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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NONSTANDARD ABBREVIATIONS: 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-napthacenedione hydrochloride; \( C_{\text{max}} \), peak plasma concentration; \( \text{O}_2^- \), superoxide anion; \( \text{H}_2\text{O}_2 \), hydrogen peroxide; ROS, Reactive Oxygen Species; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; CHF, congestive heart failure; DCFH-DA, dichlorofluorescin-diacetate; DCF, dichlorofluorescein; HPLC, high performance liquid chromatography; cTnI, cardiac troponin I.

RECOMMENDED SECTION ASSIGNMENT: Metabolism, transport, and pharmacogenomics.
ABSTRACT

Anthracycline-related cardiotoxicity correlates with cardiac anthracycline accumulation and bioactivation to secondary alcohol metabolites or Reactive Oxygen Species (ROS), like superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). We reported that in an ex vivo human myocardial strip model, 3 or 10 µM amrubicin accumulated to a lower level compared to equimolar doxorubicin or epirubicin (Salvatorelli et al., accompanying manuscript). Here, we 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 mitochondria and caused similar elevations of H$_2$O$_2$, but the mechanisms of H$_2$O$_2$ formation were different. Amrubicin produced H$_2$O$_2$ by enzymatic reduction-oxidation of its quinone moiety, while doxorubicin acted by inducing mitochondrial uncoupling. Moreover, mitochondrial aconitase assays showed that 3 µM amrubicin caused an O$_2^-$ dependent reversible inactivation, while 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 inactivated 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 favoured formation and complete or near-to-complete elimination of 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 plasma $C_{\text{max}}$ 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 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 with formation of superoxide anion ($O_2^-$) and its dismutation product, hydrogen peroxide ($H_2O_2$), members of the broad family of Reactive Oxygen Species (ROS) that cause oxidative stress. Cardiomyocytes would be particularly vulnerable by ROS as they are ill-equipped with ROS detoxifying enzymes in comparison to 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 (Vasquez-Vivar et al., 1997), the multi-subunit NADPH oxidase (Deng et al., 2007). On balance, however, more numerous lines of evidence suggest that anthracyclines would be reduced primarily by the NADH dehydrogenase of Complex I of mitochondrial electron transport chain (Davies and Doroshow, 1986; Gille and Nohl, 1997; Marcillat et al., 1989; 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 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; Minotti et al., 2010). In the case of doxorubicin and epirubicin, secondary alcohol metabolites
(doxorubicinol, epirubicinol) were also slightly but appreciably more polar than their parent drugs; accordingly, studies of post-mortem 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 (Menna et al., 2008; Stewart et al., 1993). Two-electron carbonyl reduction of anthracycline is catalyzed by heterogeneous superfamilies 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 to doxorubicin or epirubicin, amrubicin exhibited a reduced accumulation in an ex vivo human myocardial strip model. Amrubicin myocardial accumulation was limited primarily by formation of amrubicin metabolites, like 9-deaminoamrubicin and 9-deaminoamrubicinol, that diffused from the strips in plasma (Salvatorelli et al., accompanying manuscript). 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 (NSCLC) or small cell lung carcinoma (SCLC) (Ogawara et al., 2010; Kurata, 2009). However, a global preclinical assessment of the risk of cardiotoxicity associated with 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; Minotti et al., 2010; Peng et al., 2005).

Here, we characterized how amrubicin compared to 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-deoxyamrubicinolone, were provided by Celgene Corporation (Summit, NJ). 9-Deaminoamrubicinol was identified by liquid chromatography-mass spectroscopy, as described (Salvatorelli et al., accompanying manuscript). Dichlorofluorescin-diacetate (DCFH-DA) and dichlorofluorescein (DCF) were obtained from Molecular Probes, Eugene, OR. Cysteine, 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 (Salvatorelli et al., accompanying manuscript). The strips were incubated for 4h in fresh human plasma that contained anthracyclines at the pharmacokinetically relevant concentrations of 3 or 10 µM. These standard 4h 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 HPLC as described (Salvatorelli et al., accompanying paper), with modifications. In the assays for doxorubicin and epirubicin the extracts were loaded onto a (250 x 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% \( \text{CH}_3\text{CN}-10 \text{ mM NaH}_2\text{PO}_4 \), followed by a 10-min isocratic elution with (50%-50%) \( \text{CH}_3\text{CN}-10 \text{ mM NaH}_2\text{PO}_4 \). Doxorubicin(ol) and epirubicin(ol) were detected fluorimetrically (excitation at 480 nm/emission at 560 nm), identified by co-chromatography with authentic standards, and quantified against appropriate standard curves (lowest detection limit usually between 0.001 and 0.002 \( \mu \text{M} \) for each analyte). Retention times (min) were: 13.1 (epirubicinol), 13.3 (doxorubicinol), 14.4 (doxorubicin), 14.5 (epirubicin). In the assays for amrubucin and amrubucinol, the column was eluted isocratically for 18 min with (75%-25%) 10 mM NaH\text{2PO}_4, pH 4.0-\text{CH}_3\text{CN}, followed by a 2 min linear gradient to (15%-85%) 10 mM NaH\text{2PO}_4, pH 4.0-\text{CH}_3\text{CN}. The latter was maintained isocratic for 2 more min, followed by a 5 min linear gradient back to (75%-25%) 10 mM NaH\text{2PO}_4, pH 4.0-\text{CH}_3\text{CN}, which was maintained isocratic for 3 more min according to a chromatographic total run of 30 min. Amrubucin and amrubucinol were detected fluorimetrically (excitation at 465 nm/emission at 560 nm), identified by co-chromatography with authentic standards, and quantified against appropriate standard curves. Retention times (min) were 12.1 (amrubucin) and 13.8 (amrubucinol). In the assays for 7-deoxyamrubucinolone, 9-deaminoamrubucin, and 9-deaminoamrubucinol, the column was eluted with a 10 min linear gradient from 10 mM NaH\text{2PO}_4, pH 4.0 to (50%-50%) 10 mM NaH\text{2PO}_4, pH 4.0-\text{CH}_3\text{CN}, which was maintained isocratic for 4 min and then switched to a 3 min linear gradient to (15%-85%) 10 mM NaH\text{2PO}_4, pH 4.0-\text{CH}_3\text{CN}. The latter was maintained isocratic for 6 min followed by a 3 min linear gradient to 10 mM NaH\text{2PO}_4, pH 4.0, which was maintained isocratic for 4 more min according to a chromatographic total run of 30 min. Retention times (min) were 12.6 (7-deoxyamrubucinolone), 18.6 (9-deaminoamrubucinol), 19.6 min (9-deaminoamrubucin). Detection limits, coefficients of variations, and normalization of values to micromolar equivalents, were as described (Salvatorelli et al., accompanying manuscript).

**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 by cellular esterases, and the oxidation of DCFH to fluorescent DCF; the latter was detected by fluorescence microscopy or FACScan flow cytometry (Kalivendi et al., 2001; L’Ecuyer et al., 2004). Here, we adopted an HPLC modification of the DCF assay (Salvatorelli et al., 2006; Salvatorelli et
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 50 μM to ensure that DCFH distributed homogeneously to both soluble and membrane fractions of the strips (Salvatorelli et al., 2006). After 1h in the dark, the strips were washed with 0.3 M NaCl and subjected to standard 4h 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 DCFH to DCF. Soluble and membrane fractions were extracted with 2 volumes of (1:1) chloroform:methanol (CH₃OH-CHCl₃) and 25 μl of the upper phase was loaded onto a (250 x 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 [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 co-chromatography 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 said 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 (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-fold or ∼2.5-fold elevations of DCF in strips incubated for 4h in plasma added with 100 μM antimycin A (which induces a mitochondrial leakage of H₂O₂ by inhibiting complex III in respiratory chain) or 50 mM aminotriazole (which reduces H₂O₂ decomposition by inhibiting catalase) (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 H$_2$O$_2$ caused the formation of 0.43 ± 0.06 nmol of DCF/g of human myocardium (n=5). This stoichiometry was routinely adopted to quantify H$_2$O$_2$ formation in myocardial strips incubated in plasma with anthracyclines. The values (nmol H$_2$O$_2$/g) were normalized to micromolar equivalents; anthracycline-induced H$_2$O$_2$ formation was expressed as Δ H$_2$O$_2$ over basal H$_2$O$_2$ 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 O$_2^-$ 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 8453 spectrophotometer equipped with computer-assisted corrections for turbidity and scatter. The incubations (1 ml, final volume) contained 30-60 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 min. 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 4h incubations or during sample homogenization and centrifugation.

Other conditions and assays

Anthracycline metabolism was reconstituted in isolated soluble fractions as described (Salvatorelli et al., accompanying manuscript). Proteins were measured by the bicinchoninic acid method (Stoscheck, 1990). Cardiac troponin I (cTnI) was determined immunoenzymatically (Siemens Medical Diagnostics Solutions) in a Dade Behring Vista Dimension Analyzer®. cTnI release was expressed as [100 x (cTnI in plasma)/(cTnI in plasma + cTnI in the strips)]. In all of the experiments the values were means ± SE 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 non linear fittings were obtained by GraphPad Prism Software®, version 5 (GraphPad Software Inc., La Jolla, CA). Other details are given in legends for Figures and Tables.
RESULTS

Amrubicin-dependent $H_2O_2$ formation in human myocardial strips

Human myocardial strips that had been loaded with DCFH-DA and then incubated in human plasma for 4h were shown to form $H_2O_2$ ($\mu$M: $0.27 \pm 0.03$, range 0.07-0.8, n=16 triplicate experiments). In the 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 $0.27 \mu$M $H_2O_2$ compared reasonably well with the upper limit of the physiologic range of $H_2O_2$ 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 $H_2O_2$, which localized to the membrane fraction only. Epirubicin did not induce a measurable increase of $H_2O_2$ at either 3 or 10 µM concentrations (FIGURE 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, Golgi vesicles) and failed to partition toward mitochondrial sites of redox activation (Salvatorelli et al., 2006; Salvatorelli et al., 2007). Lack of anthracycline-induced $\Delta H_2O_2$ in the soluble fraction probably denoted $H_2O_2$ detoxification through the pseudoperoxidase activity of myoglobin, that is abundantly expressed in the cytoplasm of cardiomyocytes (Cartoni et al., 2004; Menna et al., 2007).

The observation that amrubicin produced as much $H_2O_2$ as did doxorubicin, had to be reconciled with our previous demonstration that amrubicin attained very low steady-state levels in the membrane fraction of the strips as compared to doxorubicin (Salvatorelli et al., accompanying manuscript). 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., accompanying manuscript). 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 $\Delta H_2O_2$ with 9-
deaminoamrubicin at 3 µM and very little Δ H2O2 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 Δ H2O2. These results showed that ROS were formed by the limited pool of unmodified amrubicin that was recovered from the membrane fraction of strips (see also TABLE 1).

Further characterization of H2O2 formation

We considered that doxorubicin and amrubicin produced H2O2 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, that is 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 H2O2 by a typical reduction-oxidation cycle of the quinone moiety.

We next characterized how doxorubicin- or amrubicin- induced Δ H2O2 correlated with mitochondrial electron transport and its potential for reducing the anthracycline quinone. Basal levels of H2O2 were used as a surrogate marker of mitochondrial electron transport in the strips. Doxorubicin-induced Δ H2O2 did not correlate with, but actually increased non-linearly over the basal levels of H2O2. In contrast, amrubicin-induced Δ H2O2 correlated linearly with a broad range of basal levels of H2O2, and began increasing non-linearly only in some strips incubated with amrubicin at 10 µM (FIGURE 2). In the light of this latter finding, we calculated the ratio of 7-deoxyamrubicinolone to Δ H2O2 as an index of H2O2 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 Δ H_2O_2 with basal levels of H_2O_2, helped to demonstrate that i) doxorubicin formed H_2O_2 by mechanisms other than oxidation-reduction of its quinone moiety, ii) amrubicin formed H_2O_2 by quinone reduction-oxidation, but 10 µM amrubicin began inducing also quinone-independent mechanism(s).

Inactivation of mitochondrial aconitase

Mitochondrial aconitase rate limits the Krebs cycle by catalyzing reversible isomerisation 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_2O_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). Interestingly, however, treatment with iron under reducing conditions caused aconitase reactivation effects that depended on the anthracycline analogue and its concentration.

In experiments with anthracyclines at 3 µM, doxorubicin samples lacked aconitase reactivation, while epirubicin or amrubicin samples showed an aconitase reactivation that nearly approached that of reactivated control samples. In experiments with anthracyclines at 10 µM, 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 µM or to doxorubicin at 10 µM.

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 µM, developed levels of secondary alcohol metabolites in a concentration-dependent manner. Epirubicin formed less alcohol metabolite than doxorubicin at both 3 and 10 µM 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 µM amrubicin did not develop measurable levels of its secondary alcohol metabolite, amrubicinol (FIGURE 3). The apparent lack of amrubicinol 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 amrubicinol (see also FIGURE 3).

Determinants of the lack of amrubicinol formation in human myocardial strips

We attributed the lack of amrubicinol formation to the very low levels attained by amrubicin in the soluble fraction of the strips in comparison to doxorubicin or epirubicin (Salvatorelli et al., accompanying manuscript). However, separate analyses of strips exposed to anthracyclines at 3 or 10 µM showed that at both concentrations there were a few strips in which the levels attained by amrubicin in the soluble fraction were similar to those attained by doxorubicin or epirubicin (FIGURE 4/A-B). This suggested that other factors contributed to limiting amrubicin conversion to amrubicinol.

We next considered that amrubicin 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 µM 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 µM anthracycline; interestingly, however, amrubicinol formation was similar to epirubicinol formation at both 5 or 10 µM anthracycline (TABLE 3). These results denoted that amrubicin was not fully resistant to cytoplasmic reductases; hence, some amrubicinol should have been measured in those strips whose soluble fraction contained as much amrubicin as doxorubicin or epirubicin.

We eventually considered that amrubicinol formation could be precluded by amrubicin metabolites that competed with unmodified amrubicin for reduction by cytoplasmic reductases. We
focused on 9-deaminoamrubicin, which was formed in the membrane fraction of the strips but diffused in the soluble fraction and then, in plasma (Salvatorelli et al., accompanying manuscript). Studies of isolated soluble fractions incubated with NADPH and amrubicin or 9-deaminoamrubicin showed that the levels of formation of 9-deaminoamrubicin were many times higher than those of amrubicin (FIGURE 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., accompanying manuscript). By extrapolating data from the ascending portion of the plot of 9-deaminoamrubicinol vs 9-deaminoamrubicin, the apparent $K_m$ of 9-deaminoamrubicin for cytoplasmic reductases was approximated to ≤10 μM; in contrast, the apparent $K_m$ of amrubicin was approximated to ≥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 (FIGURE 6A). 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 (Salvatorelli et al., accompanying manuscript), 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 (FIGURE 6B).

Inactivation of cytoplasmic aconitase

Similar to what 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), while 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 or 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 to 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, while 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., accompanying manuscript). 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, while samples with a complete clearance showed the highest level of aconitase reactivation. In the latter samples, the magnitude of aconitase reactivation compared to that of strips exposed to amrubicin at 10 µM, which in fact exhibited a complete clearance of 9-deaminoamrubicinol (FIGURE 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 4h 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 H₂O₂, regardless of whether the catalase inhibitor, aminotriazole, was included to diminish H₂O₂ detoxification (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 to doxorubicin, amrubicin attained much lower levels in the membrane fraction but produced comparable amounts of H$_2$O$_2$. The unusual behaviour 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-deoxyaglycone metabolites), and by correlating anthracycline-induced $\Delta$ H$_2$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. Doxorubicin 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.

Redox cycling-independent mechanisms of H$_2$O$_2$ formation rest with 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 Complex I, III and IV, eventually inducing a passive leakage of reducing equivalents toward molecular oxygen (Marcillat et al., 1989; Simůnek 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 favourable 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 amphipatic enough to engage in electrostatic interactions with cardiolipin (Goormaghtigh et al., 1990; Marcillat et al., 1989). Doxorubicin exhibited these characteristics (Xu et al, 2011; Salvatorelli et al., accompanying manuscript). Amrubicin attained much lower levels in the membrane and showed considerably less
polar than doxorubicin (Salvatorelli et al., accompanying manuscript); therefore, amrubicin had greater chances to produce H$_2$O$_2$ through a canonical redox cycling mechanism.

The different behaviour of doxorubicin and amrubicin was also characterized by measuring reversible inactivation of mitochondrial aconitase as a marker of anthracycline mitochondrial localization and O$_2^-$ formation. Our data show that 3 or 10 µM doxorubicin always caused irreversible rather than reversible inactivation of mitochondrial aconitase. This observation lends support to the concept that doxorubicin caused membrane effects that favoured other mechanisms of ROS formation and aconitase inactivation. In contrast, 3 µM amrubicin caused reversible aconitase inactivation that was consistent with canonical mechanisms of O$_2^-$ formation and reactivity. Irreversible aconitase inactivation only occurred when amrubicin was used at 10 µM, 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 µM, 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, of epirubicinol; in contrast, strips exposed to amrubicin never developed measurable levels of amrubicinol. This latter finding was not caused by amrubicinol 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 to doxorubicin, amrubicin exhibited the same moderate resistance to carbonyl reduction as that of epirubicin; moreover, the apparent $K_m$ of amrubicin for cytoplasmic reductases was approximated to >100 µM, which was also similar to epirubicin as opposed to a doxorubicin apparent $K_m$ of <100 µM (Salvatorelli et al., 2007). Our data suggest that amrubicinol formation was precluded by competing pathways that favoured 9-deaminoamrubicinol formation. Based on our previous studies of amrubicin deamination in the membrane fraction of the strips
(Salvatorelli et al., accompanying manuscript), we calculate that the apparent $K_m$ of amrubicin for carbon-nitrogen lyases was significantly lower than its apparent $K_m$ for cytoplasmic reductases (∼50 vs >100 µM). Here we have shown that in diffusing from the membrane fraction to the soluble fraction, 9-deaminoamrubicin exceeded the levels of amrubicin in that fraction and reacted with cytoplasmic reductases according to an apparent $K_m$ of as low as <10 µM. Thus, efficient amrubicin deamination and 9-deaminoamrubicinol formation outcompeted amrubicinol formation.

Differences in alcohol metabolite formation reflected in different patterns of cytoplasmic aconitase inactivation by doxorubicin or epirubicin or amrubicin. In strips exposed to doxorubicin or epirubicin at 3 µM, 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-deaminoamrubicinol. 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., accompanying manuscript), it can now be said that formation of diffusible 9-deaminoamrubicin(ol) induced the dual effect of improving amrubicin clearance while also sparing possible targets of amrubicinol toxicity.

Inactivation of mitochondrial or cytoplasmic aconitase was reported to dysregulate energy metabolism, iron homeostasis, and redox balance (Minotti et al., 2004a; Narahari et al., 2000), eventually inducing tissue damage in a number of pathologic conditions (Recalcati et al., 2010). Here, reversible or irreversible inactivation of the two aconitases was used as a surrogate marker of formation and reactivity of anthracycline-derived $O_2^-$ or secondary alcohol metabolites, but strips exposed to anthracyclines at 3 or 10 µM did not release cTnI during the course of the experiments. Troponin release only occurred when the strips were exposed to 100 µM H$_2$O$_2$, which was >2 orders of magnitude higher than basal or anthracycline-induced H$_2$O$_2$ levels in the strips. Similar observations were done with myoglobin release (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 to control strips; myocardial damage only occurred when anthracyclines were used at 100 µM (Cartoni et al., 2004; Salvatorelli et al.,
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., accompanying manuscript).

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 amrubicinol, but previous preclinical studies showed that amrubicinol formed abundantly in tumor cells and proved ∼5-50 times more active than amrubicin (Ogawara et al., 2010; Noguchi et al., 1998; Yamaoka et al., 1998). Selective formation of amrubicinol in tumors but not in heart, may have contributed to the encouraging therapeutic index of amrubicin in cancer patients. On the other hand, our data show that 9-deaminoamrubicin formation and elimination was an important factor of amrubicin clearance from the strips, and other studies showed that purified 9-deaminoamrubicin was also less active than amrubicin in tumor cells (Yamaoka et al., 1998). Having said that the mechanisms and relative abundance of amrubicin deamination in the heart or tumors will require ad hoc investigations, these facts denote that 9-deaminoamrubicin may represent a common metabolic determinant of amrubicin detoxification or inactivation.

Attempts to incorporate ROS and secondary alcohol metabolites in an 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 surface 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 (Menna et al., 2011; Minotti et al., 2010). The cardiac safety of epirubicin was nonetheless questioned by recent 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;
Salvatorelli et al., accompanying manuscript). The search for active but tolerable anthracyclines should therefore be directed toward analogues that exhibited both a reduced accumulation and a less damaging profile of bioactivation in the heart. Amrubicin seems to meet both such requirements.

In conclusion, the pharmacokinetic and biotransformation characteristics of amrubicin, as described here and in the accompanying report, provide mechanistic explanations supporting the lack of cardiotoxicity in preclinical models and clinical trials. Amrubicin appears 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.
AUTORSHIP CONTRIBUTIONS

Participated in research design: Salvatorelli, Menna, Surapaneni, Aukerman, Sung, Minotti

Conducted experiments: Salvatorelli, Menna, Gonzalez Paz

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

Performed data analysis: Salvatorelli, Menna, Minotti.

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


Salvatorelli E, Menna P, Surapaneni S, Aukerman SL, Chello M, Covino E. Sung V, and Minotti G. Pharmacokinetic characterization of amrubicin cardiac safety in an ex vivo human myocardial strip model I. Amrubicin accumulates to a lower level than doxorubicin or epirubicin (accompanying manuscript)


FOOTNOTES

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1Bioavailability 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).

2Control experiments, performed by incubating isolated soluble fractions with NADPH and amrubicinol, ruled out the possibility that 9-deaminoamrubicinol could be formed through deamination of amrubicinol.

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LEGENDS FOR FIGURES

Figure 1  **ROS formation in human myocardial strips exposed to anthracyclines at 3 or 10 µM.**

Human myocardial strips were loaded with DCFH-DA and then incubated with anthracyclines at 3 or 10 µM, as described in Materials and Methods. At the end of incubations, membrane or soluble fractions of the strips were assayed for ROS by DCF-detectable H$_2$O$_2$ (Δ over basal levels). Values were means ± SE of 3-5 triplicate experiments.

Figure 2  **Δ H$_2$O$_2$ vs basal H$_2$O$_2$ in human myocardial strips exposed to doxorubicin or epirubicin at 3 or 10 µM.**

Δ 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 also text for explanations.

Figure 3  **Secondary alcohol metabolites in human myocardial strips incubated with anthracyclines at 3 or 10 µM**

Human myocardial strips were incubated with doxorubicin or epirubicin or amrubicin at 3 or 10 µM. At the end of standard 4h incubations, plasma and strips (membrane and soluble fractions) were assayed for doxorubicinol, epirubicinol, or amrubcinol. The values were means ± SE of 4-11 experiments, with each experiment having been performed in triplicate.

*Denotes epirubicinol significantly lower than doxorubicinol (P<0.01)

Figure 4  **Secondary alcohol metabolites vs parent anthracyclines in the soluble fraction of human myocardial strips incubated with anthracyclines at 3 or 10 µM**

Levels of secondary alcohol metabolites in the soluble fraction of human myocardial strips were plotted against levels of parent anthracyclines in the same fraction. Shaded boxes delimit samples in which the soluble fraction of myocardial strips contained similar levels of doxorubicin or epirubicin or amrubicin.
Figure 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 prot./ml), NADPH (0.25 mM), and increasing concentrations of amrubicin or 9-deaminoamrubicin, in 0.3 M NaCl, pH 7.0. After 4h at 37 °C, incubations were extracted and assayed for amrubicinol or 9-deaminoamrubicinol as described in Materials and Methods. The values were taken from representative titrations.

Figure 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 4h 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). In panel A, the soluble fraction of strips was assayed for amrubicin (squares) and 9-deaminoamrubicin (circles); the latter was added with 9-deaminoamrubicin or 9-deaminoamrubicinol that had diffused from the soluble fraction in plasma. In panel 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.

Figure 7 9-Deaminoamrubicinol clearance and cytoplasmic aconitase reactivation in human myocardial strips exposed to amrubicin at 3 or 10 μM

Reactivated cytoplasmic aconitase (solid circles) was measured in soluble fractions from myocardial strips that had been incubated with amrubicin at 3 or 10 μM (mean ± SE of 3-6 determinations). Where indicated (open circles), some of the strips incubated with 3 μM amrubicin exhibited an incomplete 9-deaminoamrubicinol clearance (70 ± 5%, corresponding to an accumulation of 0.11 ± 0.03 μM 9-deaminoamrubicinol in the soluble fraction of strips).
Deaminoamrubicinol clearance was calculated by the formula \[100 \times \frac{(9\text{-deaminoamrubicinol in plasma})}{(9\text{ deaminoamrubicinol in strips}) + (9\text{-deaminoamrubicinol in plasma})}\].
Table 1

Anthracycline content and distribution and ROS formation in membrane fractions of human myocardial strips incubated with amrubicin or 9-deaminoamrubicin.

<table>
<thead>
<tr>
<th>Anthracycline in plasma</th>
<th>µM</th>
<th>anthracycline in membrane fraction</th>
<th>Δ H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>9-deaminoamrubicin (µM)</td>
<td>amrubicin (µM)</td>
</tr>
<tr>
<td>9-deaminoamrubicin</td>
<td>3</td>
<td>1.3 ± 0.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.1 ± 0.2*</td>
<td>n.d.</td>
</tr>
<tr>
<td>amrubicin</td>
<td>10</td>
<td>1.4 ± 0.1**</td>
<td>2.9 ± 0.9</td>
</tr>
</tbody>
</table>

DCFH-DA loaded human myocardial strips were subjected to standard 4h incubations in plasma that contained 3 or 10 µM 9-deaminoamrubicin or 10 µM amrubicin. At the end of incubations, membrane fractions of the strips were assayed for anthracycline content and composition and ROS formation (Δ H₂O₂) as described in Materials and Methods. The values were means ± SE of 3-6 experiments.

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

**9-deaminoamrubicin significantly lower in strips incubated with 10 µM amrubicin compared to strips incubated with 10 µM 9-deaminoamrubicin (P < 0.01).
Table 2
Basal and reactivated mitochondrial aconitase in membrane fractions of human myocardial strips exposed to anthracyclines

<table>
<thead>
<tr>
<th>aconitase (U/g)</th>
<th>control</th>
<th>anthracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µM</td>
</tr>
<tr>
<td>basal</td>
<td>0.41 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>reactivated</td>
<td>0.78 ± 0.09*</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>basal</td>
<td>0.39 ± 0.03</td>
<td>10</td>
</tr>
<tr>
<td>reactivated</td>
<td>0.74 ± 0.08*</td>
<td>0.31 ± 0.07</td>
</tr>
</tbody>
</table>

Basal and reactivated mitochondrial aconitase was measured in membrane fractions of strips incubated in plasma only (control strips) or in plasma with anthracyclines at 3 or 10 µM. Values were means ± SE of 3-7 experiments.

*Denotes reactivated aconitase significantly higher (P<0.01) than basal aconitase.
Table 3

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

<table>
<thead>
<tr>
<th>Anthracycline</th>
<th>Alcohol metabolite (nmol/mg prot./4h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 µM</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>0.044 ± 0.007</td>
</tr>
<tr>
<td>epirubicin</td>
<td>0.007 ± 0.001*</td>
</tr>
<tr>
<td>amrubicin</td>
<td>0.004 ± 0.001*</td>
</tr>
</tbody>
</table>

Incubations (0.25 ml, final volume) contained isolated soluble fractions of human myocardial strips (0.6 mg prot./ml), NADPH (0.25 mM), and anthracyclines (5 or 10 µM), in 0.3 M NaCl, pH 7.0. After 4h at 37 °C, incubations were extracted and assayed for alcohol metabolites as described in Materials and Methods. The values were means ± SE of 4-8 experiments.

*Denotes epirubicinol and amrubicinol significantly lower than doxorubicinol (P<0.001).
Table 4

Basal and reactivated cytoplasmic aconitase in soluble fractions of human myocardial strips exposed to anthracyclines.

<table>
<thead>
<tr>
<th>aconitase (U/g)</th>
<th>control</th>
<th>anthracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>doxorubicin</td>
</tr>
<tr>
<td>basal</td>
<td>0.12 ± 0.003</td>
<td>0.023 ± 0.005</td>
</tr>
<tr>
<td>reactivated</td>
<td>0.18 ± 0.01*</td>
<td>0.015 ± 0.01</td>
</tr>
</tbody>
</table>

Basal and reactivated aconitase was measured in soluble fractions of human myocardial strips incubated in plasma only (control strips) or in plasma with anthracyclines at 3 µM. Values were means ± SE of 3-6 experiments.

*Denotes reactivated aconitase significantly higher than basal aconitase (P<0.001).
Figure 1

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Figure 2
Figure 3

3 μM anthracycline

10 μM anthracycline

alcohol metabolite (μM)

membrane fraction

soluble fraction

plasma

* doxorubicin

epirubicin

amrubicin

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Figure 4
Figure 5

- 9-deaminoamrubicinol
- amrubicinol

Alcohol metabolite (nmol/mg prot./4h)

Amrubicin or 9-deaminoamrubicin (μM)
Figure 6

A

Cytoplasmic anthracycline (μM)

0.01
0.1
1
10
100

0 10 20 30 40

Amrubicin bioavailability (μM)

9-deamino amrubicin

Amrubicin

B

Alcohol metabolite (μM)

0.0
0.5
1.0
1.5

0 10 20 30 40

Amrubicin bioavailability (μM)

9-deamino amrubicinol

Amrubicinol

Figure 6
Figure 7