C and D, DOX degradation (initial DOX-residual DOX) and 3-methoxyphthalic acid formation were measured in incubations containing 500 μM H2O2 and 3 mg of protein/ml of Mb−cytosol, Mb−cytosol, or Mb−cytosol reconstituted with its salted out myoglobin (2.3 nmol of MbIII/mg of protein, dialyzed just prior to experiments to remove ammonium sulfate). Values were means ± S.E. of triplicate experiments.

Fig. 10. Doxorubicin has long been known to undergo one-electron reduction of its quinone, two-electron reduction of its side chain C-13 carbonyl group, or reductive cleavage of the glycosidic bond linking its tetracyclic ring with an amino sugar (cf. Fig. 1). While forming semiquinone free radicals and secondary alcohol or deoxyaglycone derivatives that have been implicated to explain, at least in part, the cardiotoxic properties of DOX (25, 38, 39), these reductive processes do not induce any important or irreversible loss of the optical and fluorescent properties of unchanged DOX. However, the administration of DOX to humans and laboratory animals was shown to generate also non-fluorescent compounds that were tentatively attributed to oxidative degradation rather than reductive biotransformation of the drug (40, 41). Our results indicate that 3-methoxyphthalic acid is formed not only in reconstituted chemical systems but also in the heart of mice treated with DOX or in human myocardial biopsies exposed to concentrations of DOX similar to those found in the plasma of patients (cf. Tables II and III). In mice, the heart generates considerably more 3-methoxyphthalic acid than organs like liver or kidneys, as one would expect if MbIV were more effective than other peroxidases at degrading DOX. Comparisons between human heart homogenate and Mb−or Mb+ cytosol also confirmed that cytosol served a preferred site of DOX degradation and 3-methoxyphthalic acid formation, and that both processes could be abolished or reactivated by removing or reintroducing myoglobin (cf. Fig. 10). Our results therefore identify MbIV and 3-methoxyphthalic acid among the long sought catalyst(s) and product(s) of anthracycline oxidation in biologic systems.

In re-attempting characterization of other products of DOX degradation products by ESI(−)MS, we occasionally detected ions at m/z = 203 or 237, 242, and 265. However, further characterizations and unambiguous assignment of these ions were not possible.

Fig. 11. Toxicity of DOX or 3-methoxyphthalic acid to H9c2 cardiomyocytes. Toxicity was evaluated as the percentage of viable cells (MTT assay) after an overnight exposure to DOX or 3-methoxyphthalic acid. Values were means ± S.E. of four separate determinations.
Experiments with DOX-treated mice or human myocardial strips exposed to DOX show that there may be conditions when the cardiac levels of 3-methoxyphthalic acid exceed those of unchanged DOX or fluorescent metabolites (cf. the heart of mice 24 h after DOX administration or myocardial strips exposed to 1 μg DOX) (cf. Tables II and III). These observations suggest that 3-methoxyphthalic acid might be an important determinant of the mode of action of anthracyclines and offered a rationale to see how it compared with DOX in inducing toxicity to cardiomyocytes. We demonstrate that concentrations of 3-methoxyphthalic acid equal to or several times higher than those found in whole cardiac tissues lack toxicity to isolated cardiomyocytes; under comparable conditions, however, low concentrations of DOX are highly toxic to cardiomyocytes (cf. Fig. 11). Although the biologic action(s) of other currently unknown degradation products cannot be disregarded, these results raise the possibility that the pseudoperoxidase activity of MbIV may serve an important mechanism to diminish cellular levels of anthracyclines and to divert them from formation of toxic reduced metabolites toward formation of non-toxic oxidized products.

In summary, we have shown the following. (i) MbIV is a very good catalyst of anthracycline degradation. (ii) 3-Methoxyphthalic acid and simple phthalic acid are common products of the oxidative degradation of methoxy-substituted or demethoxy analogs like DOX and DNR or IDA, respectively. (iii) The compounds I and II of MbIV are sequentially involved in oxidizing DOX, although with different stoichiometries of 3-methoxyphthalic acid formation versus anthracycline degradation. (iv) 3-Methoxyphthalic acid is an abundant product of DOX degradation in the heart of laboratory animals or in human myocardium. (v) 3-Methoxyphthalic acid does not induce toxicity to cardiomyocytes. These results unravel novel functions for MbIV and pose mechanism-based foundations to see whether reactions of MbIV with anthracyclines may be exploited to improve cardiac tolerability of these otherwise useful agents.

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