The Novel Anthracenedione, Pixantrone, Lacks Redox Activity and Inhibits Doxorubicinol Formation in Human Myocardium: Insight to Explain the Cardiac Safety of Pixantrone in Doxorubicin-Treated Patients

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ABSTRACT
Cardiotoxicity from the antitumor anthracycline doxorubicin correlates with doxorubicin cardiac levels, redox activation to superoxide anion (O$_{2}^-$) and hydrogen peroxide (H$_2$O$_2$), and formation of the long-lived secondary alcohol metabolite doxorubicinol. Cardiotoxicity may first manifest during salvage therapy with other drugs, such as the anthracenedione mitoxantrone. Minimal evidence for cardiotoxicity in anthracycline-pretreated patients with refractory-relapsed non-Hodgkin lymphoma was observed with the novel anthracenedione pixantrone. We characterized whether pixantrone and mitoxantrone caused different effects on doxorubicin levels, redox activation, and doxorubicinol formation. Pixantrone and mitoxantrone were probed in a validated ex vivo human myocardial strip model that was either doxorubicin-naïve or preliminarily subjected to doxorubicin loading and washouts to mimic doxorubicin treatment and elimination in the clinical setting. In doxorubicin-naïve strips, pixantrone showed higher uptake than mitoxantrone; however, neither drug formed O$_2^-$ or H$_2$O$_2$. In doxorubicin-pretreated strips, neither pixantrone nor mitoxantrone altered the distribution and clearance of residual doxorubicin. Mitoxantrone showed an unchanged uptake and lacked effects on doxorubicin levels, but synergized with doxorubicin to form more O$_2^-$ and H$_2$O$_2$, as evidenced by O$_2^-$-dependent inactivation of mitochondrial aconitase or mitoxantrone oxidation by H$_2$O$_2$-activated peroxidases. In contrast, pixantrone uptake was reduced by prior doxorubicin exposure; moreover, pixantrone lacked redox synergism with doxorubicin, and formed an N-dealkylated product that inhibited metabolism of residual doxorubicin to doxorubicinol. Redox inactivity and inhibition of doxorubicinol formation correlate with the cardiac safety of pixantrone in doxorubicin-pretreated patients. Redox inactivity in the face of high cardiac uptake suggests that pixantrone might also be safe in doxorubicin-naïve patients.

Introduction
Clinical use of doxorubicin (DOX) and other topoisomerase II–inhibiting antitumor anthracyclines is limited by dose-related cardiotoxicity. In adults, cumulative doses of 400–450 mg/m$^2$ of DOX introduce a 5% risk of congestive heart failure (Swain et al., 2003). In children, even lower cumulative doses introduce a lifetime risk of cardiotoxicity (Barry et al., 2007).

Anthracycline cardiotoxicity is a multifactorial and incompletely defined process, but a few basic determinants have been identified. Cardiotoxicity correlates with the plasma peak concentration (C$_{max}$) of DOX and its accumulation in the heart, which depends on the balance between DOX uptake and clearance (Minotti et al., 2004a). Clearance seems to be incomplete, as DOX can be found in the hearts of patients expiring months or years after the last administration (Stewart et al., 1993). Moreover, the risk of cardiotoxicity increases significantly if DOX undergoes reductive bioactivation. DOX is composed of a tetracyclic quinone-hydroquinone chromophore, an aminosugar, and a short side chain with a carbonyl group (Fig. 1). One-electron reduction of the quinone yields a semiquinone that reduces oxygen to superoxide anion (O$_2^-$) and its dismutation product, hydrogen peroxide (H$_2$O$_2$), members of the broad family of reactive oxygen species (ROS) that cause oxidative stress in the relatively unprotected heart (Doroshaw, 1983; Gewirtz, 1999). Two-electron reduction of superoxide anion

ABBREVIATIONS: ANOVA, analysis of variance; C$_{max}$, peak concentration; cTnI, cardiac troponin I; DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescin-diacetate; DOX, doxorubicin; DOXOL, doxorubicinol; ESI(+)/MRM, electron spray ionization(+)/multiple reaction monitoring; eV, electron volt; H$_2$O$_2$, hydrogen peroxide; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; MDR, multidrug resistance; MITOX, mitoxantrone; MRP1, multidrug resistance protein 1; N,NH, non-Hodgkin lymphoma; O$_2^-$, superoxide anion; PIX, pixantrone; ROS, reactive oxygen species; SIM, single-ion monitoring.
the side chain carbonyl moiety generates a secondary alcohol metabolite [doxorubicinol (DOXOL); Fig. 1]. Being more polar than DOX, DOXOL shows essentially no cardiac clearance (Salvatorelli et al., 2007). DOXOL accumulation exposes the heart to a lifetime risk of congestive heart failure (Minotti et al., 2010; Blanco et al., 2012).

Cardiotoxicity also complicates the clinical management of patients who progress or relapse after first-line therapy with \( \leq 400 \text{ mg/m}^2 \) of DOX. Second- or third-line nonanthracycline chemotherapeutics might precipitate cardiotoxicity by interfering with the cardiac clearance of DOX, forming ROS, or increasing DOXOL formation. The risk of cardiotoxicity may be higher when salvage drugs share structural or functional similarities with DOX (Minotti et al., 2004a).

Anthracenediones are aglyconic quinone-hydroquinone drugs with anthracycline-like antitumor activity. Although traditionally grouped with anthracyclines, anthracenediones show important structural differences: the chromophore contains three rings only, and the side chains lack carbonyl groups precursor of secondary alcohol metabolites. In patients with diffuse large B-cell lymphoma, the prototypic anthracenedione mitoxantrone (MITOX) (Fig. 1) was less efficacious than DOX, but had similar cardiotoxicity (Sonneveld et al., 1995; Osby et al., 2003). MITOX exacerbates cardiotoxicity in anthracycline-pretreated laboratory animals (Cavalletti et al., 2007) and patients (Faulds et al., 1991).

Pixantrone (PIX) is a novel anthracenedione that differs from MITOX due to removal of the hydroquinone, insertion of a nitrogen heteroatom in the same ring, and substitution of (ethylamino)-diethylamino for (hydroxyethylamino)-ethylamino side chains (Fig. 1). PIX caused essentially no cardiotoxicity in anthracycline-naive or -pretreated animals (Cavalletti et al., 2007). In phase I studies, PIX was tolerable at doses associated with antitumor activity, and this activity was pronounced in patients with late-stage refractory/relapsed aggressive non-Hodgkin lymphoma (NHL) (Borchmann et al., 2001). Therefore, PIX was developed to treat patients with NHL who progress or relapse after DOX-containing chemotherapy regimens. In the phase III EXTEND (Expanding the reach of anthracyclines with piXanTronE in relapsed or refractory aggressive NHL Disease) study of patients with NHL who had received the cyclophosphamide/DOX/vincristine/prednisone regimen with a median cumulative dose of \( \sim 300 \text{ mg/m}^2 \) of DOX, single-agent PIX was tolerable and demonstrated a significantly higher complete response rate and progression-free survival compared with the investigators’ choice of single-agent chemotherapeutics (Pettengell et al., 2012). Rapidly reversible grade-3 reductions in left ventricular ejection fraction were observed in two of 68 (3%) PIX-treated patients. The study resulted in conditional approval by the European Medicines Agency of PIX as monotherapy in patients with relapsed aggressive B-cell NHL who are not candidates for stem cell transplantation.

Studies of cardiac safety or toxicity of antitumor drugs are limited by differences in drug metabolism between humans and laboratory animals (Mordente et al., 2003; Minotti et al., 2004a; Menna et al., 2008). We validated an ex vivo human myocardial strip model that eliminated this problem and helped characterize DOX accumulation and bioactivation to ROS or DOXOL in human myocardium (Salvatorelli et al., 2006a,b, 2007, 2009, 2012a,b). In the present study, the same model was used to explore the determinants of PIX cardiac safety in anthracycline-pretreated patients. MITOX was used as a comparator.

Materials and Methods

Chemicals. DOX and DOXOL were obtained from Nerviano Medical Sciences (Milan, Italy). PIX dimaleate and its metabolites were provided by Cell Therapeutics, Inc. (Seattle, WA). Dichlorofluorescein-diacetate (DCFH-DA) and dichlorofluorescein (DCF) were obtained from Invitrogen (Carlsbad, CA). MITOX, verapamil, and all other chemicals were obtained from Sigma-Aldrich (Milan, Italy).

Human Myocardial Strip Preparation and Incubation with Drugs. One hundred seventy-four myocardial samples were obtained from 126 male patients (69 \( \pm \) 1.3 years of age) and 48 female patients (71 \( \pm \) 2.7 years of age) undergoing aortocoronary bypass grafting. All samples were to be routinely disposed of by the surgeons during cannulation of the nonischemic, beating right atrium; therefore, the patients were not subjected to any unjustified loss of tissue (Salvatorelli et al., 2012a,b). Thin strips (\( \sim \)10 mm long, \( \sim \)2 mm wide) were dissected free of fat or grossly visible foreign tissues. The strips were incubated in 2 mL of fresh human plasma to allow for effects of binding of drugs to albumin and other proteins (Whitaker et al., 2008).

DOX was used at a final concentration of 10 \( \mu \text{M} \), similar to the plasma \( C_{\text{max}} \) produced by standard DOX infusions (Gianni et al., 1997). PIX was used at 1 \( \mu \text{M} \) (calculated as the PIX base). Thin strips (\( \sim \)10 mm long, \( \sim \)2 mm wide) were dissected free of fat or grossly visible foreign tissues. The strips were incubated in 2 mL of fresh human plasma to allow for effects of binding of drugs to albumin and other proteins (Whitaker et al., 2008). PIX was used at 1 \( \mu \text{M} \) (calculated as the PIX base) to mimic the plasma \( C_{\text{max}} \) produced by 1-hour infusion of 85 mg/m\(^2\) of PIX in the EXTEND study (Cell Therapeutics Inc., 2010). MITOX was used at 1 \( \mu \text{M} \), which is similar to the plasma \( C_{\text{max}} \) produced by 14 mg/m\(^2\) of MITOX (Canal et al., 1993), the dose level adopted for MITOX as a comparator to PIX in the EXTEND study (Pettengell et al., 2012).

Probing PIX or MITOX as Single Agents or in Sequence with DOX. Incubations were carried out at 37°C in a gently shaking Dubnoff metabolic bath under a room air atmosphere.
Unless otherwise indicated, four sets of 4-hour incubations were performed. In the first experiment, the myocardial strips were incubated with plasma for 2.5 hours. Single-agent anthracyclene was then added at 1 μM, and incubation continued for an additional 1.5 hours [Fig. 2A (a)]. In the other three experiments, PIX or MITOX was added in sequence with DOX. In the first condition, DOX was added and allowed to stay over the 4-hour incubations; anthracyclene was added at 2.5 hours [Fig. 2A (b)]. This condition (DOX loading) probed PIX or MITOX in strips subjected to a continuous uptake and retention of DOX. In the second condition, the strips were exposed to DOX for 30 minutes and then placed in fresh anthracycline-free plasma; after 2.5 hours, anthracyclene was added [Fig. 2A (c)]. This condition (DOX loading and washout) probed PIX or MITOX in strips subjected to DOX uptake and clearance. In the third condition, the strips were exposed to DOX for 30 minutes and then placed in fresh anthracycline-free plasma that was renewed every 30–60 minutes up to 2.5 hours; next, anthracyclene was added [Fig. 2A (d)]. This condition (DOX loading and multiple washouts) probed PIX or MITOX in strips subjected to DOX uptake and more extensive clearance.

**Drug Uptake, Clearance, and Accumulation.** DOX accumulation was determined by the net levels of DOX retention in the strips at the end of the 4-hour incubations. In the DOX loading/washout (s) experiments, uptake was determined as ([DOX accumulation] + [DOX efflux in plasma]); DOX clearance was determined as [100 × (efflux/uptake)] (Salvatorelli et al., 2012b). Similar procedures were adopted in ad hoc experiments with single-agent anthracyclene. Where indicated, the experimental conditions were modified to measure PIX uptake, clearance, and accumulation in strips that released increasing levels of DOX in plasma. The strips were subjected to 30 minutes of loading with DOX at 1 or 10 μM; next, plasma was replaced with fresh plasma, and PIX was added at 1 μM to measure its uptake while DOX diffused from the strips in plasma. After an additional 30 minutes, plasma was replaced fresh, and the incubations were allowed to proceed to measure PIX clearance while DOX was diffusing from the strips in plasma. Strips loaded with only PIX were used as controls.

**High-Performance Liquid Chromatography Assays for DOX and Anthracyclenes.** After incubation, the strips were washed with ice-cold 0.3 M NaCl, homogenized in 1 ml of the same medium, and centrifuged for 90 minutes at 105,000 g to separate soluble and whole-membrane fractions. These were extracted with an equal volume of (90:10) CH3OH-CH3CN and acidified with 0.2% CF3COOH. Plasma was extracted in a similar manner. After centrifugation at 25,000g for 10 minutes, 1 μl of the supernatant was analyzed by high-performance liquid chromatography (HPLC) in a Hewlett-Packard 1100 system (Palo Alto, CA).

We used a (5 μm, 250 × 2.1 mm) Supelcosil ABZ+Plus column (Supelco, Bellafonte, PA). The column was operated at 25°C and eluted at a flow rate of 0.6 ml/min for a total 30-minute run time (8-minute linear gradient from 100% 50 mM NaH2PO4, pH 3.0, to (80%–20%) 50 mM NaH2PO4-CH3CN, followed by a 7-minute linear gradient to (65%–35%) 50 mM NaH2PO4-CH3CN, which eventually was switched to a 15-minute linear gradient to (50%–50%) 50 mM NaH2PO4-CH3CN). DOX and DOXOL were detected fluorimetrically (excitation at 480 nm/emission at 560 nm) and identified by cochromatography with authentic standards. Because of differences in the fluorescence yield of one anthracycline versus another, and parent anthracyclines versus secondary alcohol metabolites, we did not use an internal standard, such as daunorubicin; instead, DOX and DOXOL were quantified against standard curves prepared on the day of the experiment (Salvatorelli et al., 2007). Retention times were 15.0 minutes for DOX and 13.8 minutes for DOXOL, with a lowest detection limit of 0.001 μM. PIX and MITOX were detected by diode array spectroscopy (λmax = >420 nm and 610 nm, respectively), identified by cochromatography with authentic standards, and quantified against appropriate standard curves. Retention times were 6.4 minutes for PIX and 10.7 minutes for MITOX; the lowest detection limit was 0.005 μM for PIX or 0.001 μM for MITOX. Extraction efficiency and HPLC recovery always averaged >90%. Within-day and between-days coefficients of variation were ~3% and ~10%, respectively. All values were normalized to tissue weight and expressed as micromolar equivalents, since the density of cardiac tissue is very similar to that of water (Muslin et al., 1993).

**HPLC Assay for DCF-Detectable H2O2.** Basal or drug-stimulated ROS formation was measured by an HPLC adaptation of the method based on the uptake of membrane-permeable DCFH-DA, its intracellular deacetylation to membrane-impermeable DCFH, and oxidation of DCFH to DCF by H2O2 (Salvatorelli et al., 2006a, 2007, 2012a).

Myocardial strips were incubated in 4 ml of 50 mM phosphate buffer/123 mM NaCl/5.6 mM glucose, pH 7.4, to which DCFF-DH-DA was added at 50 μM. After 1 hour in the dark, the strips were washed with 0.3 M NaCl and incubated with plasma with or without drugs. In experiments of DOX loading and multiple washouts, the last washout was followed by 30 minutes of re-exposure of the strips to 50 μM DCFF-DH-DA; this was done to compensate for any DCFF-DH-DA that had diffused from strips in plasma during washouts. At the end of the experiment, the strips were homogenized in 2 ml of ice-cold 123 mM NaCl, to which the antioxidant 4-hydroxytempo was added at 1 mM to prevent further oxidation of DCFH to DCF during tissue homogenization. Soluble and membrane fractions were extracted and analyzed by HPLC as described for DOX(OL) and PIX or MITOX, except that the loading volume was 25 μl and the total run time was 25 minutes (10-minute linear gradient from 100% 50 mM NaH2PO4, pH 3.0, to (30%–70%) 50 mM NaH2PO4-CH3CN). The fluorescent peak of DCF (excitation at 488 nm/emission at 525 nm) was identified by cochromatography with an authentic standard (retention time = 14.0 minutes) and quantified against a proper standard curve (lowest detection limit = 0.001 μM).
Measurements of ROS by fluorescent probes suffer from potential limitations and artifacts (Kalyanaraman et al., 2012); therefore, the DCF assay adopted in this study was validated to obtain unambiguous identification of DCF as a marker of H$_2$O$_2$-dependent DCFH oxidation, and to establish quantitative correlations between DCF and H$_2$O$_2$. Unambiguous identification of DCF as the product of H$_2$O$_2$-dependent DCFH oxidation was obtained by measuring, respectively, ~4-fold or ~2.5-fold elevations of DCF in strips incubated for 4 hours in plasma with added 100 $\mu$M antimycin A (which induces a mitochondrial leakage of H$_2$O$_2$ by inhibiting complex III in the respiratory chain) or 50 $\mu$M aminotriazole (which reduces H$_2$O$_2$ decomposition by inhibiting catalase) (Salvatorelli et al., 2006a). Quantitative correlations between DCF and H$_2$O$_2$ were obtained by measuring DCF in strips incubated for 15 minutes in 4 ml of 50 $\mu$M phosphate buffer/123 mM NaCl/5.6 mM glucose, pH 7.4, to which H$_2$O$_2$ was added at 0.1–10 $\mu$M. DCF formation in these strips was plotted against a DCF standard curve, and a DCF:H$_2$O$_2$ stoichiometry of 0.43 could be established (Salvatorelli et al., 2006a, 2012a). The lack of a 1:1 stoichiometry could be attributed to H$_2$O$_2$-independent oxidation of DCFH or side reactions of DCF with redox active-cell constituents (Rota et al., 1999).

In Vitro Oxidation of PIX or MITOX. Incubations contained 10 $\mu$M anthracenedione and 0.01 $\mu$M horseradish peroxidase (HRP) in 10 mM ammonium acetate (pH 7.0, 37°C). Oxidation was started by 100 $\mu$M H$_2$O$_2$ and was terminated after 30 minutes by inhibiting HRP with 0.5 $\mu$M methimazole (Reszka et al., 2011). Liquid Chromatography/Mass Spectrometry Analyses of PIX or MITOX Metabolites. PIX metabolites were analyzed by liquid chromatography/mass spectrometry (Agilent 1200 HPLC-6410 triple quadrupole spectrometer; Agilent Technologies, Santa Clara, CA). Cell-free reaction mixtures were injected unmodified; incubations of myocardial strips were extracted as described previously. We used an Eclipse Plus C18 column (3.5 $\mu$m, 100 × 2.1 mm; Agilent Technologies). The autosampler was set at 4°C, and the loading volume was 8 $\mu$L. For PIX metabolite characterizations, the samples were eluted at a flow rate of 200 $\mu$L/min with a mobile phase composed of (90:10) (H$_2$O-0.1% HCOOH:CH$_3$CN-0.1% HCOOH), which was changed to (20:80) in 5 minutes, maintained isocratic for 1999 minutes (Salvatorelli et al., 2006a, 2012a). The lack of a 1:1 stoichiometry could be attributed to H$_2$O$_2$-independent oxidation of DCFH or side reactions of DCF with redox active-cell constituents (Rota et al., 1999).

Results

PIX or MITOX Levels in DOX-Loaded Human Myocardial Strips. In human myocardial strips, single-agent PIX accumulated at concentrations ~3 times those of MITOX (Fig. 2B (a)). DOX loading did not affect PIX accumulation [Fig. 2B (b)]; however, DOX loading/washout and DOX loading/multiple washouts diminished PIX accumulation by 25% and 45%, respectively [Fig. 2B (c) and (d)]. This was not observed with MITOX; it follows that, after DOX loading and multiple washouts, PIX accumulated only 1.5 times more than MITOX [see also Fig. 2B (d)]. DOX loading/washout and DOX loading/multiple washouts diminished PIX accumulation in both membrane and soluble fractions of the strips; again, this did not occur with MITOX (Fig. 2, C and D).

Single and multiple washouts induced, respectively, ~40% and ~60% clearance of DOX from myocardial strips, and diminished DOX accumulation in both the membrane fraction and soluble fractions of the strips. PIX and MITOX did not affect DOX accumulation, distribution, or clearance (Table 1). In human myocardial strips, many anthracycline-related effects occur over a narrow range of anthracycline levels (Salvatorelli et al., 2006a,b, 2012a,b). Inasmuch as single or multiple washouts diminished both the accumulation of DOX and that of PIX added after DOX, we considered that PIX accumulation was limited by the presence of a critical amount of DOX in the strips. This proved not to be the case. In DOX loading experiments, decreasing the DOX concentration from 10 to 3 $\mu$M caused a decrease of ~66% in DOX accumulation in the strips, which was similar to the decrease induced by multiple washouts in experiments with DOX at 10 $\mu$M; however, PIX accumulation was not diminished in comparison with strips exposed to PIX as a single agent (not shown). We concluded that PIX accumulation was independent of the net levels of DOX in the strips. By considering that DOX loading experiments probed DOX uptake and retention but not DOX clearance, we also hypothesized that PIX accumulation was limited by effects that only occurred when washouts caused DOX clearance.
Single-agent PIX showed higher uptake and clearance than MITOX. PIX uptake was 2.8-fold higher, whereas clearance was only ~30% higher, indicating that greater accumulation of single-agent PIX was mainly driven by higher uptake (Table 2). We next characterized whether DOX clearance interfered with PIX uptake and/or augmented PIX clearance. To obtain this information, we measured PIX uptake and clearance in strips that released increasing amounts of DOX following exposure to the anthracene at 1 or 10 μM (see Materials and Methods). Under such defined conditions, PIX accumulation was limited by a selective decrease in PIX uptake (Fig. 3, A–C). Strips exposed to 1 or 10 μM DOX exhibited one order of magnitude difference in the net values of DOX efflux (not shown); nevertheless, both strips caused comparable decreases in PIX uptake and accumulation. This suggests that even minor fluxes of DOX clearance impair PIX uptake. Mammalian cardiomyocytes constitutively express multidrug resistance (MDR) proteins such as multidrug resistance protein 1 (MRP1) (Flens et al., 1996) and P-glycoprotein (Budde et al., 2011), the latter also being highly expressed in endothelial cells (Meissner et al., 2004). We characterized whether, in the strips subjected to DOX loading and washouts, MDR proteins extruded PIX and diminished its accumulation. The calcium channel blocker verapamil was used to inhibit P-glycoprotein and MRP1 (Vellonen et al., 2004). To avoid confounding effects due to calcium channel blockade, verapamil was used at a concentration level (1 μM) that was high enough to inhibit DOX extrusion by MDR proteins (Salvatorelli et al., 2012b) but was lower than verapamil plasma levels associated with calcium channel blockade and cardiovascular effects in clinical studies of tumor resistance reversal (Motzer et al., 1995; Warner et al., 1998). In the strips sequentially exposed to DOX loading/multiple washouts and PIX, 1 μM verapamil increased the accumulation of DOX but not of PIX (Fig. 3D).

**H₂O₂ Formation by PIX or MITOX in Human Myocardial Strips.** In control strips, DCF-detectable H₂O₂ averaged 0.21 ± 0.01 μM. In light of the low antioxidant defenses of the heart, this value compared reasonably well with the upper limit of the physiologic range of H₂O₂ concentrations measured by other investigators in different cells and tissues (Chance et al., 1979). DOX loading increased H₂O₂ levels by ~70%, whereas strips subjected to DOX loading/multiple washouts showed no such increases. Neither PIX nor MITOX, as single agents or in sequence with DOX, increased DCF-detectable H₂O₂ (Table 3).

**Correlations of H₂O₂ Formation with Peroxidatic PIX Metabolism.** H₂O₂-activated peroxidases contribute to the oxidizing of DCFH to DCF (LeBel et al., 1992); however, they may also oxidize anthrancenediones (Blanz et al., 1991;...
These results showed that CT-45886 formed by spontaneous hydrolysis of PIX, whereas CT-45889 and CT-45890 formed both spontaneously and by peroxidatic metabolism.

We next measured PIX metabolites in strips incubated with PIX alone or in sequence with DOX. High levels of CT-45886, the nonperoxidatic metabolite, were recovered from all incubations examined. By also measuring CT-45886 in plasma, we determined that its clearance averaged 59% ± 20%, 31% ± 12%, and 35% ± 12% in strips exposed to PIX alone or in sequence with DOX in sequence with DOX loading or DOX loading/multiple washouts, respectively. Total levels of CT-45886 diminished when PIX was added after DOX loading/multiple washouts, which is consistent with the limited accumulation of PIX in these strips (Fig. 4). CT-45889 and CT-45890, the potentially peroxidatic metabolites, formed in much lower amounts and showed near-to-complete clearance from the strips (93% ± 7% and 94% ± 6%, respectively). Comparable levels of CT-45889 and CT-45890 were measured in experiments with PIX alone or in sequence with DOX loading, which is not consistent with the fact that DOX loading caused H$_2$O$_2$ formation in the strips. Neither CT-45889 nor CT-45890 was recovered when PIX was added after DOX loading/multiple washouts, which is consistent with the limited accumulation of PIX in these strips (Fig. 4). These results suggest that the levels of potentially peroxidatic metabolites correlated poorly with H$_2$O$_2$ formation; such metabolites probably formed by spontaneous rearrangements that did not consume H$_2$O$_2$ in competition with the DCF assay. We confirmed that PIX did not form H$_2$O$_2$ in the strips.

**Correlations of H$_2$O$_2$ Formation with Peroxidatic MITOX Metabolism.** In cell-free experiments, MITOX converted to metabolites only after HRP had been activated with H$_2$O$_2$ (not shown). Single-agent MITOX was also stable in the strips, but six putative metabolites were identified when MITOX was used in sequence with DOX loading; two of these metabolites also formed when MITOX was used in sequence with DOX loading/multiple washouts. All metabolites diffused completely from strips in plasma.

MITOX metabolites showed different patterns of oxidation. In strips with high DOX levels (DOX loading), MITOX converted to both strongly and mildly oxidized metabolites that exhibited combinations of hydroquinone oxidation with side-chain N-cyclization or C-terminus peroxidation or carboxylation. In strips with low DOX levels (DOX loading/multiple washouts), MITOX converted to mildly oxidized metabolites that exhibited only hydroquinone oxidation or spontaneous hydrolysis of PIX.

**Table 3**

Effects of PIX or MITOX on H$_2$O$_2$ levels in human myocardial strips

<table>
<thead>
<tr>
<th>Anthracenedione</th>
<th>Control Hydrogen Peroxide ($\mu$M)</th>
<th>DOX Loading Hydrogen Peroxide ($\mu$M)</th>
<th>DOX Loading + Multiple Washouts Hydrogen Peroxide ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.21 ± 0.01</td>
<td>0.35 ± 0.03*</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>PIX</td>
<td>0.22 ± 0.02</td>
<td>0.32 ± 0.03*</td>
<td>0.22 ± 0.01</td>
</tr>
</tbody>
</table>

*H$_2$O$_2$: hydrogen peroxide; MITOX, mitoxantrone; PIX, pixantrone.  
* Membrane fraction + soluble fraction.  
* $P < 0.05$ versus control or DOX loading/multiple washouts (one-way analysis of variance followed by Bonferroni’s test for multiple comparisons).
reactive than \( \text{H}_2\text{O}_2 \) toward aconitase (estimated rate constants of \( \sim 10^7 \text{ M}^{-1} \text{s}^{-1} \) and \( \sim 10^3 \text{ M}^{-1} \text{s}^{-1} \), respectively). This makes mitochondrial aconitase inactivation an exquisite marker of formation of pathophysiologic amounts of \( \text{O}_2^- \) in cells and tissues (Gardner, 2002; Salvatorelli et al., 2006a, 2012a). Monitoring \( \text{O}_2^- \) formation by aconitase inactivation could also be used for correlations with, and validation of, \( \text{H}_2\text{O}_2 \) measurements by the DCF assay.

Mitochondrial aconitase was inactivated after DOX loading but not after DOX loading/multiple washouts, which correlated well with the increase in \( \text{H}_2\text{O}_2 \) after DOX loading but not after DOX loading/multiple washouts (see Table 3). MITOX alone did not inactivate mitochondrial aconitase; however, MITOX increased aconitase inactivation induced by DOX loading and caused aconitase inactivation in strips subjected to DOX loading/multiple washouts (Fig. 7C). PIX alone did not inactivate mitochondrial aconitase, did not increase aconitase inactivation from DOX loading, and did not cause aconitase inactivation in strips subjected to DOX loading/multiple washouts (Fig. 7D). These results confirmed that PIX lacks activity in ROS formation, whereas MITOX synergizes with DOX in causing more \( \text{O}_2^- \) formation.

**Effects of PIX or MITOX on DOXOL Formation.** DOX is metabolized to DOXOL by NADPH-dependent cytoplasmic reductases (Mordente et al., 2003; Minotti et al., 2004a; Slupe et al., 2005; Menna et al., 2008; Blanco et al., 2012; Kalabus et al., 2010). In the soluble fraction of strips incubated with DOX, DOXOL formation increased with DOX levels in the following order: DOX loading/multiple washouts < DOX loading/washout < DOX loading (Fig. 8A). Anthracenediones did not affect DOXOL levels after DOX loading or DOX loading/washout. In the strips subjected to DOX loading/multiple washouts, DOXOL levels were significantly diminished by PIX but not by MITOX (Fig. 8B).

PIX did not induce DOXOL efflux from strips (not shown); therefore, we characterized whether PIX decreased DOXOL levels by interfering with DOX metabolism. In isolated soluble fractions incubated with NADPH and DOX, MITOX and PIX or PIX metabolites inhibited DOXOL formation in a competitive concentration-dependent manner, i.e., inhibition was reversed upon increasing DOX concentration (not shown). With DOX at 10 \( \mu \text{M} \), 50% inhibition occurred when anthracenedione:DOX ratios were approximately 30 for MITOX, 20 for PIX and CT-45886, and 2 for CT-45889 and CT-45890 (Fig. 8C). We calculated anthracenedione:DOX ratios in the soluble fraction of strips sequentially exposed to DOX and anthracenediones. The ratios were normalized to those that caused 50% inhibition of DOXOL formation in isolated soluble fractions, and the values thereby obtained (IC\(^{50} \) percentages) were used to approximate the likelihood with which anthracenediones inhibited DOXOL formation in the strips. After DOX loading, DOX levels were too high to permit formation of significant IC\(^{50} \) percentages. After DOX loading/multiple washouts, DOX levels diminished substantially, anthracenedione:DOX ratios increased, and IC\(^{50} \) percentages could be calculated. CT-45886, the nonperoxidatic N-dealkylated metabolite of PIX, showed the highest IC\(^{50} \) percentage, followed by PIX and MITOX. CT-45889 and CT-45890 were too diffusible to generate IC\(^{50} \) percentages (Fig. 8D). MITOX metabolites diffused completely and were assumed to have minor significance in these experiments.

In the soluble fraction of strips sequentially exposed to DOX loading/multiple washouts and PIX, decreases in DOXOL
correlated with CT-45886 concentrations, but not PIX concentrations (Fig. 9A). This suggests that DOXOL formation diminished as PIX hydrolyzed to CT-45886 and the latter attained higher molar ratios to DOX. Accordingly, treating the strips with 1 mM PIX caused partial inhibition of DOXOL formation, and treating the strips with equimolar CT-45886 completely inhibited DOXOL formation (Fig. 9B).

Discussion

In comparison with MITOX, PIX exhibited higher uptake and accumulation in both DOX-naïve and DOX-loaded human myocardial strips. This cannot be explained by lipophilicity, as PIX is less lipophilic than MITOX (Colombo et al., 2009). The greater accumulation of PIX may be due to its weaker binding to plasma proteins (Cell Therapeutics Inc., 2012), resulting in a higher fraction of diffusible PIX. The uptake of PIX diminished if DOX loading and PIX administration were separated by one or more washouts that stimulated DOX clearance. The greatest reduction of PIX uptake occurred when multiple washouts were used to simulate clinical situations in which patients receive DOX and eliminate it over months or years before they undergo PIX salvage therapy (Pettengell et al., 2012).

The effects of DOX clearance on reducing PIX uptake were not primarily due to facilitated interaction of PIX with MDR drug efflux pumps. Verapamil, an inhibitor of P-glycoprotein and MRP1, increased accumulation of DOX but not of PIX, which was consistent with PIX being a poor substrate or inhibitor of MDR pumps as compared with...
DOX, MITOX, and other investigational anthracenediones (Chou et al., 2002; Cell Therapeutics Inc., 2012). It has been suggested that chemical modifications of the chromophore backbone limited PIX recognition by MDR proteins (Chou et al., 2002).

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Fig. 7. Myocardial cTnI release or mitochondrial aconitase inactivation. (A) and (B) cTnI release. (C) and (D) Mitochondrial aconitase inactivation. Experimental conditions were 1) no drug, 2) anthracenedione, 3) DOX loading, 4) DOX loading followed by anthracenedione, 5) DOX loading and multiple washouts, and 6) DOX loading and multiple washouts followed by anthracenedione. Where indicated, the strips were incubated with only 100 μM H2O2. Values are the mean ± S.E. of three experiments. (C) *P < 0.05 versus samples 1, 2, and 5 (one-way ANOVA followed by Bonferroni’s test for multiple comparisons); †P < 0.01 versus samples 3 and 6 (unpaired Student’s t test). (D) *P < 0.05 versus all other samples (one-way ANOVA followed by Bonferroni’s test for multiple comparisons). Prot., protein.

Fig. 8. Effects of anthracenediones on DOXOL formation in human myocardial strips. (A) DOX and DOXOL levels in the soluble fraction of strips exposed to DOX loading, DOX loading and washout, or DOX loading and multiple washouts. (B) After DOX loading and multiple washouts, DOXOL formation was significantly inhibited by the addition of PIX but not MITOX. Values are the mean ± S.E. of 7–12 experiments. *P < 0.05 versus all other samples (one-way ANOVA followed by Bonferroni’s test for multiple comparisons); †P < 0.05 for PIX versus MITOX (unpaired Student’s t test). (C) Isolated soluble fractions (0.6 mg prot./ml) were incubated with NADPH (0.25 mM) and DOX (10 μM) in Tris-HCl, pH 7.0, in the absence or presence of increasing concentrations of anthracenediones. After 4 hours at 37°C, DOXOL control values were 0.12 ± 0.02 nmol/mg prot. (n = 7 experiments in triplicate); the figure shows anthracenedione:DOX ratios that caused 50% inhibition of DOXOL formation (n = 3). *P < 0.05 versus MITOX (unpaired Student’s t test); **P < 0.05 versus MITOX, PIX, and CT-45886 (one-way ANOVA followed by Bonferroni’s test for multiple comparisons). (D) IC50 percentages attained by anthracenediones in the soluble fractions of strips after DOX loading or DOX loading/multiple washouts. Values are the mean ± S.E. of 3–4 experiments in triplicate. *P < 0.05 versus MITOX (unpaired Student’s t test); **P < 0.05 versus MITOX and PIX (one-way ANOVA followed by Bonferroni’s test for multiple comparisons). Prot., protein.
metabolites that reflected formation and utilization of H$_2$O$_2$ in MITOX synergized with DOX to accentuate H$_2$O$_2$ formation. It formed metabolites consistent with increased production for multiple comparisons). Nevertheless, a reduced inactivation, a highly sensitive marker of subthreshold conditions did not cause histologic damage of the strips. We reported that such patients treated with cumulative doses of DOX may develop circulating levels of cTnI (Cardinale and Sandri, 2010). Here, myocardial strips received a single, clinically relevant dose of DOX and/or anthracyclinedione, which was insufficient to induce cTnI release. We reported that such conditions did not cause histologic damage of the strips (Salvatorelli et al., 2006a, 2012a). Nonanthracycline chemotherapeutics may change DOXOL formation without altering the expression level of the reductases (Salvatorelli et al., 2006b). Drugs that occupy a positive regulatory site of the reductases increase DOXOL formation, whereas drugs that compete for the active site decrease DOXOL formation (Salvatorelli et al., 2006b). One-electron redox cycling of DOX and ROS formation is promoted by the NADH dehydrogenase of mitochondrial complex I, sarcoplasmic NADPH–cytochrome P450 reductase, NADPH oxidase, and other oxidoreductases (Doroshow, 1983; Powis, 1989; Vasquez-Vivar et al., 1997; Deng et al., 2007). The quinone moiety of MITOX is more electronegative than that of DOX ($\Delta E^{\#} = 0.79$ V versus $-0.6$ V) (Nguyen and Gutierrez, 1990). This makes MITOX a poor substrate for NAD(P)H oxidoreductases. Elimination of the electron-dense hydroquinone moiety may have rendered the quinone moiety of PIX even more electronegative and virtually resistant to one-electron reduction. On the other hand, the data suggest that a prior exposure of the strips to DOX enabled MITOX to engage in redox reactions. DOX complexation with mitochondrial cardiolipin, and changes in the architecture and redox potential of the mitochondrial electron transport system, may play a role in these settings (Salvatorelli et al., 2012a).

The lifetime risk of cardiotoxicity correlates with cardiac accumulation of DOXOL (Menna et al., 2008; Minotti et al., 2010; Blanco et al., 2012). In human myocardium, DOXOL is formed by heterogeneous families of cytoplasmic aldo-keto or carbonyl reductases (Mordente et al., 2003; Slupe et al., 2005; Salvatorelli et al., 2006a; Menna et al., 2008; Blanco et al., 2012). In human myocardium, DOXOL is accumulated in myocardial samples that mimicked prior damage from pathophysiologic levels of O$_2^+$ (Gardner, 2002; Salvatorelli et al., 2006a, 2012a). PIX did not increase aconitase inactivation in the strips loaded with DOX; in contrast, MITOX exacerbated inactivation induced by DOX loading and synergized with DOX in inducing inactivation in the strips subjected to DOX loading/multiple washouts. Peroxidatic MITOX metabolites might be electrophilic enough to substitute for O$_2^+$ in inactivating mitochondrial aconitase; however, this possibility should be weighed against complete elimination of such metabolites from the strips.

Fig. 9. Effects of PIX or CT-45886 on DOXOL formation in human myocardial strips. (A) DOXOL levels versus PIX or CT-45886 levels in the soluble fraction of strips sequentially exposed to DOX loading/multiple washouts and PIX. (B) DOXOL levels in strips sequentially exposed to DOX loading/multiple washouts and PIX or CT-45886 at 1 μM (n = 3). *P < 0.05 versus control (one-way ANOVA followed by Bonferroni’s t-test for multiple comparisons).

Neither PIX nor MITOX altered myocardial distribution or clearance of DOX; however, the two drugs were substantially different in terms of ROS formation. PIX did not increase the myocardial levels of DCF-detectable H$_2$O$_2$ and did not form metabolites that reflected formation and utilization of H$_2$O$_2$ in drug metabolism by cellular peroxidases. MITOX also failed to increase DCF-detectable H$_2$O$_2$, but in the presence of DOX, it formed metabolites consistent with increased production and utilization of H$_2$O$_2$ in peroxidatic drug metabolism, even in DOX loading/multiple washout experiments in which DOX levels were too low to form H$_2$O$_2$. These findings indicate that MITOX synergized with DOX to accentuate H$_2$O$_2$ formation.

Patients treated with cumulative doses of DOX may develop circulating levels of cTnI (Cardinale and Sandri, 2010). Here, myocardial strips received a single, clinically relevant dose of DOX and/or anthracyclinedione, which was insufficient to induce cTnI release. We reported that such conditions did not cause histologic damage of the strips (Salvatorelli et al., 2006a, 2012a). Nevertheless, a reduced cardiotoxicity of PIX versus MITOX in DOX-treated patients could be anticipated by measuring mitochondrial aconitase inactivation, a highly sensitive marker of subthreshold uptake would diminish if washouts caused DOX to diffuse and reorient in proximity to the membrane outer leaflet exposed to PIX. MITOX accumulation was not diminished by washouts. The higher lipophilicity of MITOX may have favored its penetration even in a membrane environment altered by DOX clearance.

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DOX administration, 2) lack of effects on the levels and distribution of residual DOX, 3) failure to generate ROS, and 4) inhibition of DOXOL formation. This information should be tempered by unavoidable study limitations. Months to years after the last anthracycline administration, progressive DOX clearance or conversion to DOXOL should generate roughly identical myocardial levels of DOX and DOXOL (Stewart et al., 1993); in contrast, strips subjected to DOX loading/multiple washouts still contained much more DOX than DOXOL. In addition, our model reproduces pharmacologic events associated with just one PX administration, whereas the cardiac toxicity of a drug is usually cumulative, increasing with additional doses or regimens. Multiple doses of DOX cause a mitochondrial cardiodytosis that results in ROS formation even after completion of chemotherapy (Lebrecht and Walker, 2007; Minotti et al., 2010); this cannot be characterized by our model. Nonetheless, our data show that PX was redox-inactive and inhibited DOXOL formation in the face of significant levels of residual DOX. MITOX did not inhibit DOXOL formation, but synergized with DOX to form more ROS.

These data provide a biologic rationale for the cardiac safety of PX in patients with prior DOX therapy. Moreover, it is known that, in cancer cells, the mode of action of PX was not confined to inhibiting topoisomerase II, but extended to forming PI-membrane conjugates that crosslinked to DNA much more efficaciously than equivalent DOX conjugates (Evison et al., 2009; Cell Therapeutics Inc., 2012). This anticipates that, in cancer cells altered by prior waves of DOX clearance, a limited uptake of PX would be outweighed by its greater potency in damaging DNA.

A lack of intramyocardial toxic bioactivation suggests that PX could also be of value as a first-line agent. In a phase II study that compared cyclophosphamide/DOX/vincristine/prednisone -rituximab with CP(pxantrone)/OP-rituximab in chemotherapy-naïve patients with diffuse large B-cell lymphoma, COPP-rituximab caused similar rates of progression-free survival but induced lower rates of serious cardiac events (Herbrecht et al., 2011). Interestingly, patients treated with COPP-rituximab were also shown to develop fewer troponin elevations over the course of therapy (Cell Therapeutics Inc., unpublished data). This denoted that, in patients exposed to cumulative doses of drugs, PX was safer than DOX and spared the heart from the summation of toxic insults. It will be important to establish whether PX also caused fewer elevations of natriuretic peptides that signal hemodynamic stress, cardiomyocyte stretch, and ventricle dilation or hypertrophy (Braunwald, 2008). This could not be established in our short-term studies of isolated myocardial samples. In conclusion, PX differs from both DOX and MITOX in a translational model of human myocardium. This information endorses the development of PX as a new agent for active and safe therapy for NHL.

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**Participated in research design:** Salvatorelli, Menna, Minotti.

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**Performed data analysis:** Salvatorelli, Menna, Minotti.

**Wrote or contributed to the writing of the manuscript:** Salvatorelli, Menna, Singer, Minotti.

**References**


