Doxorubicin Degradation in Cardiomyocytes

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Received March 15, 2007; accepted April 25, 2007

ABSTRACT

Antitumor therapy with the anthracycline doxorubicin is limited by a severe cardiotoxicity, which seems to correlate with the cardiac levels of doxorubicin and its metabolism to reactive oxygen species. Previous biochemical studies showed that hydrogen peroxide-activated myoglobin caused an oxidative degradation of doxorubicin; however, a pharmacological evaluation of this metabolic pathway was precluded by the lack of safe and specific inhibitors of doxorubicin degradation. We found that tert-butoxycarbonyl-alanine inhibited doxorubicin degradation induced in vitro by hydrogen peroxide-activated oxyferrous myoglobin. When assessed in H9c2 cardiomyocytes, tert-butoxycarbonyl-alanine neither stimulated the cellular uptake of doxorubicin nor diminished its efflux; moreover, tert-butoxycarbonyl-alanine did not cause toxicity per se nor did it augment the toxicity induced by hydrogen peroxide or chemical agents that increased the cellular levels of reactive oxygen species. Nonetheless, tert-butoxycarbonyl-alanine increased the cellular levels of doxorubicin, its conversion to reactive oxygen species, and its concentration-related toxicity. tert-Butoxycarbonyl-alanine also aggravated the toxicity of a degradation-prone anthracycline analog, daunorubicin, but it caused a minor effect on the toxicity of a degradation-resistant analog, aclacinomycin. These results suggested that tert-butoxycarbonyl-alanine increased the cellular levels and toxicity of doxorubicin by inhibiting its oxidative degradation to harmless products. Accordingly, doxorubicin samples that had been oxidized in vitro with hydrogen peroxide and oxyferrous myoglobin lacked toxicity to cardiomyocytes. The effects of tert-butoxycarbonyl-alanine were most evident at 0.1 to 1 μM doxorubicin, which may be relevant to clinical conditions. These studies identify an oxidative degradation of doxorubicin as a possible salvage mechanism for diminishing its levels and toxicity in cardiomyocytes.

The antitumor anthracycline doxorubicin causes a severe cardiotoxicity. Several lines of evidence indicate that cardiotoxicity correlates with the cardiac levels of doxorubicin; therefore, pharmacokinetic factors that increased or decreased the cardiac levels of doxorubicin were shown to aggravate or mitigate cardiotoxicity, respectively (Minotti et al., 2004). Doxorubicin is composed of a tetracyclic ring system with quinone and hydroquinone moieties (Fig. 1A). Numerous NAD(P)H oxidoreductases catalyze an one-electron reduction of the quinone moiety, thereby forming a semiquinone that recycles to its parent compound by reducing oxygen to reactive oxygen species (ROS) such as superoxide anion (O2-), hydrogen peroxide (H2O2), and the redox cycling of the quinone moiety does not alter the anthracycline chromophore, but it probably causes cardiotoxicity by forming ROS in excess of the detoxifying mechanisms of cardiomyocytes (Gewirtz, 1999; Minotti et al., 2004).

Previously, we showed that H2O2-activated myoglobin oxidized the hydroquinone in juxtaposition to the quinone, causing the formation of an unstable diquione and the consequent opening and degradation of the tetracyclic ring of doxorubicin (Menna et al., 2002; Cartoni et al., 2004). The importance of this alternative metabolism of doxorubicin has remained elusive, primarily because of technical difficulties in characterizing the products of doxorubicin degradation. Further complexity is introduced by the mode of action of H2O2-activated myoglobin. The physiological oxyferrous myoglobin (MbIV0) is converted by H2O2 into a ferrylmyoglobin characterized by a long-lived iron-oxo moiety (MbIV-O2); this ferrylmyoglobin is identical to the compound II of peroxidases. Metmyoglobin (MbIII0) is converted by H2O2 into a ferrylmyoglobin characterized also by a short-lived porphyrin radical that dissipates in the globin and causes the formation of amino acid radicals; this latter ferrylmyoglobin is identical to the compound I of peroxidases (Egawa et al., 2004).

This work was supported by Associazione Italiana Ricerca sul Cancro and Ministero dell’Università e Ricerca Scientifica e Tecnologica (Cofin 2004 and Center of Excellence on Aging, University of Chieti). P.M. and E.S. contributed equally to this work.

ABBREVIATIONS: ROS, reactive oxygen species; MbIII0, oxyferrous myoglobin; MbIV-O2, ferrylmyoglobin iron-oxo moiety; MbIII, metmyoglobin; t-boc-Ala, tert-butoxycarbonyl-alanine; DCFH, dichlorofluorescin; DCFH-DA, dichlorofluorescin-diacetate; DCF, dichlorofluorescein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HRP, horseradish peroxidase; LPO, lactoperoxidase; HPLC, high-performance liquid chromatography; t-boc + Ala, hydrolyzed t-boc-Ala.

JPET 322:408–419, 2007
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The hydroquinone moiety of doxorubicin proved able to oxidize with both compound I and compound II of ferrymyoglobin, but only its oxidation with compound I released measurable amounts of a stable degradation product that was identified as 3-methoxypthalic acid (Fig. 1B) (Cartoni et al., 2004). Thus, 3-methoxypthalic acid would be formed by H$_2$O$_2$/Mb$^{III}$ but not by H$_2$O$_2$/Mb$^{II}O$_2$.

Myoglobin is highly abundant in the heart, and doxorubi-
cin seems to accelerate the autoxidation of MbIII'O2 to MbII' (Trost and Wallace, 1994). We therefore hypothesized that cardiomyocytes might use MbIII'O2 and/or MbII' to degrade doxorubicin and to diminish its cellular levels and toxicity. To probe this hypothesis, we needed to modulate doxorubicin degradation without exposing cardiomyocytes to oxidants or antioxidants or heme poisons that altered the redox balance of these cells and their sensitivity to doxorubicin-derived ROS. We considered using cardiomyocytes that lacked or overexpressed myoglobin, but recent studies show that both a deficiency and a redundancy of myoglobin render cardiomyocytes hypersensitive to ROS (Flögel et al., 2004; Witting et al., 2006).

During the search for safe and specific modulators of doxorubicin degradation, we examined the possible value of an arginine derivative in which the amino group is protected by a tert-butoxycarbonyl group, whereas the guanidine moiety forms adducts with oxidized biomolecules (Oe et al., 2003). We hypothesized that tert-butoxycarbonyl-arginine reacted also with the early products of doxorubicin oxidation, thereby halting the process of ring opening and leaving more doxorubicin undegraded or only partially degraded. Contrary to our expectations, preliminary in vitro studies with H2O2/H11005 showed that tert-butoxycarbonyl-arginine neither inhibited doxorubicin degradation nor formed adducts with product(s) of doxorubicin oxidation. Nonetheless, tert-butoxycarbonyl-arginine diminished 3-methoxyphthalic acid formation, as if it altered the access of doxorubicin to ferrylmyoglobin and made it oxidize with MbIII'O (compound II) but not with porphyrin/globin radicals (compound I). A similar effect was not observed with authentic peroxidases, demonstrating that the mode of action of tert-butoxycarbonyl-arginine was specific to myoglobin.

The effects of tert-butoxycarbonyl-arginine might have been refined and eventually exploited to modulate an interaction of doxorubicin with ferrylmyoglobin in cardiomyocytes; unfortunately, however, we noticed that even minor increments of tert-butoxycarbonyl-arginine caused a rapid reduction of MbIV'O. This latter finding suggested that the free guanidine moiety of tert-butoxycarbonyl-arginine might have reacted with many cellular electrophiles, thereby confounding an interpretation of the cause-and-effect relations between doxorubicin degradation and cell damage (P. Menna, E. Salvatorelli, and G. Minotti, unpublished observations).

In the light of this exploratory work, we focused on amino acids that had their amino group protected by a tert-butoxycarbonyl group but that did not expose other redox-active moieties. We hoped that such amino acids perturbed the sterical interactions of doxorubicin with ferrylmyoglobin while not engaging redox reactions with other cellular components. tert-Butoxycarbonyl-alanine (t-boc-Ala) exposes redox-inactive methyl, carboxyl, and amide moieties (Fig. 1C); therefore, t-boc-Ala was used to modulate doxorubicin degradation and to evaluate its role in cardiomyocytes.

**Materials and Methods**

**Chemicals.** Doxorubicin [(SS-cis)-10-[(3-amino-2,3,6-trideoxy-a-L-fuco-hexopyranosanoyloxy)-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione-6,11-dione] and daunorubicin [(SS-cis)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-a-L-fuco-hexopyranosanoyloxy)-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione] (Fig. 1D) were obtained through the courtesy of Nerviano Medical Sciences (Milan, Italy). Dichlorofluorescein-diacetate (DCFH-DA) and dichlorodihydrofluorescein (DCF) were from Invitrogen (Carlsbad, CA), whereas t-boc-Ala and 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) were from Fluka (Milan, Italy); where indicated, t-boc-Ala was used after its hydrolysis in HCl-dioxane (Han et al., 2001). Sigma-Aldrich (Milan, Italy) provided aclarubacin [2-ethyl-1,2,3,4,6,11-hexa-hydro-5,7,9,11-dioxo-6,11-dioxo-4-O-[2-R-trans]-tetrahydro-6-methyl-5-oxo-2H-pyran-2-yl]-O-L-[lyxo-hexopyranosyl]-1-naphthacene carboxylic acid methyl ester] (Fig. 1E), paraguan (1,1,1-dimethyl-4-·tetrapropyridinium) (Fig. 1F), streptonigrin [5-amino-6-(7-amino-5,8-dihydro-6-methoxy-5,8-dioxo-2-guinoyl)-4-(2-hydroxy-3,4-dimethoxyphenyl)-3-methyl-2-pyrindinecarboxylic acid] (Fig. 1G), etoposide [4-demethyl-epipodophyllotoxin 9-[4,6-O-(R)-ethylidene-β-d-glucopyranoside], 4′-(dihydrogen phosphate) (Fig. 1H), horse heart cytochrome c, MbIII, type VI-A horseradish peroxidase (HRP), and bovine milk lactoperoxidase (LPO). Oxyferrous myoglobin was prepared by reducing 10 μM MbIII' with 1 mM ascorbate; unreacted ascorbate was removed by ultrafiltration and spatial immobilization ascorbate oxidase (Roche Diagnostics, Mannheim, Germany). Metmyoglobin and MbIII'O2 were quantitated by assuming ε310 nm = 3.5 mM⁻¹ cm⁻¹ and ε580 nm = 11 mM⁻¹ cm⁻¹ respectively (Prasad et al., 1989; Menna et al., 2002).

**Spectrophotometric Assays for Doxorubicin Degradation or MbIV'O Reduction.** In the assays for doxorubicin degradation, the anthracycline was added after a computer-assisted correction of the background absorbance of authentic peroxidases (HRP and LPO) or pseudo-peroxidases (MbIII,MbIIO2, type VI-A horseradish peroxidase (HRP), and bovine milk lactoperoxidase (LPO). Oxyferrous myoglobin was prepared by reducing 10 μM MbIII' with 1 mM ascorbate; unreacted ascorbate was removed by ultrafiltration and spatial immobilization ascorbate oxidase (Roche Diagnostics, Mannheim, Germany). Metmyoglobin and MbIII'O2 were quantitated by assuming ε310 nm = 3.5 mM⁻¹ cm⁻¹ and ε580 nm = 11 mM⁻¹ cm⁻¹ respectively (Prasad et al., 1989; Menna et al., 2002).

**Cell Cultures and Treatments.** We used the embryonic rat heart-derived cell line H9c2 (CRL 1446; American Type Culture Collection, Manassas, VA), which proved useful in many studies of the mechanisms of anthracycline-induced cardiotoxicity (Kluzu et al., 2004; Chua et al., 2006; L'Ecuyer et al., 2006; Merten et al., 2006; Salvatorelli et al., 2006). Cells were grown at 37°C under 5% CO₂, air or pseudo-peroxidases (MbIII,MbIIO2, cytochrome c, and hemin). Next, the absorbance of doxorubicin was recorded, H2O2 was added, and the decay of the anthracycline chromophore (λmax = 477 nm) was monitored against a blank that contained H2O2 and the (pseudo)peroxidase. Hemin was prepared as 10 mM stock solutions in 100 mM NaOH and diluted appropriately with 50 mM phosphate buffer to obtain 1 mM working solutions. In the assays for MbIII'O reduction, doxorubicin was added to cuvettes zeroed for the absorbance of MbIII' or MbIII'O2; next, H2O2 was added, and the peaks of MbIV'O were identified (at 546 and 586 nm) against a blank that contained only doxorubicin. The concentrations of H2O2, doxorubicin, and authentic or pseudo-peroxidases are indicated in the legends for figures or tables. The incubations were carried out at 37°C in 0.3 M NaCl, carefully adjusted to pH 7.0 just before the experiments; this was done to avoid interference of most common buffers with the redox reactions of anthracyclines with ferrylmyoglobin and other authentic or pseudo-peroxidases (Menna et al., 2002; Cartoni et al., 2004). Although unbuffered, the pH of incubations did not vary throughout the experiment.

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In the light of this exploratory work, we focused on amino acids that had their amino group protected by a tert-butoxycarbonyl group but that did not expose other redox-active moieties. We hoped that such amino acids perturbed the sterical interactions of doxorubicin with ferrylmyoglobin while not engaging redox reactions with other cellular components. tert-Butoxycarbonyl-alanine (t-boc-Ala) exposes redox-inactive methyl, carboxyl, and amide moieties (Fig. 1C); therefore, t-boc-Ala was used to modulate doxorubicin degradation and to evaluate its role in cardiomyocytes.
specified in the legends for figures and tables. In preliminary experiments with serum-supplemented medium, we found that the concentration-related effects of doxorubicin on cell viability were highly variable; hence, the IC50 value of doxorubicin could not be determined accurately. Because anthracyclines bind avidly to serum proteins, we considered that omitting serum facilitated a concentration-dependent diffusion of doxorubicin in cardiomyocytes. Cardiomyocytes were therefore exposed to doxorubicin or other test compounds in serum-free medium for 16 h, which was shorter than the time required for serum withdrawal to reduce the viability of H9c2 and other cardiac cell lines (24–72 h) (Bonavita et al., 2003; Granata et al., 2003; Alcendor et al., 2004). Viability assays confirmed that a 16- to 19-h incubation in serum-free medium did not reduce the viability of H9c2 cells compared with cells maintained in serum-supplemented medium. In selected experiments, cardiomyocytes were incubated with doxorubicin that had been oxidized with an excess of H2O2-activated MnO2. In brief, 20 μM doxorubicin was incubated with 80 μM MnO2 and 160 μM H2O2. After 20 min, the reaction mixtures were added with catalase (2600 U) to decompose any residual H2O2, and they were ultrafiltered through YM 10 Centricon (Millipore Corporation, Bedford, MA). Aliquots of the ultrafiltrates were diluted as appropriate with serum-free medium and delivered to cardiomyocytes at a formal concentration of 0.1 to 10 μM degraded doxorubicin. Where indicated, cardiomyocytes were loaded for 2 h with t-boc-Ala, Ala, or hydrolyzed t-boc-Ala, all in serum-free medium. Next, the medium was changed, and the cardiomyocytes were exposed to doxorubicin or other test compounds. At the end of the treatment, cells from 10 identical dishes were scraped with H2O and added with 10 mM 4-hydroxytempo at 1 mM to prevent further oxidation of DCDF by DCDF during cell disruption and homogenization (Salvatorelli et al., 2006). The homogenate was extracted with a 2-fold volume of CHCl3/CH3OH (1:1), and 100 μl of the upper phase was analyzed by HPLC as described for doxorubicin and 3-methoxyphthalic acid. The fluorescent peak of DCDF (excitation at 488 nm, emission at 525 nm) was identified by cochromatography with a DCDF standard (retention time 18.3 min), and it was quantified against an appropriate standard curve. The lowest detection limit for DCDF was 1 × 10−5 μM, 3 times below the levels of DCDF recovered from untreated cells. Basal or doxorubicin-induced H2O2 formation was quantified by assuming that 1 nmol of H2O2 formed 0.43 nmol of DCF; this stoichiometry was calculated by correcting total DCF fluorescence for H2O2-independent DCFH oxidation or side reactions of DCF with cellular redox agents other than H2O2 (Rota et al., 1999; Salvatorelli et al., 2006).

**Viability Assay.** Cell viability was determined by the MTT reduction assay, an indicator of mitochondrial function. The assay was performed directly on the cell cultures at the end of their exposure to doxorubicin or other test compounds. After removing the medium, the cells were added with 1 ml of 1.2 mM MTT (dissolved in fresh serum-free medium), and they were incubated for 3 h at 37 °C under 5% CO2, air. Next, 1 ml of dimethyl sulfoxide was added to dissolve the water-insoluble formazan salt derived from MTT reduction. After an additional 30 min at 37 °C, aliquots of the mixture were taken and assayed spectrally for formazan (λmax = 560 nm). The mitochondrial dependence of the assay was probed by adding parallel cell cultures with MTT and the respiratory chain inhibitor antimycin A (used at 100 μM) (Berridge and Tan, 1995). This procedure showed that, in control cells, mitochondria contributed to MTT reduction by 65 ± 3% (n = 16). At the IC50 values indicated in this study, doxorubicin and other toxic stimuli diminished the mitochondrion-specific antimycin A-inhibitable MTT reduction, but they caused little or no effect on the extramitochondrial antimycin A-insensitive reduction. Viability was therefore expressed as the percentage of antimycin A-inhibitable MTT reduction that occurred in treated cells compared with control cells.

**Other Assays and Conditions.** The peroxidatic activity of authentic or pseudo-peroxidases was measured by the guaiacol assay (Landino et al., 1997). Proteins were measured by the bicinchoninic acid method. Myoglobin was determined by an electrochemiluminescence immunoassay with an Elecsys 10 Analyzer (Roche Diagnostics), according to the manufacturer’s instructions. Unless otherwise indicated, all the values were means ± S.E. of at least three experiments. Data were analyzed by one-way analysis of variance followed by Bonferroni’s post hoc test. Where indicated, differences were considered statistically significant if p < 0.05.
by Bonferroni’s test for multiple comparisons; where indicated, unpaired Student’s *t* test was also applied. Differences were considered significant when *p* < 0.05. Other details are given under Results and in the legends for figures and tables.

**Results**

**Effects of t-boc-Ala on Doxorubicin Levels and Toxicity in H9c2 Cardiomyocytes.** One to 10 millimolar t-boc-Ala did not reduce the viability of H9c2 cardiomyocytes (Fig. 2A); however, 5 and 10 mM t-boc-Ala aggravated the toxicity of doxorubicin in a concentration-dependent manner. The toxic synergism of 5 mM t-boc-Ala with doxorubicin became evident when the anthracycline was used at 1 μM, whereas the effect of 10 mM t-boc-Ala became evident at 0.5 μM doxorubicin (Fig. 2B). Five millimolar t-boc-Ala diminished the IC_{50} value of doxorubicin from 5.6 ± 1.3 to 0.8 ± 0.1 μM (*p* < 0.001), whereas 10 mM t-boc-Ala diminished it to 0.4 ± 0.2 μM (*p* < 0.05 versus 5 mM t-boc-Ala; unpaired Student’s *t* test). Similar effects were not induced by 10 mM Ala or hydrolyzed t-boc-Ala, referred to as (t-boc + Ala) (Fig. 2C).

We next performed experiments at 0.5 μM doxorubicin. At this concentration level, neither doxorubicin alone nor [doxorubicin + 1 mM t-boc-Ala] could induce a measurable toxicity, but an ~10 or ~50% loss of viability occurred with [doxorubicin + 5 mM t-boc-Ala] or [doxorubicin + 10 mM t-boc-Ala], respectively (see Fig. 2B). Under such defined conditions, 5 mM t-boc-Ala increased the cellular levels of doxorubicin by ~4-fold compared with doxorubicin alone or (doxorubicin + 1 mM t-boc-Ala), whereas 10 mM t-boc-Ala caused an ~20-fold increase; again, 10 mM Ala or (t-boc + Ala) lacked effects on the cellular levels of doxorubicin (Fig. 3). Collectively, these results suggested that 5 to 10 mM t-boc-Ala was able to increase the cellular levels and toxicity of doxorubicin in a concentration-dependent manner.

We considered the possibility that t-boc-Ala acted by increasing the cellular uptake of doxorubicin or by diminishing its efflux. Experiments at 0.5 or 2.5 μM doxorubicin showed that doxorubicin uptake or efflux followed a concentration-dependent pattern, but neither process was altered by 10 mM t-boc-Ala (Fig. 4, A and B).

**Effects of t-boc-Ala on Myoglobin-Dependent Doxorubicin Degradation.** We performed in vitro experiments to characterize whether t-boc-Ala acted by inhibiting doxorubicin degradation. In the first set of experiments, 10 μM doxorubicin was incubated with equimolar Mb^O_2 and 20 μM H_2O_2. Neither H_2O_2 nor Mb^O_2 was able to degrade doxorubicin (data not shown); however, a 20-min incubation of doxorubicin with both H_2O_2 and Mb^O_2 caused the degradation of ~50% of the anthracycline chromophore measured at 477 nm. t-boc-Ala inhibited the H_2O_2/Mb^O_2-dependent degradation of doxorubicin in a concentration-dependent manner, with a near-to-complete inhibition occurring at 50 μM t-boc-Ala (Fig. 5A). HPLC analyses showed that doxorubicin extracted from incubations with Mb^O_2/H_2O_2/t-boc-Ala coeluted with an authentic anthracycline standard or with the limited amount of doxorubicin that remained undegraded after 20-min incubations with Mb^O_2/H_2O_2 in the absence of t-boc-Ala; moreover, the spectra associated with the three HPLC peaks showed that t-boc-Ala did not change the optical properties of the anthracycline chromophore (Fig. 5B).

The H_2O_2/Mb^O_2 system gave rise to an Mb^{IV=O} spectrum with peaks at 546 and 586 nm (Menna et al., 2002; Cartoni et al., 2004). In the absence of doxorubicin, the spectrum of Mb^{IV=O} reached its maximum in ~3 min and remained stable for at least ~20 more minutes (data not shown). In the presence of doxorubicin, the spectrum exhibited a time-dependent decay of the peaks at 546 and 586 nm, coupled with...
the appearance of a peak at 500 nm that indicated the regeneration of MbIII (Menna et al., 2002; Cartoni et al., 2004) (Fig. 5C). This latter finding showed that doxorubicin oxidation/degradation was coupled with the reduction of MbIV→O to MbIII. It is noteworthy that the doxorubicin-dependent reduction of MbIV→O to MbIII was diminished and eventually abolished by t-boc-Ala in a concentration-dependent manner (Fig. 5, D and E). Ala and (t-boc + Ala) lacked effects on the redox reactions between doxorubicin and MbIV→O (data not shown). These results showed that t-boc-Ala did not reduce MbIV→O in competition with doxorubicin nor formed adducts with doxorubicin oxidation product(s); instead, t-boc-Ala interfered with doxorubicin-MbIV→O interactions, thereby blocking both the oxidation/degradation of doxorubicin and the reduction of MbIV→O to MbIII.

The concentrations of t-boc-Ala that inhibited doxorubicin oxidation/degradation in cell-free systems were many times lower than those that increased doxorubicin levels and toxicity in cardiomyocytes (10–50 μM versus 5–10 mM). To resolve such a discrepancy, we measured how t-boc-Ala partitioned from the medium into cardiomyocytes and vice versa. We adopted the same experimental conditions as those described for the uptake/efflux of doxorubicin; t-boc-Ala was used at 5 mM, the highest concentration level at which its UV absorbance (λmax = 203 nm) could be reliably identified and quantified by HPLC. These experiments showed that a 2-h loading with 5 mM t-boc-Ala resulted in a cellular uptake of 618 nmol of t-boc-Ala/mg of cellular proteins, a value corresponding to as little as ~2% of the total t-boc-Ala available to cellular proteins. We also found that cardiomyocytes released t-boc-Ala back into the medium at the rapid initial rate of 410 nmol/mg protein/h. This latter finding demonstrated that, during the course of the exposure to doxorubicin, the cellular levels of t-boc-Ala decreased to values comparable with those that inhibited doxorubicin oxidation/degradation in the cell-free systems.

Under the experimental conditions of this study, neither t-boc-Ala nor doxorubicin changed the spectrum of MbIII; moreover, experiments at 50 μM t-boc-Ala showed that doxorubicin degradation could be gradually reactivated by increasing doxorubicin concentration from 10 μM up to 100 μM (data not shown). This suggested that doxorubicin and t-boc-Ala reversibly competed for sites at a distance from the heme pocket of myoglobin.

**Comparisons of MbIII with Other (Pseudo)Peroxidases.** Previous studies showed that myoglobin was the main catalyst of the oxidation of typical peroxidatic substrates in cardiomyocytes (Witting et al., 2006); nonetheless, we performed in vitro experiments to characterize whether t-boc-Ala increased the cellular levels and toxicity of doxorubicin also by inhibiting doxorubicin oxidation with (pseudo)peroxidases other than MbIII. Horseradish peroxidase and LPO were used as prototypic models of authentic peroxidases; MbIII, cytochrome c, and hemin were used as pseudo-peroxidases of pathophysiological relevance, with the H2O2/hemin system serving as a surrogate for compound I (Cartoni et al., 2004).

As shown in Table 1, the peroxidatic activity of authentic peroxidases, measured by guaiacol oxidation, was 3 to 4 orders of magnitude higher than that of MbIII and other pseudo-peroxidases; however, the pseudo-peroxidases were far superior to authentic peroxidases at inducing doxorubicin degradation normalized to peroxidatic activity. Further inspections showed that LPO exhibited ~16% of the peroxidatic activity of HRP, but it degraded ~2 times more doxorubicin/U of peroxidatic activity. Oxyferrous myoglobin oxidized less guaiacol and degraded less doxorubicin than did MbIII, but a similar relation between peroxidatic activity and doxorubicin degradation was not observed with the other pseudo-peroxidases. Cytochrome c or hemin exhibited less than one third of the peroxidatic activity of MbIII; however, cytochrome c degraded as much doxorubicin as did MbIII, whereas hemin degraded more doxorubicin than any other peroxidase or pseudo-peroxidase examined in this study. These observations supported the notion that the anthracycline-degrading activity of a (pseudo)peroxidase did not correlate with its ability to oxidize a canonical peroxidatic substrate such as guaiacol (Cartoni et al., 2004).

As mentioned, a compound I-dependent degradation of doxorubicin is accomplished by the formation of 3-methoxypthalic acid, an oxidatively modified remnant of the D ring of the anthracycline (see Fig. 2B). Pseudo-peroxidases such as MbIII, cytochrome c, and hemin formed much more 3-methoxypthalic acid/unit of peroxidatic activity compared with HRP or LPO; this was in agreement with the measurements of doxorubicin degradation. Oxyferrous myoglobin, which only forms a compound II, did not release measurable 3-methoxypthalic acid (Table 1).

We next determined the effects of t-boc-Ala on doxorubicin degradation induced by authentic or pseudo-peroxidases. To permit direct comparisons, 1) all the (pseudo)peroxidases were used in equiperoxidatic amounts (3.6 mU/ml), 2) t-boc-Ala was adjusted to achieve increasing ratios to the peroxi-
Peroxidatic activity vs. doxorubicin degradation: comparisons between authentic peroxidases and myoglobin or other pseudo-peroxidases

**TABLE 1**

Peroxidatic activity vs. doxorubicin degradation: comparisons between authentic peroxidases and myoglobin or other pseudo-peroxidases

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Pseudo-Peroxidase</th>
<th>Peroxidatic Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Doxorubicin Degradation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>3-Methoxypythalic Acid&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>HRP</td>
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<td>LPO</td>
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<tr>
<td>Mb&lt;sup&gt;III&lt;/sup&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td>20.8 ± 3.9</td>
<td>0.05 ± 0.002</td>
<td>0.003 ± 0.0003</td>
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<tr>
<td>Mb&lt;sup&gt;III&lt;/sup&gt;I&lt;sub&gt;IO2&lt;/sub&gt;</td>
<td></td>
<td>3.4 ± 0.4</td>
<td>0.1 ± 0.02</td>
<td>0.005 ± 0.0005</td>
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<td>Cytochrome c</td>
<td></td>
<td>1.1 ± 0.1 (&lt;x&gt;10&lt;sup&gt;-3&lt;/sup&gt;&lt;/x&gt;)</td>
<td>26 ± 3</td>
<td>2.4 ± 0.3</td>
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<tr>
<td>Hemin</td>
<td></td>
<td>0.9 ± 0.1 (&lt;x&gt;10&lt;sup&gt;-3&lt;/sup&gt;&lt;/x&gt;)</td>
<td>36 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 ± 0.6</td>
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N.D., not detectable.

<sup>a</sup> <i>p</i> < 0.05 for HRP vs. Mb<sup>III</sup>O<sub>2</sub>.

<sup>b</sup> <i>p</i> < 0.05 for cytochrome c vs. Mb<sup>III</sup>O<sub>2</sub> (unpaired Student's t test) or <i>p</i> > 0.05 for cytochrome c vs. Mb<sup>III</sup>I.

<sup>c</sup> <i>p</i> < 0.001 for Mb<sup>III</sup>I vs. Mb<sup>III</sup>O<sub>2</sub>.

* The incubations contained 500 μM guaiacol in 0.3 M NaCl, pH 7.0, 37 °C. HRP and LPO were used over a 0.05 to 0.5 nM range, whereas Mb<sup>III</sup>O<sub>2</sub>, Mb<sup>III</sup>I, and cytochrome c were used over a 0.1 to 10 μM range; hemin was used at 0.25 to 1 μM. The reactions were started by adding 400 μM H<sub>2</sub>O<sub>2</sub> and the peroxidatic activities were calculated by normalizing the initial rates of guaiacol oxidation to 1 nmol of authentic or pseudo-peroxidase. One unit (U) of activity was defined as the amount of (pseudo)peroxidase that oxidized 1 μmol of guaiacol/ml/min.

<sup>d</sup> In the experiments with authentic peroxidases, the incubations contained 1 μM HRP or LPO, 25 μM doxorubicin, and 400 μM H<sub>2</sub>O<sub>2</sub>. In the experiments with pseudo-peroxidases, Mb<sup>III</sup>O<sub>2</sub> and Mb<sup>III</sup>I were at 10 μM and were reacted with 10 μM doxorubicin (20 μM H<sub>2</sub>O<sub>2</sub>); cytochrome c was used at 2.5 μM and was reacted with 25 μM doxorubicin/400 μM H<sub>2</sub>O<sub>2</sub>; and hemin was used at 4 μM and was reacted with 4 μM doxorubicin/80 μM H<sub>2</sub>O<sub>2</sub>. Doxorubicin degradation was monitored at 477 nm, and the initial rates were normalized to the peroxidatic activity used in the system.

<sup>e</sup> In the experiments with authentic peroxidases the incubations contained 5 μM HRP or LPO, 50 μM doxorubicin, and 400 μM H<sub>2</sub>O<sub>2</sub>. In the experiments with pseudo-peroxidases, Mb<sup>III</sup>O<sub>2</sub> and Mb<sup>III</sup>I were at 10 μM and were reacted with 25 μM doxorubicin/200 μM H<sub>2</sub>O<sub>2</sub>; hemin was at 100 μM and was reacted with 50 μM doxorubicin/2 mM H<sub>2</sub>O<sub>2</sub>. Mb<sup>III</sup>O<sub>2</sub> and Mb<sup>III</sup>I were used as doxorubicin degradation. 3-Methoxypythalic acid was measured by HPLC and normalized to the peroxidatic activity used in the system.

**Fig. 5.** Effects of t-boc-Ala on the redox coupling of doxorubicin with Mb<sup>III</sup>O<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>. A, 10 μM doxorubicin was added to cuvettes zeroed for the absorbance of 10 μM Mb<sup>III</sup>O<sub>2</sub>, and the anthracycline chromophore was immediately recorded at 477 nm. Next, 20 μM H<sub>2</sub>O<sub>2</sub> was added, and the changes in absorbance at 477 nm were recorded every minute against a blank that contained 10 μM Mb<sup>III</sup>O<sub>2</sub>/20 μM H<sub>2</sub>O<sub>2</sub> but not doxorubicin. Where indicated, the incubations contained also 10 to 50 μM t-boc-Ala. B, HPLC peak of a 10 μM doxorubicin standard (trace a), the coelution and near-to-complete recovery of 10 μM doxorubicin extracted from incubations with 10 μM Mb<sup>III</sup>O<sub>2</sub>/20 μM H<sub>2</sub>O<sub>2</sub>/50 μM t-boc-Ala (trace b), and the coelution and limited recovery of 10 μM doxorubicin extracted from incubations with 10 μM Mb<sup>III</sup>O<sub>2</sub>/20 μM H<sub>2</sub>O<sub>2</sub> but not t-boc-Ala (trace c). Inset, anthraclyne spectrum associated with each peak. C, spectrum of Mb<sup>III</sup>O<sub>2</sub> generated through Mb<sup>III</sup>O<sub>2</sub>/20 μM, characterized by peaks at 546 and 586 nm; doxorubicin induced a decrease of the two peaks of Mb<sup>III</sup>O<sub>2</sub> and the appearance of an Mb<sup>III</sup> peak at 500 nm. D and E, t-boc-Ala concentration-dependently abolished the effects of doxorubicin on Mb<sup>III</sup>O<sub>2</sub>. The boldface spectra were recorded 3 min after adding H<sub>2</sub>O<sub>2</sub>; the other spectra were taken 10, 15, and 20 min after adding H<sub>2</sub>O<sub>2</sub>.

datric activity (nanomoles per milliunit), and 3) doxorubicin degradation that occurred in the presence of t-boc-Ala was expressed as the percentage of doxorubicin degradation that occurred in the absence of t-boc-Ala. Under such defined conditions, doxorubicin degradation induced by H<sub>2</sub>O<sub>2</sub>/Mb<sup>III</sup>O<sub>2</sub> began decreasing at (t-boc-Ala):peroxidatic activity ratios as low as 1:1; in contrast, doxorubicin degradation induced by all other (pseudo)peroxidases remained essentially unchanged at (t-boc-Ala):peroxidatic activity ratios as high as 10:1 (Fig. 6). Under comparable experimental conditions, t-boc-Ala never inhibited guaiacol oxidation induced by peroxidases or pseudo-peroxidases (Fig. 6, inset). Thus, t-boc-Ala was highly specific at inhibiting doxorubicin degradation induced by H<sub>2</sub>O<sub>2</sub>/Mb<sup>III</sup>O<sub>2</sub>, and such a specificity could not be attributed to any particular interference of t-boc-Ala with the peroxidatic activity of Mb<sup>III</sup>O<sub>2</sub> compared with other (pseudo)peroxidases.

**Toxicity of Degraded versus Undegraded Doxorubicin.** In some experiments, 20 μM doxorubicin was incubated with a 4-fold excess of H<sub>2</sub>O<sub>2</sub>-activated Mb<sup>III</sup>O<sub>2</sub>. HPLC analy-
Doxorubicin degradation was monitored at 477 nm in the absence or presence of 0.5 to 36 μM t-boc-Ala (giving 0.14:1 to 10:1 ratios of t-boc-Ala to peroxidatic activity (nanomole:milliunit). Inset, experiments in which the (pseudo)peroxidases (same symbols) were used under comparable conditions and assayed for peroxidatic activity at increasing ratios of t-boc-Ala to peroxidatic activity. One milliunit of peroxidatic activity was defined as the amount of (pseudo)peroxidase that oxidized 1 nmol of guaiacol per milliliter per minute.

Fig. 6. t-boc-Ala inhibition of doxorubicin degradation: comparisons between MbHIVO2 and MbIII or other authentic or pseudo-peroxidases. Doxorubicin (10 μM) was incubated with 3.6 mU of peroxidatic activity (corresponding to the following conditions: 6.6 μM H2O2/3.3 μM MbHIVO2, 2 μM H2O2/1 μM MbIII, 400 μM H2O2/0.17 nM HRP, 400 μM H2O2/1 nM LPO, 400 μM H2O2/3.3 μM cytochrome c, and 80 μM H2O2/4 μM hemin). Doxorubicin degradation was monitored at 477 nm in the absence or presence of 0.5 to 36 μM t-boc-Ala [giving 0.14:1 to 10:1 ratios of t-boc-Ala to peroxidatic activity (nanomole:milliunit)]. Inset, experiments in which the (pseudo)peroxidases (same symbols) were used under comparable conditions and assayed for peroxidatic activity at increasing ratios of t-boc-Ala to peroxidatic activity. One milliunit of peroxidatic activity was defined as the amount of (pseudo)peroxidase that oxidized 1 nmol of guaiacol per milliliter per minute.

Fig. 7. Effects of t-boc-Ala on the Cellular Pharmacokinetics and Toxicity of Doxorubicin: Dependence on Doxorubicin Concentration. The experiments described in the preceding sections suggested that t-boc-Ala increased the cellular levels and toxicity of doxorubicin by blocking its oxidation with H2O2/MbIVO2 and the consequent formation of nontoxic degradation products. We determined whether t-boc-Ala caused also an increased exposure of cardiomyocytes to ROS. In the first set of experiments, doxorubicin was used at 0.5 μM, a concentration that caused toxicity only if the cardiomyocytes had been preloaded with 5 or 10 mM t-boc-Ala (see Fig. 2B). Under such defined conditions, neither 0.5 μM doxorubicin nor 10 mM t-boc-Ala increased the cellular levels of H2O2; however, a sizable increase occurred in cardiomyocytes that had been preloaded with 10 mM t-boc-Ala and then exposed to 0.5 μM doxorubicin (Fig. 8). These results suggested that the t-boc-Ala-dependent inhibition of doxorubicin degradation enabled the anthracycline to accumulate and to generate ROS in excess of the detoxifying mechanisms of cardiomyocytes.

We next determined the effects of t-boc-Ala in cardiomyocytes exposed to 5 μM doxorubicin. This latter concentration of doxorubicin was high enough to induce the same toxicity as that induced by a combination of 0.5 μM doxorubicin with 10 mM t-boc-Ala (~50% loss of viability); however, the toxicity induced by 5 μM doxorubicin showed little or no aggravation by 10 mM t-boc-Ala (Fig. 2B). By comparing [0.5 μM doxorubicin ≥ 10 mM t-boc-Ala] with [5 μM doxorubicin ≥ 10 mM t-boc-Ala], we could therefore characterize whether the toxicity of doxorubicin or doxorubicin/t-boc-Ala combinations correlated with the intracellular levels of the anthracycline and the net values of anthracycline-derived H2O2. As shown in Table 2, the intracellular levels of doxorubicin and doxorubicin-derived H2O2 followed exactly the same rank order as that described for cardiomyocyte toxicity, i.e., 0.5 μM doxorubicin < [0.5 μM doxorubicin + 10 mM t-boc-Ala] = 5 μM doxorubicin = [5 μM doxorubicin + 10 mM t-boc-Ala]. These results illustrated a direct relation between the toxicity of doxorubicin, its intracellular levels, and the formation of doxorubicin-derived ROS; they also established that the presence or absence of a toxic synergism between t-boc-Ala and 0.5 or 5 μM doxorubicin correlated with the ability or the failure of t-boc-Ala to increase the cellular levels of doxorubicin and ROS at the two anthracycline concentrations. From a mechanistic viewpoint, the lack of a toxic synergism of t-boc-Ala with 5 μM doxorubicin probably indicated that the latter was high enough to saturate the myoglobin-mediated mechanism of degradation and/or to displace t-boc-Ala from myoglobin, thereby relieving its inhibition of doxorubicin degradation. Control experiments showed that t-boc-Ala or t-boc-Ala/doxorubicin combinations did not change the levels of myoglobin compared with control cells (always 1.2 to 1.6 nmol of myoglobin/mg of cytoplasmic proteins); however, competition experiments between 5 μM doxorubicin and >10

Fig. 8. Effects of t-boc-Ala on H2O2 formation in cardiomyocytes. Subconfluent H9c2 cardiomyocytes (50 × 10^6) were loaded with 10 μM DCFH-DA and then incubated for 16 h under control conditions. Where indicated the cells were exposed to 0.5 μM doxorubicin, or they were loaded with 10 mM t-boc-Ala, or they were sequentially loaded with t-boc-Ala and then exposed to doxorubicin. At the end of the experiments, cardiomyocytes were assayed by HPLC for DCF-detectable H2O2, as described under Materials and Methods. Values are means ± S.E. of three to four experiments. *p < 0.01 for (t-boc-Ala + doxorubicin) versus all the other samples.
Effects of t-boc-Ala on doxorubicin content and H$_2$O$_2$ formation in H9c2 cardiomyocytes: dependence on doxorubicin concentration

Subconfluent H9c2 cardiomyocytes ($5 \times 10^4$) were exposed for 16 h to one of the following agents: H$_2$O$_2$ (0.1–250 μM), antimycin A (0.05–25 μM), aminotriazole (0.1–50 μM), ferric ammonium citrate (0.1 μM–1 mM), streptonigrin (0.001–10 μM), paraquat (0.1–250 μM), doxorubicin (0.1–50 μM), aclarubicin (0.05–25 μM), aclarubicin (0.1–50 μM), and etoposide (0.1–100 μM). Cell viability was assessed by the antimycin A-inhibitable MTT reduction assay. All the IC$_{50}$ values are means ± S.E. of three to four experiments.

### TABLE 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability</th>
<th>Intracellular Doxorubicin</th>
<th>Doxorubicin-Derived H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 μM Doxorubicin</td>
<td>105 ± 3</td>
<td>0.02 ± 0.004</td>
<td>0.007 ± 0.002</td>
</tr>
<tr>
<td>+ 10 mM t-boc-Ala</td>
<td>44 ± 8*</td>
<td>0.38 ± 0.10*</td>
<td>0.033 ± 0.002*</td>
</tr>
<tr>
<td>5 μM Doxorubicin</td>
<td>51 ± 7*</td>
<td>0.37 ± 0.08*</td>
<td>0.029 ± 0.003*</td>
</tr>
<tr>
<td>+ 10 mM t-boc-Ala</td>
<td>33 ± 13*</td>
<td>0.30 ± 0.06**</td>
<td>0.035 ± 0.005**</td>
</tr>
</tbody>
</table>

* p < 0.01 for (0.5 μM + 10 mM t-boc-Ala) and 5 μM doxorubicin or (5 μM doxorubicin + 10 mM t-boc-Ala) vs. 0.5 μM doxorubicin alone.

** p > 0.05 for (5 μM + 10 mM t-boc-Ala) vs. 5 μM doxorubicin alone.

mM t-boc-Ala could not be performed because of an inadequate buffering of >10 mM t-boc-Ala by the minimal essential medium used in the experiments.

**Effects of t-boc-Ala on Anthracycline-Dependent and Nonoxidant Acl.** We evaluated whether t-boc-Ala exacerbated cardiomyocyte damage in a manner that was specific to doxorubicin or extended to other toxic agents. Inasmuch as the toxicity of doxorubicin to cardiomyocytes involves ROS and iron-dependent reactions (Gewirtz, 1999; Minotti et al., 2004), we exposed cardiomyocytes to H$_2$O$_2$, iron, or nonantracycline drugs that form O$_2^-$ and H$_2$O$_2$. As a negative control, we used cardiomyocytes treated with a nonoxidant drug. The H$_2$O$_2$-related stress was induced by treating cardiomyocytes with bolus H$_2$O$_2$, aminotriazole (to inhibit catalase decomposition of endogenous H$_2$O$_2$), or antimycin A (to promote a mitochondrial leakage of H$_2$O$_2$ via the inhibition of complex III) (Salvatorelli et al., 2006). The iron-related stress was induced by treating cardiomyocytes with ferric ammonium citrate or hemin. An elevation of the cellular levels of both O$_2^-$ and H$_2$O$_2$ was induced with paraquat (a bipiridyl compound) or streptonigrin (an aminooquinone), which are known to undergo a cyclic reduction-oxidation with cellular reductases and molecular oxygen (Thomas and Aust, 1986). The negative control was run by treating cardiomyocytes with etoposide, an epipodophyllotoxin derivative that induces apoptosis in a free radical-independent manner (Sentürker et al., 2002). All such toxic stimuli were compared with doxorubicin and related analogs such as daunorubicin or aclarubicin. Daunorubicin was used because it contains the same quinone-hydroquinone chromophore as that of doxorubicin; thus, it undergoes oxidation/degradation with ferrylmyoglobin (daunorubicin only differs from doxorubicin in the side chain terminus; Fig. 1D). Aclarubicin was used because it lacks a hydroquinone in its chromophore (Fig. 1E); consequently, it exhibits a limited oxidation/degradation with ferrylmyoglobin (Menna et al., 2002; Cartoni et al., 2004). Under the experimental conditions of Fig. 5, daunorubicin exhibited an oxidative degradation comparable with that of doxorubicin, whereas aclarubicin was ~50% less susceptible to an oxidative degradation. Table 3 reports the IC$_{50}$ values calculated for each test compound: they varied from ~0.01 μM (streptonigrin) up to ~10 μM (aminotriazole), with the three anthracyclines exhibiting IC$_{50}$ values over an ~5 to 10 μM range.

We next delivered the aforesaid test compounds to cardiomyocytes preloaded with 10 mM t-boc-Ala. The toxic synergism of t-boc-Ala with a given test compound was expressed as the ratio between the IC$_{50}$ value calculated for that compound in the absence or presence of t-boc-Ala; ratios above the unity indicated a toxic synergism. t-Boc-Ala aggravated the toxicity of doxorubicin and daunorubicin, but it caused a minor effect on the toxicity of aclarubicin and essentially no effect on the toxicity of the other agents (Fig. 9). These results suggested that t-boc-Ala was highly specific at aggravating the toxicity of anthracyclines, particularly of those analogs that exhibited a high oxidation/degradation with ferrylmyoglobin.

### Discussion

Previous attempts to elucidate the pharmacological relevance of doxorubicin degradation were indirect or of limited preclinical value. Some reports showed the presence of 3-methoxyphthalic acid, a product of doxorubicin degradation, in the heart of mice treated with a single dose of doxorubicin or...
in human myocardial samples exposed to doxorubicin in vitro; the same reports showed that the viability of isolated cardiomyocytes was diminished by doxorubicin but not by purified 3-methoxypthalic acid or doxorubicin samples that had been subjected to a prior oxidative degradation in vitro (Cartoni et al., 2004; Reszka et al., 2005). These experiments uncovered the biochemical feasibility of doxorubicin degradation in tissues, and they anticipated that the products of doxorubicin degradation were essentially nontoxic compared with intact doxorubicin; however, neither approach characterized whether doxorubicin degradation influenced the levels and toxicity of the anthracycline inside cardiomyocytes.

In other experiments, human leukemia HL-60 cells were shown to degrade extracellular doxorubicin, presumably by secreting peroxidases, but doxorubicin degradation only occurred if the cells were treated with (sub)millimolar H₂O₂ and nitrite. Hydrogen peroxide served to activate the peroxidases, whereas nitrite served to accelerate the catalytic turnover of the peroxidases or to form an ·NO₂ radical that also oxidized and degraded doxorubicin (Reszka et al., 2005). These conditions were of limited pathophysiological relevance; (sub)millimolar H₂O₂ or nitrite may be used for biochemical studies in vitro, but they would hardly occur in the plasma and intercellular fluids of a patient. Doxorubicin toxicity was used at concentrations 1.5 to 2 times higher than its maximal plasma level in patients (15–20 μM (Reszka et al., 2005) versus a clinical plasma peak ≤10 μM (Gianni et al., 1990); moreover, there was no evidence that an extracellular degradation determined the amount of doxorubicin that diffused and caused toxicity in the cells.

Here, we used t-boc-Ala to probe the role of doxorubicin degradation in cardiomyocytes. t-Boc-Ala was chosen during the search for a compound that modulated myoglobin-dependent doxorubicin degradation but neither caused toxicity per se nor altered the redox balance of cardiomyocytes and their sensitivity to ROS. t-Boc-Ala met all such requirements: it did not reduce cardiomyocyte viability when it was used as a single agent nor did it render cardiomyocytes more sensitive to H₂O₂, iron, or ROS produced by nonanthracycline redox cycling agents such as paraquat or streptonigrin. Nonetheless, t-boc-Ala increased the cellular levels of doxorubicin and doxorubicin-derived H₂O₂, thereby aggravating the toxicity induced by doxorubicin concentrations as low as 0.1 to 1 μM. Studies with cell-free systems suggested that t-boc-Ala caused all such effects by inhibiting doxorubicin oxidation with MbIV = O, a compound II-like species formed through H₂O₂ and Mb³O₂.

Fig. 9. Toxic synergism of t-boc-Ala with oxidant and nonoxidant agents. Cardiomyocytes were preloaded with 10 mM t-boc-Ala and then exposed to the same toxic compounds as those used in Table 3. The toxic synergism of t-boc-Ala with each compound was expressed as the ratio of (IC₅₀ without t-boc-Ala preloading) to (IC₅₀ with t-boc-Ala preloading); values above the unity indicated a toxic synergism. Cell viability was determined by the antimycin A-inhibitable MTT reduction assay. All the above the unity indicated a toxic synergism.

Anthracyclines are polar and bulky molecules that cannot enter the hydrophobic heme pocket of myoglobin or equivalent (pseudo)peroxidases; as suggested by others (Yackzan and Wingo, 1982), bulky molecules would react with myoglobin through indirect electron tunneling mechanisms. This probably occurred at sites different from those available to typical peroxidatic substrates, because t-boc-Ala did not inhibit an MbIV = O-dependent oxidation of guaiacol; moreover, the anthracycline-degrading activity of Mb³O₂ and other (pseudo)peroxidases did not correlate with their peroxidatic activity, and the pseudo-peroxidases actually degraded more doxorubicin than did the authentic peroxidases.

Being a redox-inactive molecule, t-boc-Ala inhibited doxorubicin degradation without reducing MbIV = O or forming adducts with the anthracycline chromophore; instead, t-boc-Ala prevented both doxorubicin oxidation and MbIV = O reduction. An attractive hypothesis is that t-boc-Ala reversibly competed for, and introduced sterical barriers at those myoglobin sites that relayed electrons from the hydroquinone moiety of doxorubicin to MbIV = O. Two more lines of evidence suggest that an interference of t-boc-Ala with an Mb³O₂-dependent doxorubicin degradation was governed by discrete sterical determinants. First, Ala or hydrolyzed t-boc-Ala did not inhibit doxorubicin degradation in vitro nor increase doxorubicin levels and toxicity in cardiomyocytes; and second, t-boc-Ala caused no effect on doxorubicin degradation induced by authentic peroxidases or pseudo-peroxidases such as Mb³I and others. The different behavior of Mb³O₂ or Mb³II versus t-boc-Ala was striking, but not conceptually unprecedented; other investigators found that Mb³O₂ and Mb³II, and the oxyferrous or ferric form of related neuroglobins, exhibited subtle conformational differences that influenced reversible interactions with ligands at a distance from the heme pocket (Kitahara et al., 1990; Wakasugi et al., 2003).

During the course of this study, we were unable to recover 3-methoxypthalic acid from doxorubicin-treated cardiomyocytes, despite that our fluorimetric assay would have been sensitive enough to measure <0.01 nmol of 3-methoxypthalic acid/mg protein. This finding was in conceptual agreement with a prevailing oxidation of doxorubicin with the compound II of Mb³O₂. At a same time, the lack of 3-methoxypthalic acid formation raised possible concerns about the mechanism(s) by which t-boc-Ala increased doxorubicin levels and toxicity in the cells, because we could not demonstrate that an intra-cellular accumulation of doxorubicin was paralleled by a diminished formation of a nontoxic product of anthracycline degradation. To obviate such uncertainties, we conducted experiments that further clarified how t-boc-Ala augmented the cellular levels and toxicity of doxorubicin. Our data show that 1) t-boc-Ala neither stimulated the cellular uptake of doxorubicin nor diminished its efflux; 2) t-boc-Ala aggravated the toxicity of a doxorubicin analog that was liable to oxidative degradation (daunorubicin), but it caused a minor effect.
on the toxicity of a hydroquinone-defective anthracycline that exhibited a limited oxidation/degradation (aclarubicin); and (3) doxorubicin samples that had been oxidized and degraded with H$_2$O$_2$/Mb$^{3+}$ lacked toxicity to cardiomyocytes, similar to what observed with 3-methoxyanthraquinone in previous studies and in the present report. All such findings lent credibility to the hypothesis that t-boc-Ala acted by blocking the oxidation/degradation of doxorubicin with H$_2$O$_2$/Mb$^{3+}$, regardless of the precise structure of the degradation products that were formed by this reaction.

When reexamined in the aforesaid context, the recovery of 3-methoxyanthraquinone from the heart of doxorubicin-treated mice probably reflected the high toxicity of doxorubicin usually adopted in acute models with small rodents (>2 orders of magnitude more doxorubicin/body weight compared with a standard clinical dose); this condition may have favored a doxorubicin-dependent autoxidation of Mb$^{3+}$ to Mb$^{4+}$, and the consequent degradation of doxorubicin by a compound 1-dependent mechanism. Likewise, the recovery of 3-methoxyanthraquinone from human myocardial samples incubated with doxorubicin probably reflected an unavoidable formation of Mb$^{4+}$ during the ex vivo sampling, storage, or in vitro manipulation of the myocardial specimens.

Doxorubicin-induced cardiomyopathy has long been known to follow a dose-dependent pattern. A lifetime cumulative dose of 450 to 500 mg of doxorubicin/m$^2$ is considered to be safe (Swain et al., 2003); Nonetheless, some patients develop cardiac events at lower than expected cumulative doses, an observation that not always is explained by the presence of cardiac risk factors or by the coadministration of drugs with a known effect on the redox balance of the heart (Minotti et al., 2004; Ewer and Lippman, 2005; Salvatorelli et al., 2007). Here, we have shown that a nontoxic and redox-inactive molecule such as t-boc-Ala may aggravate cardiotoxicity by competing with doxorubicin for an anthracycline-degrading factor such as Mb$^{4+}$. This finding anticipates that many drugs, currently considered to be suitable for a combination therapy with doxorubicin, might have the structural prerequisites to induce a similar effect, thereby inhibiting doxorubicin degradation and exacerbating its dose-related cardiotoxicity.

Clinically, doxorubicin cardiotoxicity may be diminished by replacing a bolus administration with a slow infusion, reportedly because the prolonged infusion schedules generate a slower plasma and cardiac exposure to doxorubicin (Berrak et al., 2001). Here, we have shown that t-boc-Ala caused a severe aggravation of the toxicity induced by 0.1 to 1 μM doxorubicin, a concentration range that compares with the plasma levels of doxorubicin during the course of slow infusion schedules. These observations suggest that the improved cardiac tolerability of slow infusion schedules might be caused also by a more efficient detoxification of doxorubicin through an oxidation-degradation mechanism.

In conclusion, we have characterized that doxorubicin degradation may serve as a salvage mechanism for diminishing the levels and toxicity of doxorubicin in cardiomyocytes. This concept may be of relevance to many clinical aspects of doxorubicin-induced cardiotoxicity.

Acknowledgments

We thank Dr. Raimondo De Cristofaro (Catholic University School of Medicine, Rome, Italy) for helpful discussions.

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In conclusion, we have characterized that doxorubicin deg- radation through an oxidation-degradation mechanism. Likewise, the recovery of 3-methoxyanthraquinone...
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