Pharmacokinetic Characterization of Amrubicin Cardiac Safety in an Ex Vivo Human Myocardial Strip Model. II. Amrubicin Shows Metabolic Advantages over Doxorubicin and Epirubicin

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Received November 26, 2011; accepted February 14, 2012

ABSTRACT
Anthracycline-related cardiotoxicity correlates with cardiac anthracycline accumulation and bioactivation to secondary alcohol metabolites or reactive oxygen species (ROS), such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂). We reported that in an ex vivo human myocardial strip model, 3 or 10 μM amrubicin [(7S,9S)-9-acetyl-9-amino-7-[2-deoxy-β-D-erythro-pentopyranosyl]oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-5,12-naphthacenedione hydrochloride] accumulated to a lower level compared with equimolar doxorubicin or epirubicin (J Pharmacol Exp Ther 341: 464–473, 2012). We have characterized how amrubicin converted to ROS or secondary alcohol metabolite in comparison with doxorubicin (that formed both toxic species) or epirubicin (that lacked ROS formation and showed an impaired conversion to alcohol metabolite). Amrubicin and doxorubicin partitioned to mitochondrial aconitase assays showed that 3 μM amrubicin caused an O₂⁻-dependent reversible inactivation, whereas doxorubicin always caused an irreversible inactivation. Low concentrations of amrubicin therefore proved similar to epirubicin in sparing mitochondrial aconitase from irreversible inactivation. The soluble fraction of human myocardial strips converted doxorubicin and epirubicin to secondary alcohol metabolites that irreversibly activated cytoplasmic aconitase; in contrast, strips exposed to amrubicin failed to generate its secondary alcohol metabolite, amrubicinol, and only occasionally exhibited an irreversible inactivation of cytoplasmic aconitase. This was caused by competing pathways that favored formation and completely eliminated 9-deaminoamrubicinol. These results characterize amrubicin metabolic advantages over doxorubicin and epirubicin, which may correlate with amrubicin cardiac safety in preclinical or clinical settings.

Introduction
Cardiotoxicity induced by doxorubicin and other antitumor anthracyclines correlates with anthracycline peak plasma concentration and accumulation in the heart; however, the risk of cardiotoxicity from a given anthracycline may increase if that anthracycline underwent one- or two-electron reduction of its quinone or carbonyl moieties.

One-electron reduction of the quinone moiety results in the formation of a semiquinone free radical that regenerates the parent quinone by oxidizing with molecular oxygen; one-electron redox cycling of the quinone moiety therefore accompanies formation of superoxide anion (O₂⁻) and its dismutation product, hydrogen peroxide (H₂O₂), which are members of the broad family of reactive oxygen species (ROS) that cause oxidative stress. Cardiomyocytes would be particularly vulnerable to ROS because they are ill equipped with ROS-detoxifying enzymes in comparison with other cell types (Gewirtz, 1999; Minotti et al., 2004a). Several oxido-reductases were shown to reduce anthracyclines to semiquinone free radicals under defined experimental conditions; these included sarcoplasmic NADPH-cytochrome P450 reductase and cytoplasmic xanthine oxidase (Powis, 1989), the reductase domain of endothelial nitric-oxide synthase (Vásquez-Vivar et al., 1997), and the multisubunit NADPH oxidase (Deng et al., 2007). On balance, however, more numerous lines of evidence suggest that anthracyclines would be re-
duced primarily by the NADH dehydrogenase of complex I of mitochondrial electron transport chain (Davies and Doroshow, 1986; Marcillat et al., 1989; Gille and Nohl, 1997; Wallace, 2003). Confocal microscopy studies confirmed that in isolated cardiomyocytes, mitochondria were the primary site of anthracycline-induced ROS formation (Salvatorelli et al., 2006).

Two-electron reduction of the side-chain carbonyl moiety results in the formation of anthracycline secondary alcohol metabolites that are less potent than their parent anthracyclines in forming ROS, but are more potent in inactivating Ca^{2+}-handling proteins of the contraction-relaxation cycle or key regulators of energy metabolism and redox balance (Minotti et al., 2004a, 2010). In the case of doxorubicin and epirubicin, secondary alcohol metabolites (doxorubicinol and epirubicinol) were also slightly but appreciably more polar than their parent drugs; accordingly, studies of postmortem or ex vivo human myocardial samples demonstrated that doxorubicinol and epirubicinol were poorly cleared from the heart and accumulated to form a long-lived toxic anthracycline reservoir (Stewart et al., 1993; Menna et al., 2008). Two-electron carbonyl reduction of anthracyline is catalyzed by heterogeneous superfamily of cytoplasmic, NADPH-dependent carbonyl reductases (EC 1.1.1.184) or aldo-keto reductases (EC 1.1.1.145–151) (Minotti et al., 2004b).

We previously characterized that, in comparison with doxorubicin or epirubicin, amrubicin [(7S,9S)-9-acetyl-9-aminoo-7-[(2-deoxy-β-D-erythro-pentopyranosyl)oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-5,12-naphthacenedione hydrochloride] exhibited a reduced accumulation in an ex vivo human myocardial strip model. Amrubicin myocardial accumulation was limited primarily by formation of amrubicin metabolites, such as 9-deaminoamrubicin and 9-deaminoamrubicinol, that diffused from the strips in plasma (Salvatorelli et al., 2012). These findings correlated with reports of amrubicin cardiac tolerability in preclinical models or in approved or investigational clinical settings that adopted cumulative doses of amrubicin in the treatment of refractory/relapsed non-small-cell lung carcinoma or small cell lung carcinoma (Kurata, 2009; Ogawara et al., 2010). However, a global preclinical assessment of the risk of cardiotoxicity associated with the clinical use of amrubicin requires that amrubicin conversion to ROS or secondary alcohol metabolite be investigated. In fact, multiple mechanisms of toxicity induced by ROS or secondary alcohol metabolites have been implicated to explain how anthracyclines may cause acute cardiotoxicity or chronic cardiomyopathy and congestive heart failure (CHF) (Gewirtz, 1999; Peng et al., 2005; Minotti et al., 2010).

Here, we characterized how amrubicin compared with doxorubicin or epirubicin in regard to formation of ROS or secondary alcohol metabolite in human myocardial strips.

Materials and Methods

Chemicals. Doxorubicin, doxorubicinol (C-13 dihydroxydoxorubicin), 7-deoxydoxorubicinolone, epirubicin, and epirubicinol (C-13 dihydroxyepirubicin) were obtained through the courtesy of Nerviano Medical Sciences (Milan, Italy). Amrubicin, amrubicinol (C-13 dihydroxyamrubicin), 9-deaminoamrubicin, and 7-deaminoamrubicinolone were provided by Celgene Corporation (Summit, NJ). 9-Deaminoamrubicinolone was identified by liquid chromatography-mass spectrometry as described previously (Salvatorelli et al., 2012). Dichlorofluorescin-diacetate (DCFH-DA) and dichlorofluorescein (DCF) were obtained from Invitrogen (Carlsbad, CA). Cystine and ferrous ammonium sulfate were obtained from Merck (Darmstadt, Germany). NADPH, cis-aconitate, and all other chemicals were obtained from Sigma-Aldrich (Milan, Italy).

Incubations of Human Myocardial Strips with Anthracyclines. Thin strips were prepared from myocardial samples that had been collected from the nonischemic right atrium of patients undergoing aorto-coronary bypass grafting precisely as described previously (Salvatorelli et al., 2012). The strips were incubated for 4 h in fresh human plasma that contained anthracyclines at the pharmacokinetically relevant concentrations of 3 or 10 μM. These standard 4-h incubations were adopted to obtain maximum anthracycline metabolism in equilibrium with anthracycline uptake and efflux from the strips (Salvatorelli et al., 2009). At the end of the incubations, the strips were processed by homogenization and ultracentrifugation to separate soluble or membrane fractions; aliquots of plasma (200 μl) were also taken and assayed for anthracyclines.

High-Performance Liquid Chromatography for Anthracyclines. Plasma and soluble or membrane fractions of human myocardial strips were extracted and assayed by high-performance liquid chromatography as described previously (Salvatorelli et al., 2012) with modifications. In the assays for doxorubicin and epirubicin the extracts were loaded onto a (250 × 4.6 mm, 5 μm) HxSil RP C-18 column (Hamilton Co., Reno, NV) operated at 25°C and eluted at the flow rate of 1 ml/min for a total 30-min run time [20-min linear gradient from 10 mM NaH2PO4, pH 4.0, to (50%–50%) CH3CN-10 mM NaH2PO4, followed by a 10-min isocratic elution with (50%–50%) CH3CN-10 mM NaH2PO4]. Doxorubicin(o) and epirubicin(o) were detected fluorimetrically (excitation at 480 nm/emission at 560 nm), identified by cochromatography with authentic standards, and quantified against appropriate standard curves (lowest detection limit usually between 0.001 and 0.002 μM for each analyte). Retention times were: 13.1 min (epirubicinol), 13.3 min (doxorubicinol), 14.4 min (doxorubicin), and 14.5 min (epirubicin). In the assays for amrubicin and amrubicinol, the column was eluted isocratically for 18 min with (75%–25%) 10 mM NaH2PO4, pH 4.0, CH3CN, followed by a 2-min linear gradient to (15%–85%) 10 mM NaH2PO4, pH 4.0, CH3CN. The latter was maintained isocratic for 2 more min, followed by a 5-min linear gradient back to (75%–25%) 10 mM NaH2PO4, pH 4.0, CH3CN, which was maintained isocratically for 4 min and then switched to a 15-min linear gradient to (15%–85%) 10 mM NaH2PO4, pH 4.0, CH3CN. The latter was maintained isocratically for 6 min followed by a 3-min linear gradient to 10 mM NaH2PO4, pH 4.0, which was maintained isocratically for 4 more min according to a chromatographic total run of 30 min. Amrubicin and amrubicinol were detected fluorimetrically (excitation at 465 nm/emission at 560 nm), identified by cochromatography with authentic standards, and quantified against appropriate standard curves. Retention times were 12.1 min (amrubicin) and 13.5 min (amrubicinol). In the assays for 7-deoxyamrubicinolone, 9-deaminoamrubicin, and 9-deaminoamrubicinol, the column was eluted with a 10-min linear gradient from 10 mM NaH2PO4, pH 4.0 to (50%–50%) 10 mM NaH2PO4, pH 4.0, CH3CN, which was maintained isocratically for 4 min and then switched to a 15-min linear gradient to (15%–85%) 10 mM NaH2PO4, pH 4.0, CH3CN. The latter was maintained isocratically for 6 min followed by a 3-min linear gradient to 10 mM NaH2PO4, pH 4.0, which was maintained isocratically for 4 more min according to a chromatographic total run of 30 min. Retention times were 12.6 min (7-deoxyamrubicinolone), 18.6 min (9-deaminoamrubicin), and 19.6 min (9-deaminoamrubicinol). Detection limits, coefficients of variations, and normalization of values to micromolar equivalents were as described previously (Salvatorelli et al., 2012).

High-Performance Liquid Chromatography for Dichlorofluorescein. In previous studies of cardiomyocytes exposed to anthracyclines, ROS formation was determined by the method based on the sequential uptake of DCFH-DA, its deacetylation to DCFH (deacetylated DCFH-DA) by cellular esterases, and the oxidation of DCFH to fluorescent DCF; the latter was detected by fluorescence microscopy or FACSan flow cytometry (Kalivendi et al., 2001; L’Ecuyer et al., 2004). Here, we adopted an high-performance liquid chromatography modification of the DCF assay (Salvatorelli et al., 2006, 2007). The strips were incubated in 4 ml of 50 mM phosphate
buffer/123 mM NaCl/5.6 mM glucose, pH 7.4, added with the membrane-permeable DCFH-DA. The latter was used at 50 µM to ensure that it distributed homogeneously to both soluble and membrane fractions of the strips (Salvatorelli et al., 2006). After 1 h in the dark, the strips were washed with 0.3 M NaCl and subjected to standard 4-h incubations in plasma with anthracyclines. At the end of incubations, the strips were homogenized in 1 ml of ice-cold 123 mM NaCl to which the antioxidant, 4-hydroxytempo (1 mM), had been added to prevent further oxidation of DCIF to DCF. Soluble and membrane fractions were extracted with 2 volumes of (1:1) chloroform/methanol (CHCl₃/CH₃OH), and 25 µl of the upper phase was loaded onto a (250 × 4.6 mm, 5 µm) HSi RP C-18 column (Hamilton Co.) operated at 25°C and eluted at the flow rate of 1 ml/min for a total 30-min run time (15-min linear gradient from 10 mM NaH₂PO₄, pH 4.0, to (50%–50%) CH₃CN-10 mM NaH₂PO₄, followed by a 15-min isocratic elution with (50%–50%) CH₃CN-10 mM NaH₂PO₄). The fluorescent peak of DCF (excitation at 488 nm/emission at 525 nm) was identified by cochromatography with an authentic standard (retention time 18.3 min) and quantified against a proper standard curve (lowest detection limit 0.001 µM).

Assignment of DCF to Hydrogen Peroxide. The DCF assay is used to monitor oxidation of DCFH by H₂O₂ and cellular peroxidases or trace amounts of iron (LeBel et al., 1992; Kalivendi et al., 2001); however, H₂O₂-independent DCFH oxidation and/or side reactions of DCF with cellular redox agents other than H₂O₂ have been described previously (LeBel et al., 1992; Rota et al., 1999). Here, as in previous studies, unambiguous identification of DCF as the product of H₂O₂-dependent DCFH oxidation was obtained by measuring, respectively, ~4- or 2.5-fold elevations of DCF in strips incubated for 4 h in plasma added with 100 µM amitriazole (which induces a mitochondrial leakage of H₂O₂ by inhibiting complex III in respiratory chain) or 50 mM aminotriazole (which reduces H₂O₂ decomposition by inhibiting catalasal) (Salvatorelli et al., 2006). To establish quantitative correlations between DCF and H₂O₂, we measured DCF in strips incubated for 15 min in 4 ml of 50 mM phosphate buffer/123 mM NaCl/5.6 mM glucose, pH 7.4, to which H₂O₂ was added at concentrations of 0.1 to 10 µM (Salvatorelli et al., 2006). DCF levels correlated linearly with the concentration of H₂O₂ delivered to the strips and were plotted against a standard curve of authentic DCF. On the basis of this procedure, we calculated that 1 nmol of H₂O₂ caused the formation of 0.43 ± 0.06 nmol of DCF/g of human myocardium (n = 5). This stoichiometry was routinely adopted to quantify H₂O₂ formation in myocardial strips incubated in plasma with anthracyclines. The values (nmol H₂O₂/g) were normalized to micromolar equivalents; anthracycline-induced H₂O₂ formation was expressed as nmol of H₂O₂ over basal H₂O₂ formation that occurred in strips incubated in plasma only.

Aconitase Assay. Inactivation of mitochondrial or cytoplasmic aconitase was used as a marker of formation and reactivity of O₂ or anthracycline secondary alcohol metabolites, respectively (Salvatorelli et al., 2006). Membrane and soluble fractions of myocardial strips were assayed for aconitase activity by monitoring the consumption of cis-aconitate at 240 nm (ε = 3.6 mM⁻¹·cm⁻¹) in a Hewlett Packard (Palo Alto, CA) 8453 spectrophotometer equipped with computer-assisted corrections for turbidity and scatter. The incubations (1 ml, final volume) contained 30 to 60 µg of protein and 0.1 mM cis-aconitate in 0.3M NaCl, pH 7.0, 37°C. One unit of aconitase activity was defined as the amount catalyzing the consumption of 1 µmol of cis-aconitate per minute. Where indicated, the proteins were preincubated for 5 min with cysteine and ferrous ammonium sulfate (1100 or 50 nmol/mg of protein, respectively) (Minotti et al., 2004b). This was done to reactivate aconitase that had been inactivated during standard 4-h incubations or sample homogenization and centrifugation.

Other Conditions and Assays. Anthracycline metabolism was reconstituted in isolated soluble fractions as described previously (Salvatorelli et al., 2012). Proteins were measured by the bicinchoninic acid method (Stoscheck, 1990). Cardiac troponin I (cTnI) was determined immunoenzymatically (Siemens Medical Diagnostics Solutions, Los Angeles, CA) in a Vista Dimension Analyzer (Dade Behring, Inc., Deerfield, IL). cTnI release was expressed as (100 × cTnI in plasma/cTnI in plasma + cTnI in the strips). In all of the experiments the values were means ± S.E. of at least three experiments. Data were analyzed by unpaired Student’s t test, and differences were considered significant when P was <0.05. Kinetic analyses and nonlinear fittings were obtained by Prism version 5 (GraphPad Software Inc., San Diego, CA). Other details are given in legends for figures and tables.

Results

Amrubicin-Dependent H₂O₂ Formation in Human Myocardial Strips. Human myocardial strips that had been loaded with DCFH-DA and then incubated in human plasma for 4 h were shown to form H₂O₂ (0.27 ± 0.03 µM; range 0.07–0.8; n = 16 triplicate experiments). In light of the low antioxidant defenses of the heart and of the different procedures adopted by other investigators in their studies of other cells, our value of 0.27 µM H₂O₂ compared reasonably well with the upper limit of the physiologic range of H₂O₂ concentrations (0.01–0.1 µM) in many tissues (Chance et al., 1979).

Strips exposed to 3 or 10 µM doxorubicin or amrubicin showed concentration-dependent comparable elevations of DCF-detectable H₂O₂, which localized only to the membrane fraction. Epirubicin did not induce a measurable increase of H₂O₂ at either 3 or 10 µM concentrations (Fig. 1). Lack of ROS formation by epirubicin was in agreement with previous reports where it was shown that epirubicin was sequestered in cytoplasmic acidic organelles (lysosomes, endosomes, and Golgi vesicles) and failed to partition toward mitochondrial sites of redox activation (Salvatorelli et al., 2006, 2007). Lack of anthracycline-induced ∆H₂O₂ in the soluble fraction probably denoted H₂O₂ detoxification through the pseudoperoxi-

Fig. 1. ROS formation in human myocardial strips exposed to anthracyclines at 3 µM (top) or 10 µM (bottom). Human myocardial strips were loaded with DCFH-DA and then incubated with anthracyclines at 3 or 10 µM as described under Materials and Methods. At the end of the incubations, membrane or soluble fractions of the strips were assayed for ROS by DCF-detectable H₂O₂ (± over basal levels). Values are means ± S.E. of three to five triplicate experiments.
The observation that amrubicin produced as much H$_2$O$_2$ as doxorubicin had to be reconciled with our previous demonstration that amrubicin attained very low steady-state levels in the membrane fraction of the strips compared with doxorubicin (Salvatorelli et al., 2012). We considered that ROS were produced by the amrubicin metabolite 9-deaminoamrubicin, which was formed in high amounts in the membrane fraction of the strips (Salvatorelli et al., 2012). To probe this possibility, we measured ROS and anthracyclines in membrane fractions of myocardial strips incubated in plasma to which 9-deaminoamrubicin had been added at concentrations of 3 or 10 µM. As shown in Table 1, 9-deaminoamrubicin diffused from plasma and accumulated in the membrane fraction of the strips in a concentration-dependent manner; however, there was no measurable $\Delta$H$_2$O$_2$ with 9-deaminoamrubicin at 3 µM and very little $\Delta$H$_2$O$_2$ with 9-deaminoamrubicin at 10 µM. Membrane fractions of strips exposed to 10 µM amrubicin showed 9-deaminoamrubicin levels that were similar to or lower than those attained by exogenously added 3 or 10 µM 9-deaminoamrubicin; nevertheless, these strips exhibited a sizeable $\Delta$H$_2$O$_2$. These results showed that ROS were formed by the limited pool of unmodified amrubicin that was recovered from the membrane fraction of strips (Table 1).

Further Characterization of H$_2$O$_2$ Formation. We considered that doxorubicin and amrubicin produced H$_2$O$_2$ by different mechanisms. We therefore measured 7-deoxyaglycone alcohol metabolites that originate from side reactions of semiquinone free radicals and fingerprint anthracycline redox cycling in mitochondria (Gille and Nohl, 1997). Strips exposed to amrubicin developed measurable levels of 7-deoxyamrubicinolone, the 7-deoxyaglycone alcohol metabolite of amrubicin (0.03 ± 0.01 or 0.07 ± 0.02 µM in strips exposed to 3 or 10 µM amrubicin, respectively; n = 12 or 9 triplicate experiments; P < 0.05 between the two groups). In contrast, doxorubicin did not generate its corresponding metabolite, 7-deoxydoxorubicinolone. These findings suggested that only amrubicin produced H$_2$O$_2$ by a typical reduction-oxidation cycle of the quinone moiety.

We next characterized how doxorubicin- or amrubicin-induced ∆H$_2$O$_2$ correlated with mitochondrial electron transport and its potential for reducing the anthracycline quinone. Basal levels of H$_2$O$_2$ were used as a surrogate marker of mitochondrial electron transport in the strips. Doxorubicin-induced ∆H$_2$O$_2$ did not correlate with but actually increased nonlinearly over the basal levels of H$_2$O$_2$. In contrast, amrubicin-induced ∆H$_2$O$_2$ correlated linearly with a broad range of basal levels of H$_2$O$_2$ and began increasing nonlinearly only in some strips incubated with amrubicin at 10 µM (Fig. 2). In light of this latter finding, we calculated the ratio of 7-deoxyamrubicinolone to ∆H$_2$O$_2$ as an index of H$_2$O$_2$ formation that was produced by amrubicin through the reduction-oxidation of its quinone. This ratio was 0.38 ± 0.06 at 3 µM amrubicin, but diminished to 0.16 ± 0.02 at 10 µM amrubicin (n = 9–12; P < 0.01).

Measuring 7-deoxyaglycone metabolites, and correlating anthracycline-induced ∆H$_2$O$_2$ with basal levels of H$_2$O$_2$, helped to demonstrate that 1) doxorubicin formed H$_2$O$_2$ by mechanisms other than oxidation-reduction of its quinone moiety, and 2) amrubicin formed H$_2$O$_2$ by quinone reduction-oxidation, but 10 µM amrubicin began also inducing quinone-independent mechanisms.

### Inactivation of Mitochondrial Aconitase. Mitochondrial aconitase rate limits the Kreb’s cycle by catalyzing reversible isomerization of citrate to isocitrate via cis-aconitate (Beinert and Kennedy, 1993); it works by virtue of a labile [4Fe–4S] cluster from which O$_2$ removes one iron atom much more specifically than does H$_2$O$_2$ (Hausladen and Fridovich, 1994). Iron removal is accompanied by aconitase inactivation, but enzyme activity can be rescued by reconstituting [4Fe–4S] clusters with iron under reducing conditions (Minotti et al., 2004b). Reversible inactivation of mitochondrial aconitase therefore serves as a marker of basal or anthracycline-augmented O$_2$ formation in mitochondria (Gardner et al., 1995; Salvatorelli et al., 2006). Membrane fractions of strips incubated with plasma only showed a basal aconitase activity that could be increased by reconstituting [4Fe–4S] clusters. Membrane fractions from strips exposed to anthracyclines at 3 or 10 µM exhibited lower basal levels of aconitase activity, presumably because both doxorubicin and epirubicin or amrubicin made the clusters more liable to nonspecific decay during strip homogenization and centrifugation (Minotti et al., 2004b). It is noteworthy, however, that

### Table 1

<table>
<thead>
<tr>
<th>Anthracycline in Plasma</th>
<th>Anthracycline in Membrane Fraction</th>
<th>$\Delta$H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9-Deaminoamrubicin</td>
<td>Amrubicin</td>
</tr>
<tr>
<td>9-Deaminoamrubicin, 3 µM</td>
<td>1.3 ± 0.3</td>
<td>N.D.</td>
</tr>
<tr>
<td>9-Deaminoamrubicin, 10 µM</td>
<td>2.1 ± 0.2*</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Amrubicin, 10 µM</td>
<td>1.4 ± 0.1**</td>
<td>2.9 ± 0.9</td>
</tr>
</tbody>
</table>

N.D., not detected.

* 9-Deaminoamrubicin significantly higher at 10 vs. 3 µM (P < 0.01).

** 9-Deaminoamrubicin significantly lower in strips incubated with 10 µM amrubicin compared with strips incubated with 10 µM 9-deaminoamrubicin (P < 0.01).

![Fig. 2. $\Delta$H$_2$O$_2$ versus basal H$_2$O$_2$ in human myocardial strips exposed to doxorubicin or epirubicin at 3 or 10 µM. ∆H$_2$O$_2$ induced by anthracyclines at 3 or 10 µM in a given myocardial strip was plotted against the basal level of H$_2$O$_2$ formation in that strip. See Results for explanation.](image-url)
treatment with iron under reducing conditions caused aconitase reactivation effects that depended on the anthracycline analog and its concentration. In experiments with anthracyclines at 3 \( \mu \text{M} \), doxorubicin samples lacked aconitase reactivation, whereas epirubicin or amrubicin samples showed an aconitase reactivation that nearly approached that of reactivated control samples. In experiments with anthracyclines at 10 \( \mu \text{M} \), only epirubicin samples showed an aconitase reactivation similar to that of reactivated control samples (Table 2). Thus, amrubicin caused a pattern of aconitase inactivation that was similar to epirubicin at 3 \( \mu \text{M} \) or doxorubicin at 10 \( \mu \text{M} \).

**Lack of Conversion of Amrubicin to Amrubcinol in Human Myocardial Strips.** The soluble fraction of myocardial strips exposed to doxorubicin or epirubicin at 3 or 10 \( \mu \text{M} \) developed levels of secondary alcohol metabolites in a concentration-dependent manner. Epirubicin formed less alcohol metabolite than doxorubicin at both 3 and 10 \( \mu \text{M} \) anthracycline, which was in agreement with our previous demonstration of a moderate resistance of epirubicin to cytoplasmic two-electron reductases (Salvatorelli et al., 2007). Strips incubated with 3 or 10 \( \mu \text{M} \) amrubicin did not develop measurable levels of its secondary alcohol metabolite, amrubcinol (Fig. 3). The apparent lack of amrubcinol formation could not be attributed to its diffusion from the soluble fraction toward membranes or plasma; in fact, neither the membrane fraction of strips nor plasma contained measurable levels of amrubcinol (Fig. 3).

**Determinants of the Lack of Amrubcinol Formation in Human Myocardial Strips.** We attributed the lack of amrubcinol formation to the very low levels attained by amrubicin in the soluble fraction of the strips in comparison with doxorubicin or epirubicin (Salvatorelli et al., 2012). However, separate analyses of strips exposed to anthracyclines at 3 or 10 \( \mu \text{M} \) showed that at both concentrations there were a few strips in which the levels attained by amrubcin in the soluble fraction were similar to those attained by doxorubicin or epirubicin (Fig. 4). This suggested that other factors contributed to limiting amrubcinol conversion to amrubcinol.

We next considered that amrubcin was intrinsically resistant to cytoplasmic reductases; therefore, we measured alcohol metabolite formation in NADPH-supplemented isolated soluble fractions to which anthracycline concentrations of 5 or 10 \( \mu \text{M} \) had been added. Under such defined conditions, alcohol metabolites were formed in a concentration-dependent manner. Epirubicinol formation remained lower than doxorubicinol formation at both 5 or 10 \( \mu \text{M} \) anthracycline; it is noteworthy, however, that amrubcinol formation was similar to epirubicinol formation at both 5 or 10 \( \mu \text{M} \) anthracycline (Table 3). These results indicated that amrubcin was not fully resistant to cytoplasmic reductases; hence, some amrubcinol should have been measured in those strips whose soluble fraction contained as much amrubcin as doxorubicin or epirubicin.

We eventually considered that amrubcinol formation could be precluded by amrubcin metabolites that competed with unmodified amrubcin for reduction by cytoplasmic reductases. We focused on 9-deaminoamrubcin, which was formed in the membrane fraction of the strips but diffused in the soluble fraction and then in plasma (Salvatorelli et al., 2012). Studies of isolated soluble fractions incubated with

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**TABLE 2**

Basal and reactivated mitochondrial aconitase in membrane fractions of human myocardial strips exposed to anthracyclines

<table>
<thead>
<tr>
<th>Aconitase</th>
<th>Control</th>
<th>Anthracycline</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>Doxorubicin</td>
<td>Epirubicin</td>
<td>Amrubcin</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.41 ± 0.02</td>
<td>0.29 ± 0.04</td>
<td>0.31 ± 0.01</td>
<td>0.33 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Reactivated</td>
<td>0.78 ± 0.09*</td>
<td>0.30 ± 0.08</td>
<td>0.50 ± 0.06*</td>
<td>0.51 ± 0.06*</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.39 ± 0.03</td>
<td>0.26 ± 0.05</td>
<td>0.36 ± 0.09</td>
<td>0.30 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Reactivated</td>
<td>0.74 ± 0.08*</td>
<td>0.31 ± 0.07</td>
<td>0.68 ± 0.11*</td>
<td>0.43 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

* Reactivated aconitase significantly higher (\( P < 0.01 \)) than basal aconitase.
doxorubicin  ε  epirubicin  ε  amrubicin

Fig. 4. Secondary alcohol metabolites versus parent anthracyclines in the soluble fraction of human myocardial strips incubated with anthracyclines at 3 μM (top) or 10 μM (bottom). Levels of secondary alcohol metabolites in the soluble fraction of human myocardial strips were plotted against levels of parent anthracyclines in the same fraction. Boxes delimit samples in which the soluble fraction of myocardial strips contained similar levels of doxorubicin, epirubicin, or amrubicin.

Table 3

Anthraclyline secondary alcohol metabolite formation in isolated soluble fractions of human myocardial strips

<table>
<thead>
<tr>
<th>Anthracycline</th>
<th>Alcohol Metabolite</th>
<th>5 μM</th>
<th>10 μM</th>
</tr>
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<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.044 ± 0.007</td>
<td>0.083 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>Epirubicin</td>
<td>0.007 ± 0.001*</td>
<td>0.020 ± 0.007*</td>
<td></td>
</tr>
<tr>
<td>Amrubicin</td>
<td>0.004 ± 0.001*</td>
<td>0.015 ± 0.003*</td>
<td></td>
</tr>
</tbody>
</table>

* Epirubicinol and amrubicinol significantly lower than doxorubicinol (P < 0.001).

NADPH and amrubicin or 9-deaminoamrubicin showed that the levels of formation of 9-deaminoamrubicinol were many times higher than those of amrubicinol (Fig. 5). The dependence of 9-deaminoamrubicinol formation on 9-deaminoamrubicin concentration followed the bell-shaped pattern that we previously attributed to substrate and/or product inhibition (Salvatorelli et al., 2012). By extrapolating data from the ascending portion of the plot of 9-deaminoamrubicinol versus 9-deaminoamrubicin, the apparent $K_m$ of 9-deaminoamrubicin for cytoplasmic reductases was approximated to $\approx 10$ μM; in contrast, the apparent $K_m$ of amrubicin was approximated to $\approx 100$ μM. Cumulative analysis of strips exposed to amrubicin at 3 or 10 μM showed that at any given level of myocardial anthracycline bioavailability, the amount of 9-deaminoamrubicin that accumulated in the soluble fraction before diffusing in plasma was one or more orders of magnitude higher than unmodified amrubicin (Fig. 6A).

Fig. 5. Reduction of amrubicin or 9-deaminoamrubicin to secondary alcohol metabolite in isolated soluble fractions of human myocardial strips. Incubations (0.25 ml, final volume) contained isolated soluble fractions of human myocardial strips (0.6 mg of protein/ml), NADPH (0.25 mM), and increasing concentrations of amrubicin or 9-deaminoamrubicin in 0.3 M NaCl, pH 7.0. After 4 h at 37°C, incubations were extracted and assayed for amrubicinol or 9-deaminoamrubicinol as described under Materials and Methods. The values were taken from representative titrations.

Fig. 6. Levels of amrubicin, 9-deaminoamrubicin, and secondary alcohol metabolites in human myocardial strips exposed to amrubicin at 3 or 10 μM. Human myocardial strips were subjected to standard 4-h incubations with amrubicin at 3 μM (open symbols) or 10 μM (solid symbols). Amrubicin bioavailability was calculated as the net amount of amrubicin that had been incorporated in myocardial strips (amrubicin in strips + 9-deaminated metabolites in strips + 9-deaminated metabolites that diffused from strips in plasma). A, the soluble fraction of strips was assayed for amrubicin (squares) and 9-deaminoamrubicin (circles); the latter was added with 9-deaminoamrubicin that had diffused from the soluble fraction in plasma. B, the soluble fraction was assayed for amrubicinol (squares) and 9-deaminoamrubicinol (circles); the latter was added with 9-deaminoamrubicinol that had diffused from the soluble fraction in plasma.
Bioavailability indicated the net amount of amrubicin that had been incorporated in myocardial strips (amrubicin in strips + metabolites in strips + metabolites that diffused from strips in plasma). It follows that at any given level of amrubicin bioavailability a combination of higher concentration and lower $K_m$ rendered 9-deaminoamrubicin the preferred substrate for cytoplasmic reductases, making the strips generate 9-deaminoamrubicinol but not amrubicinol. As reported previously (Salvatorelli et al., 2012), 9-deaminoamrubicinol, too, diffused from the soluble fraction of strips in plasma. The actual levels of 9-deaminoamrubicinol formation were therefore calculated by adding 9-deaminoamrubicinol in the soluble fraction with 9-deaminoamrubicinol in plasma. Under such defined conditions, the pattern of formation of 9-deaminoamrubicinol in the strips followed the same bell-shaped pattern as that characterized in isolated soluble fractions (Fig. 6B). Control experiments, performed by incubating isolated soluble fractions with NADPH and amrubicin, ruled out the possibility that 9-deaminoamrubicinol could be formed through deamination of amrubicin.

**Inactivation of Cytoplasmic Aconitase.** Similar to what was reported for mitochondrial aconitase, the highly homologous cytoplasmic aconitase works by virtue of a labile [4Fe-4S] cluster (Beinert and Kennedy, 1993). Anthracyclines slowly remove iron from [4Fe-4S] clusters (Brazzolotto et al., 2003), whereas secondary alcohol metabolites irreversibly inactivate cytoplasmic aconitase by inducing mechanisms of cluster disassembly that preclude cluster reassembly (Minotti et al., 1998). Even trace amounts of secondary alcohol metabolites proved able to attack and inactivate cytoplasmic aconitase (Sacco et al., 2003). We therefore measured cytoplasmic aconitase activity in soluble fractions of strips that had been incubated with doxorubicin, epirubicin, or amrubicin at 3 μM. Soluble fractions from strips incubated with plasma only showed a basal level of aconitase activity that could be increased by reconstituting [4Fe-4S] clusters. After reactivation, cytoplasmic aconitase activity remained ~76% lower than mitochondrial aconitase activity, which was in keeping with the high density of mitochondria in the heart (Salvatorelli et al., 2006). Compared with control samples, the soluble fractions from strips exposed to anthracyclines showed a lower basal level of aconitase activity. After reconstitution with iron under reducing conditions, doxorubicin and epirubicin samples lacked aconitase reactivation, whereas amrubicin samples showed a measurable, but statistically, insignificant trend toward aconitase reactivation (Table 4).

Having considered that amrubicin lacked amrubicinol formation in myocardial strips, failure to reactivate cytoplasmic aconitase in amrubicin-treated samples required further insight. We considered that in the strips exposed to amrubicin cytoplasmic aconitase could be irreversibly inactivated by 9-deaminoamrubicinol; in experiments with amrubicin at 3 μM there were in fact a few strips that showed an incomplete 9-deaminoamrubicinol clearance (Salvatorelli et al., 2012). Samples that showed incomplete or complete 9-deaminoamrubicinol clearance were therefore examined separately. We found that samples with an incomplete clearance exhibited the lowest level of aconitase reactivation, whereas samples with a complete clearance showed the highest level of aconitase reactivation. In the latter samples, the magnitude of aconitase reactivation compared with that of strips exposed to amrubicin at 10 μM, which in fact exhibited a complete clearance of 9-deaminoamrubicinol (Fig. 7).

**Cardiac Troponin I Release.** Increased plasma levels of cardiac TnI have been measured in patients who developed cardiotoxicity from antitumor drugs (Cardinale and Sandri, 2010). At the end of standard 4-h incubations with anthracyclines at 10 μM, there was little or no cTnI release from myocardial strips in plasma (<2.5% of total myocardial cTnI). An extensive cTnI release (~35%) occurred when the strips were challenged with 100 μM H$_2$O$_2$, regardless of whether the catalase inhibitor, aminotriazole, was included to diminish H$_2$O$_2$ detoxification (data not shown). These results denoted that human myocardial strips were vulnerable by a robust oxidant stress that exceeded defense mechanisms; however, the anthracycline concentrations adopted in this study did not cause significant damage to human myocardial strips.

**Discussion**

We characterized amrubicin bioactivation to ROS and secondary alcohol metabolites in an ex vivo human myocardial strip model. Doxorubicin was used as a comparator that formed both ROS and alcohol metabolite; epirubicin was used as a comparator that exhibited varying levels of defective conversion to ROS or alcohol metabolite.

In comparison with doxorubicin, amrubicin attained much lower levels in the membrane fraction but produced comparable amounts of H$_2$O$_2$. The unusual behavior of amrubicin did not reflect membrane formation and redox cycling of the more abundant metabolite, 9-deaminoamrubicin. By measuring stable end products of semiquinone free radicals (7-deoxyxaglycone metabolites) and correlating anthracycline-induced ΔH$_2$O$_2$ with the basal levels of H$_2$O$_2$ in the strips, we characterized that amrubicin and doxorubicin produced H$_2$O$_2$ by different mechanisms. Dxorubicin acted by redox cycling-independent mechanisms; in contrast, amrubicin acted by a typical redox cycling mechanism, although redox cycling-independent H$_2$O$_2$ formation began occurring as amrubicin concentration was raised from 3 to 10 μM.

**Table 4**
Basal and reactivated cytoplasmic aconitase in soluble fractions of human myocardial strips exposed to anthracyclines

<table>
<thead>
<tr>
<th>Anthracycline</th>
<th>Control</th>
<th>Reactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.23 ± 0.01</td>
<td>0.017 ± 0.01</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>0.015 ± 0.01</td>
<td>0.017 ± 0.01</td>
</tr>
<tr>
<td>Amrubicin</td>
<td>0.023 ± 0.005</td>
<td>0.023 ± 0.005</td>
</tr>
</tbody>
</table>

* Reactivated aconitase significantly higher than basal aconitase ($P < 0.001$).
Redox cycling-independent mechanisms of $\text{H}_2\text{O}_2$ formation rest with the formation of complexes between anthracyclines and the mitochondrial phospholipid cardiolipin. These complexes cause functional uncoupling of the mitochondrial electron transport system at the level of complexes I, III, and IV, eventually inducing a passive leakage of reducing equivalents toward molecular oxygen (Marcillat et al., 1989; Simunek et al., 2009). It is worth noting that cardiolipin deteriorates under ischemic conditions (Sparagna and Lesnefsky, 2009); in the present study, however, myocardial samples had been collected under nonischemic conditions and, hence, we were in a favorable position to unravel processes that depended on doxorubicin-cardiolipin interactions.

Binding to cardiolipin and membrane chaotropic effects are more likely to occur when anthracyclines attained high levels in mitochondria and showed amphiphilic enough to engage in electrostatic interactions with cardiolipin (Marcillat et al., 1989; Goormaghtig et al., 1990). Doxorubicin exhibited these characteristics (Xu et al., 2011; Salvatorelli et al., 2012). Amrubicin attained much lower levels in the membrane and was considerably less polar than doxorubicin (Salvatorelli et al., 2012); therefore, amrubicin had more chances to produce $\text{H}_2\text{O}_2$ through a canonical redox cycling mechanism.

The different behaviors of doxorubicin and amrubicin were also characterized by measuring reversible inactivation of mitochondrial aconitase as a marker of anthracycline mitochondrial localization and $\text{O}_2^-$ formation. Our data show that 3 or 10 $\mu$M doxorubicin always caused irreversible rather than reversible inactivation of mitochondrial aconitase. This observation lends support to the concept that doxorubicin caused membrane effects that favored other mechanisms of ROS formation and aconitase inactivation. In contrast, 3 $\mu$M amrubicin caused reversible aconitase inactivation that was consistent with canonical mechanisms of $\text{O}_2^-$ formation and reactivity. Irreversible aconitase inactivation only occurred when amrubicin was used at 10 $\mu$M, a concentration level at which amrubicin began inducing redox cycling-independent processes. Thus, both doxorubicin and amrubicin localized to mitochondria, but there were conditions in which amrubicin could generate ROS in a more tolerable manner than did doxorubicin. In the experiments with anthracyclines at 3 $\mu$M, mitochondrial aconitase could be reactivated equally well in amrubicin and epirubicin samples, despite the fact that only amrubicin formed ROS in these samples.

The soluble fraction of myocardial strips exposed to doxorubicin or epirubicin developed measurable levels of doxorubicinol and, to a lesser extent, epirubicinol; in contrast, strips exposed to amrubicin never developed measurable levels of amrubicinol. This latter finding was not caused by amrubcinol diffusing from strips in plasma, nor was it caused by amrubicin showing a complete resistance to side-chain carbonyl reduction. Studies with isolated soluble fractions demonstrate that in comparison with doxorubicin, amrubicin exhibited the same moderate resistance to carbonyl reduction as that of epirubicin; moreover, the apparent $K_m$ of amrubcin for cytoplasmic reductases was approximated to $>100$ $\mu$M, which was also similar to epirubicin as opposed to a doxorubicin apparent $K_m$ of $<100$ $\mu$M (Salvatorelli et al., 2007). Our data suggest that amrubicinol formation was precluded by competing pathways that favored 9-deaminoamrubcinol formation. Based on our previous studies of amrubicin deamination in the membrane fraction of the strips (Salvatorelli et al., 2012), we calculate that the apparent $K_m$ of amrubcin for carbon-nitrogen lyases was significantly lower than its apparent $K_m$ for cytoplasmic reductases ($\approx 50$ versus $>100$ $\mu$M). Here, we have shown that in diffusing from the membrane fraction to the soluble fraction, 9-deaminoamrubcin exceeded the levels of amrubcin in that fraction and reacted with cytoplasmic reductases according to an apparent $K_m$ as low as $<10$ $\mu$M. Thus, efficient amrubcin deamination and 9-deaminoamrubcinol formation outcompeted amrubcinol formation.

Differences in alcohol metabolite formation were reflected in different patterns of cytoplasmic aconitase inactivation by doxorubicin, epirubicin, or amrubicin. In strips exposed to doxorubicin or epirubicin at 3 $\mu$M, doxorubicinol or epirubicinol caused irreversible inactivation of cytoplasmic aconitase; in strips exposed to amrubicin, irreversible inactivation of cytoplasmic aconitase was confined to those few samples that showed an incomplete clearance of 9-deaminoamrubcinol. Amrubicin metabolism therefore spared cytoplasmic aconitase in the majority of experimental conditions. Based on our previous characterization of limited amrubicin accumulation in human myocardial strips (Salvatorelli et al., 2012), it can now be said that formation of diffusible 9-deaminoamrubcinol(ol) induced the dual effect of improving amrubcinol clearance while also sparing possible targets of amrubcinol toxicity.

Inactivation of mitochondrial or cytoplasmic aconitase was reported to dysregulate energy metabolism, iron homeostasis, and redox balance (Narhar et al., 2000; Minotti et al., 2004a), eventually inducing tissue damage in a number of pathologic conditions (Recalcati et al., 2010). Here, reversible or irreversible inactivation of the two aconitas was used as a surrogate marker of formation and reactivity of anthracycline-derived $\text{O}_2^-$ or secondary alcohol metabolites, but strips exposed to anthracyclines at 3 or 10 $\mu$M did not release cTnI during the course of the experiments. Troponin release only occurred when the strips were exposed to 100 $\mu$M $\text{H}_2\text{O}_2$, which was $>2$ orders of magnitude higher than basal or anthracycline-induced $\text{H}_2\text{O}_2$ levels in the strips. Similar observations were done with myoglobin release (data not shown). In developing the human myocardial strip model adopted in this study, we never obtained convincing evidence...
that ≤10 μM doxorubicin or other anthracyclines caused histologic damage compared with control strips; myocardial damage only occurred when anthracyclines were used at 100 μM (Cartoni et al., 2004; Salvatorelli et al., 2006). Collectively, these findings confirmed that our model was tailored to characterizing authentic anthracycline pharmacokinetics without confounding factors and artifacts caused by a concomitant tissue damage (Salvatorelli et al., 2012).

The metabolic fate of amrubicin in cardiomyocytes seems to be both different from and similar to that characterized in tumor cells. Our data show that human myocardial strips failed to generate amrubcinol, but previous preclinical studies showed that amrubcinol formed abundantly in tumor cells and proved to be both different from and similar to that characterized in tumor cells (Yamaoka et al., 1998). Having said that the mechanisms and relative abundance of amrubcinol deamination in the heart or tumors will require ad hoc investigations, these facts indicate that 9-deaminoamrubcin may represent a common metabolic determinant of amrubcin detoxification or inactivation.

Attempts to incorporate ROS and secondary alcohol metabolites in a unifying picture of cardiotoxicity suggested that ROS might induce transient arrhythmias or blood pressure disorders during the course of chemotherapy; in contrast, long-lived secondary alcohol metabolites were implicated to cause cardiomyopathy and CHF that may surfaces anytime after completing chemotherapy (Carver et al., 2007; Gianni et al., 2008; Minotti et al., 2010). The reduced cardiotoxicity of epirubicin in comparison with doxorubicin was therefore attributed to its defective conversion to ROS or epirubicinol in human myocardium. In particular, a defective conversion of epirubicin to the longer lived epirubicinol was implicated to explain the reduced risk of delayed CHF from cumulative doses of epirubicin (Minotti et al., 2010; Menna et al., 2012).

The cardiac safety of epirubicin was nonetheless questioned by reports that reviewed data from very large cohorts of patients (Ryberg et al., 2008; van Dalen et al., 2010). These cautionary reports raise the possibility that in certain vulnerable individuals the risk of cardiotoxicity would be determined primarily by the high levels of epirubicin uptake and retention in the heart (Salvatorelli et al., 2009, 2012). The search for active but tolerable anthracyclines therefore should be directed toward analogs that exhibit both a reduced accumulation and a less damaging profile of bioactivation in the heart. Amrubcin seems to meet both such requirements.

In conclusion, the pharmacokinetic and biotransformation characteristics of amrubcin, as described here and in Salvatorelli et al. (2012), provide mechanistic explanations supporting the lack of cardiotoxicity in preclinical models and clinical trials. Amrubcin seems to offer safety advantages over older anthracycline analogs and is worthy of further study in malignant clinical indications where cumulative dose restrictions may limit efficacy.

Authorship Contributions
Participated in research design: Salvatorelli, Menna, Surapaneni, Aukenman, Sung, and Minotti.

Conducted experiments: Salvatorelli, Menna, and Gonzalez Paz.

Contributed new reagents or analytic tools: Menna, Chello, and Covino.

Performed data analysis: Salvatorelli, Menna, and Minotti.

Wrote or contributed to the writing of the manuscript: Salvatorelli, Sung, and Minotti.

References


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