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

Emanuela Salvatorelli, Pierantonio Menna, Sekhar Surapaneni, Sharon L. Aukerman, Massimo Chello, Elvio Covino, Victoria Sung, and Giorgio Minotti

Drug Sciences (E.S., P.M, G.M.) and Cardiac Surgery (M.C., E.C.), Center for Integrated Research, University Campus Bio-Medico, Rome, Italy; Drug Metabolism and Pharmacokinetics (S.S.) and Translational Development (S.L.A.), Celgene Corporation, Summit, New Jersey; and Translational Development, Celgene Corporation, San Francisco, California (V.S.)

Received November 26, 2011; accepted February 14, 2012

ABSTRACT

Antitumor anthracyclines such as doxorubicin and epirubicin are known to cause cardiotoxicity that correlates with anthracycline accumulation in the heart. The anthracycline 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-naphthacenenedione hydrochloride] has not shown cardiotoxicity in laboratory animals or patients in approved or investigational settings; therefore, we conducted preclinical work to characterize whether amrubicin attained lower levels than doxorubicin or epirubicin in the heart. Anthracyclines were evaluated in ex vivo human myocardial strips incubated in plasma to which anthracycline concentrations of 3 or 10 μM were added. Four-hour incubations were performed to characterize myocardial anthracycline accumulation derived from anthracycline uptake in equilibrium with anthracycline clearance. Short-term incubations followed by multiple washouts were performed to obtain independent measurements of anthracycline uptake or clearance. In comparison with doxorubicin or epirubicin, amrubicin attained very low levels in the soluble and membrane fractions of human myocardial strips. This occurred at both 3 and 10 μM anthracycline concentrations and was caused primarily by a highly favorable clearance of amrubicin. Amrubicin clearance was facilitated by formation and elimination of sizeable levels of 9-deaminoamrubicin and 9-deaminoamrubicinol. Amrubicin clearance was not mediated by P glycoprotein or other drug efflux pumps, as judged from the lack of effect of verapamil on the partitioning of amrubicin and its deaminated metabolites across myocardial strips and plasma. Limited accumulation of amrubicin in an ex vivo human myocardial strip model may therefore correlate with the improved cardiac tolerability observed with the use of amrubicin in preclinical or clinical settings.

Introduction

The anthracyclines are DNA intercalators and topoisomerase II inhibitors that show activity in many human tumors; unfortunately, however, anthracyclines cause a dose-related cardiomyopathy that limits their clinical use. Among the anthracyclines approved for clinical use, doxorubicin shows activity in solid tumors and lymphomas. Cumulative doxorubicin doses of 400 to 450 mg/m² introduce a 5% risk of congestive heart failure (Swain et al., 2003). Doxorubicin is composed of an aglycone and a sugar. The aglycone (doxorubicinone) is a tetracyclic ring system with adjacent quinone-hydroquinone moieties and a short side chain with a carbonyl group at C-13 and a primary alcohol terminus at C-14; the sugar (daunosamine) is an amino-substituted trideoxy fucosyl moiety. The closely related analog, epirubicin, differs from doxorubicin in an axial-to-equatorial epimerization of the hydroxyl group at C-4 in the amino sugar; such a minor modification improves glucuronidation and systemic elimination, which require administering epirubicin at doses ~1.5 times higher than those of doxorubicin. Cardiomyopathy and congestive heart failure usually occur when epirubicin is used at cumulative doses ~2 times higher than those of doxorubicin, which suggests that epirubicin shows a ~30 to 35% reduction in cardiotoxicity (Ewer and Benjamin, 2006; Smith et al., 2010). Other anthracyclines, such as daunorubicin and idarubicin, are used

ABBREVIATIONS: SCLC, small-cell lung cancer; Cmax, peak plasma concentration; HPLC, high-performance liquid chromatography; MRP1, multidrug resistance protein 1.
at different dose levels and by different schedules to treat acute leukemias. Both of them are cardiotoxic, but dose-adjusted comparisons with cardiotoxicity from doxorubicin or epirubicin are difficult to establish (Minotti et al., 2004). These facts illustrate the need for active but less cardiotoxic anthracycline analogs (Gianni et al., 2008).

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] is a totally synthetic anthracycline that differs from doxorubicin in the following chemical features: 1) substitution of a methyl group for a primary alcohol at the side-chain terminus, 2) lack of a methoxy group at C-4 in the D ring, 3) dislocation of an -NH₂ residue from C-3’ in the sugar to C-9 in the A ring, 4) symmetrical dislocation of an -OH residue from C-9 in the A ring to C-3’ in the amino sugar, and 5) lack of a methyl group at C-5 in the amino sugar (Ishizumi et al., 1987) (Fig. 1). Removal of the amino group and double-bond addition in the A ring convert amrubicin to 9-deaminoamrubicin (see Fig. 1).

In human tumor-nude mouse systems, amrubicin showed more activity than doxorubicin when the two drugs were administered at their maximum tolerated doses (25 or 12.5 mg/kg, respectively) (Morisada et al., 1989b); however, amrubicin neither caused morphologic or functional indices of cardiotoxicity in anthracycline-naive rabbits or dogs nor aggravated cardiomyopathy in doxorubicin-pretreated dogs (Suzuki et al., 1997; Noda et al., 1998). Amrubicin did not cause serious cardiac events in clinical trials and postmarketing surveillance related to its approved indication in Japan for the treatment of both small-cell lung cancer (SCLC) and non-SCLC.

Amrubicin is undergoing a global development program with plans for regulatory submissions and approval in several countries. In a phase II study of this program, patients with refractory SCLC received cumulative doses of amrubicin equal to or higher than 750 mg/m² without experiencing systolic dysfunction over a 6-month survival period (Ettinger et al., 2010). There is a lack of clinical studies that compare amrubicin with doxorubicin with regard to activity and cardiotoxicity in a given oncologic setting; however, preclinical toxicology studies showed that amrubicin myelotoxicity was more severe even at half of the maximum tolerated dose than doxorubicin myelotoxicity at the maximum tolerated dose (Morisada et al., 1989a). Because myelotoxicity represents a surrogate of antiproliferative activity, one can approximate that in humans, cumulative amrubicin doses of ≥750 mg/m² would be at least as active as cumulative doxorubicin doses that are used in most clinical settings. Amrubicin therefore holds promise for both reduced cardiotoxicity and preserved or improved antitumor activity.

Cardiotoxicity is a multifactorial process that proceeds through the uptake and accumulation of anthracyclines in cardiomyocytes; unmodified anthracyclines or their metabolites or by-products then engage in toxic interactions with cell constituents and functions (Tokarska-Schlattner et al., 2006). The net level of anthracycline accumulation in cardiomyocytes therefore represents a primary determinant of anthracycline-related cardiotoxicity (Minotti et al., 2004).

![Fig. 1. Structures of doxorubicin, epirubicin, amrubicin, and 9-deaminoamrubicin. Epirubicin differs from doxorubicin in an axial-to-equatorial epimerization of the hydroxyl group at C-4’ in the amino sugar. Differences between amrubicin and doxorubicin are indicated by arrows: substitution of a methyl group for a primary alcohol at the side-chain terminus, lack of a methoxy group at C-4 in the D ring, dislocation of an -NH₂ residue from C-3’ in the sugar to C-9 in the A ring, symmetrical dislocation of an -OH group from C-9 in the A ring to C-3’ in the amino sugar, and lack of a methyl group at C-5 in the amino sugar. 9-Deaminoamrubicin is formed by removal of the amino group at C-9 and double-bond addition in the A ring (red circle).](image-url)
Here, we characterized whether the cardiac tolerability of amrubicin could be determined by its reduced accumulation in the heart. The studies were carried out with an ex vivo human myocardial strip model that obviated pitfalls of laboratory animal models and proved useful for deciphering the clinical pattern of cardiac safety or toxicity of anthracycline-containing regimens (Salvatorelli et al., 2006, 2007).

Materials and Methods

Chemicals. Doxorubicin and epirubicin were obtained through the courtesy of Novo Nordic Medical Sciences (Milan, Italy). Amrubicin and 9-deaminoamrubicin were provided by Celgene Corporation (Summit, NJ). (-)-Verapamil and all other chemicals were obtained from Sigma-Aldrich (Milan, Italy).

Human Myocardial Strips. Fifty nine anonymous myocardial samples were obtained from 39 male patients (68 ± 1.5 years, range 42–82 years) and 20 female patients (72.2 ± 1.4 years, range 44–86 years) undergoing aorto-coronary bypass grafting (Salvatorelli et al., 2006, 2007). To improve myocardial preservation, in the present study all samples were obtained during cannulation of beating, nonischemic right atrium. This was a standard procedure, and all samples were to be routinely discarded by the surgeons; therefore, the patients were not subjected to any unjustified loss of tissue. At the time of the experiments thin strips (10 mm long, 2 mm wide) were carefully dissected free of fat or grossly visible foreign tissues. Next, the strips were incubated in 2 ml of fresh human plasma that contained test compounds. Plasma was used to account for the binding of anthracyclines to albumin and other proteins; it was prepared before each experiment by 1500g centrifugation of blood samples obtained from healthy donors and anticoagulated with sodium citrate. Because of its poor solubility in water, amrubicin was prepared concentrated in dimethyl sulfoxide and then diluted serially in 0.3 M NaCl. In the final incubation, the volume of dimethyl sulfoxide never exceeded 2 to 4 μl, which was shown not to affect the pharmacokinetics of any of the anthracyclines tested in this study.

Standard 4-h Incubations. Human myocardial strips were incubated in plasma added with amrubicin at 3 or 10 μM, two concentration levels that spanned plasma C_max values (3.9–10.1 μM) produced in patients by a bolus intravenous injection of 30 to 45 mg of amrubicin/m² (Matsuura et al., 2006). Recommended starting dose is 45 mg/m² on days 1 to 3 of a 3- to 4-week cycle, according to the approved dosage for lung cancer in Japan. Doxorubicin and epirubicin were also used at 3 or 10 μM to obtain direct comparisons with amrubicin; however, 10 μM doxorubicin or epirubicin was similar to the plasma C_max value produced by clinically approved bolus infusions of 60 mg of doxorubicin/m² or 90 mg of epirubicin/m² (Gianni et al., 1997; Grasselli et al., 2001). After 4-h incubation at 37°C in a gently shaking Dubnoff metabolic bath under a room air atmosphere, the strips were washed with ice-cold 0.3 M NaCl homogenized in a minimum volume of the same medium, and centrifuged for 90 min at 105,000g to separate soluble and whole membrane fractions which were assayed for anthracyclines. Aliquots of plasma (200 μl) were also taken and assayed for anthracyclines. We reported previously that under such defined conditions human myocardial strips retained their viability and functions throughout the incubation time (Salvatorelli et al., 2006, 2007); therefore, anthracycline pharmacokinetics could be assessed without confounding effects caused by an acute damage and perturbations of membrane permeability or drug metabolism.

Loading and Multiple Washout Experiments. Human myocardial strips were incubated for 30 min in plasma that contained anthracycline at a concentration of 3 or 10 μM. Next, the strips were placed in fresh anthracycline-free plasma that was replaced every 30 to 60 min up to 4 h to promote the highest possible anthracycline efflux from the strips. During the experiment, plasma samples (200 μl) were taken at regular times and assayed for parent anthracyclines or metabolites. The values determined at each time point were added with values measured at the preceding time point: this provided the time course of the cumulative efflux of anthracyclines or their metabolites, without confounding effects caused by their competing reuptake in the strips (Salvatorelli et al., 2009). At the end of the experiments, the strips were assayed for anthracyline that had not been released during washout (anthracyline retention). By adding anthracyline retention with cumulative anthracyline efflux, the amount of anthracyline that had been taken up during the 30-min loading could be calculated. Anthracyline clearance was calculated by the formula [100 × (cumulative efflux/uptake)] (Salvatorelli et al., 2009).

High-Performance Liquid Chromatography and Liquid Chromatography-Tandem Mass Spectrometry Assays for Anthracyclines. Plasma and soluble or membrane fractions of human myocardial strips were extracted with a 4-fold volume of (1:1) chloroform and methanol (CHCl₃-CH₂OH). The organic phases were combined to obtain an extract that was analyzed by reversed-phase high-performance liquid chromatography (HPLC) in a Hewlett Packard (Palo Alto, CA) 1100 system. 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 and epirubicin were detected fluorimetrically (excitation at 480 nm/emission at 560 nm), identified by cochromatography with authentic standards, and quantified against appropriate standard curves. The retention times were 14.4 min for doxorubicin or 14.5 min for epirubicin.

In the assays for amrubicin and 9-deaminoamrubicin, 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 isocratic for 4 min and then switched to a 3-min linear gradient to (15–85%) 10 mM NaH₂PO₄, pH 4.0, CH₃CN. The latter was maintained isocratic 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 9-deaminoamrubicin were detected fluorimetrically (excitation at 465 nm/emission at 560 nm), identified by cochromatography with authentic standards, and quantified against appropriate standard curves. The retention times were 12 min for amrubicin and 19.6 min for 9-deaminoamrubicin. Chromatograms of the organic extracts of the soluble fractions of myocardial strips, and especially of plasma, contained a fluorescent peak that eluted 1 min before 9-deaminoamrubicin; this peak did not cochromatograph with any of the amrubicin metabolite standards available to us. We tentatively attributed this peak to 9-deaminoamrubicinol, a hydroxymetabolite (or secondary alcohol metabolite) formed by a two-equivalent reduction of the side-chain C-13 carbonyl group of 9-deaminoamrubicin. Peak assignment to 9-deaminoamrubicinol was obtained by the following procedure: 1) incubation of 9-deaminoamrubicin with isolated soluble fractions and NADPH resulted in formation of a fluorescent HPLC peak that eluted 1 min before 9-deaminoamrubicin; and 2) liquid chromatography-tandem mass spectrometry of the organic extracts showed that 9-deaminoamrubicin and the presumed 9-deaminoamrubicinol peak gave 465.4 m/z and 467.4 m/z, respectively, which was consistent with a two-equivalent reduction of the side-chain carbonyl group of 9-deaminoamrubicin. Liquid chromatography-tandem mass spectrometry was carried out with an Agilent Technologies (Santa Clara, CA) 6410 triple quadrupole operated in electron spray ionization (negative ion mode) and single-charged ion monitoring.

In the HPLC assays of myocardial strips, all of the values were expressed as nanomole/gram of tissue; in the assays of plasma, the values (nmol/ml) were normalized to the weight of the strips (Salvatorelli et al., 2007). Next, all of the values were expressed as micro-
molar equivalents considering that the cardiac tissue has a density very similar to that of water (Salvatorelli et al., 2007). The lowest detection limit was 0.001 µM; within-day and between-days coefficients of variation were <3 and <10%, respectively.

Other Conditions and Assays. Where indicated, anthracycline metabolism was characterized in 0.25-ml incubations that contained isolated soluble fractions from myocardial strips (0.15 mg of protein), 0.25 mM NADPH, and known amounts of anthracyclines in 0.3 M NaCl, pH 7.0. After 4 h at 37°C, the incubations were extracted and assayed by HPLC as described above. Proteins were measured by the bicinchoninic acid method (Stoscheck, 1990). 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.

![Fig. 2](image-url) Limited steady-state accumulation of amrubicin in human myocardial strips in comparison with doxorubicin and epirubicin. Human myocardial strips were incubated with 3 or 10 µM anthracycline under conditions of standard 4-h incubations. The values are means ± S.E. of three experiments with doxorubicin, four experiments with epirubicin, and six experiments with amrubicin, with each experiment performed in triplicate. P < 0.001 for amrubicin versus doxorubicin or epirubicin at both 3 and 10 µM anthracycline; P < 0.001 for amrubicin at 10 versus 3 µM.

### TABLE 1
Limited steady-state accumulation of amrubicin in both soluble and membrane fractions of human myocardial strips in comparison with doxorubicin and epirubicin

<table>
<thead>
<tr>
<th>Anthracycline</th>
<th>Membrane Fraction</th>
<th>Soluble Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin, 3 µM</td>
<td>5.4 ± 0.5</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>Epirubicin, 3 µM</td>
<td>4.5 ± 0.5</td>
<td>0.54 ± 0.13</td>
</tr>
<tr>
<td>Amrubicin, 3 µM</td>
<td>0.6 ± 0.1*</td>
<td>0.11 ± 0.02†</td>
</tr>
<tr>
<td>Doxorubicin, 10 µM</td>
<td>12.3 ± 0.7</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>Epirubicin, 10 µM</td>
<td>14.2 ± 1.5</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Amrubicin, 10 µM</td>
<td>1.5 ± 0.2†</td>
<td>0.4 ± 0.07†</td>
</tr>
</tbody>
</table>

* Significantly lower (P < 0.001) than doxorubicin or epirubicin.
† Significantly higher (P < 0.001) vs. 3 µM amrubicin.

### TABLE 2
Anthracycline recovery at the end of standard 4-h incubations

Anthracycline recovery was calculated by the sum of anthracycline in strips with anthracycline in plasma. Where indicated, amrubicin recovery was calculated separately for unmodified amrubicin or the sum of unmodified amrubicin with 9-deaminoamrubicin (9-deaminoamrubicin + 9-deaminoamrubicinol). Sample sizes were the same as those reported in the legend for Fig. 2. Values in parentheses indicate the percentages of recovery.

<table>
<thead>
<tr>
<th>Anthracycline</th>
<th>Membrane Fraction</th>
<th>Soluble Fraction</th>
<th>Amrubicin</th>
<th>Amrubicin + Dimetabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 µM</td>
<td></td>
<td></td>
<td>3.8 ± 0.3* (40 ± 5%)</td>
<td>9.0 ± 0.6 (109 ± 7%)</td>
</tr>
<tr>
<td>10 µM</td>
<td></td>
<td></td>
<td>22 ± 1.2** (79 ± 4%)</td>
<td>31 ± 1.3 (113 ± 6%)</td>
</tr>
</tbody>
</table>

* Amrubicin recovery significantly lower (P < 0.05) than doxorubicin or epirubicin recovery at 3 or 10 µM, respectively.

### Results

Anthracycline Recovery and Distribution after Standard 4-h Incubations. Human myocardial strips were exposed to concentrations of 3 or 10 µM anthracycline in standard 4-h incubations that allowed evaluation of anthracycline uptake from plasma in equilibrium with anthracycline efflux in plasma (clearance). At the end of incubations, the strips exhibited a high steady-state accumulation of doxorubicin or epirubicin; the strips actually accumulated both anthracyclines in excess of their concentration in plasma, which was in agreement with the known ability of the cardiac tissue to concentrate anthracyclines (Olson et al., 1988). The strips accumulated much less amrubicin (approximately 12% in comparison with doxorubicin and epirubicin at both 3 and 10 µM anthracycline) (Fig. 2). Such a limited steady-state accumulation of amrubicin occurred in both membrane and soluble fractions of the myocardial strips (Table 1). In principle, these results suggested that the low steady-state accumulation of amrubicin could be caused by its limited partitioning from plasma in strips.

At the end of experiments with 3 or 10 µM anthracycline, total doxorubicin or epirubicin recovery (measured by the sum of anthracycline in the strips in anthracycline in plasma) averaged ≥90%; amrubicin recovery was significantly lower, but it approached ~100% if we considered the recovery of 9-deaminated metabolites such as 9-deaminoamrubicin and its secondary alcohol metabolite 9-deaminoamrubicinol (Table 2). The majority of 9-deaminated metabolites was recovered from plasma (~50 or ~90% in experiments with 3 or 10 µM amrubicin, respectively); however, plasma alone did not generate any such compound, regardless of whether NADPH was added as enzymatic cofactor (Fig. 3).

The relative abundance and distribution of 9-deaminated metabolites were characterized. 9-Deaminoamrubicin always ranked 10 or more times higher than 9-deaminoamrubicinol, both in the strips and in plasma. In the strips, 9-deaminoamrubicin localized mainly to the membrane fraction, whereas 9-deaminoamrubicinol was recovered from the soluble fraction of myocardial strips that had been exposed to 3 µM amrubicin but not in those exposed to 10 µM amrubicin. Total levels of 9-deaminoamrubicin (strips + plasma) increased in a concentration-dependent manner, whereas total levels of 9-deaminoamrubicinol did not (Table 3).

Having shown that plasma alone failed to generate 9-deaminated metabolites, we characterized whether these compounds were formed in the strips through the action of enzymes of the membrane or soluble fraction. Isolated membrane fractions converted amrubicin to 9-deaminoamrubicin in a concentration-related manner, regardless of the presence or absence of enzyme cofactors such as NADPH; however, heat-inactivated membrane fractions showed much less formation of...
9-deaminoamrubicin, which indicated the involvement of heat-labile enzymes. Isolated soluble fractions did not convert amrubicin to 9-deaminoamrubicin (Fig. 4A). Isolated soluble fractions reduced 9-deaminoamrubicin to 9-deaminoamrubicinol, which was subject to the availability of NADPH; it is noteworthy that the dependence of 9-deaminoamrubicinol formation on 9-deaminoamrubicin concentration followed a bell-shaped pattern that made 9-deaminoamrubicinol reach its maximum level of formation over a narrow range of 9-deaminoamrubicin concentrations. Isolated membrane fractions did not reduce 9-deaminoamrubicin to 9-deaminoamrubicinol (Fig. 4B).

Collectively, these results suggested that the low steady-state accumulation of amrubicin in myocardial strips could also be caused by intramyocardial formation of deaminated metabolites that eventually diffused in plasma.

**Apparent or Theoretical Uptake and Clearance of Amrubicin in Human Myocardial Strips.** Standard 4-h incubations did not allow us to distinguish anthracycline uptake from anthracycline clearance; therefore, we performed loading and multiple washout experiments that allowed us to calculate anthracycline retention or efflux and, hence, anthracycline uptake or clearance (see Materials and Methods). In comparison with doxorubicin, amrubicin showed both a reduced apparent uptake (~75% decrease at both anthracycline concentration levels) and an increased apparent clearance (~65% or ~125% increase at 3 or 10 μM anthracycline) (Table 4). In comparison with doxorubicin, epirubicin showed a higher uptake but a similar clearance, such that the net retention of epirubicin in the strips exceeded that of doxorubicin (see Table 4). In standard 4-h incubations, the strips did not accumulate more epirubicin than doxorubicin (Fig. 2; Table 1). This discrepancy is explained by the fact that in standard 4-h incubations, longer equilibration, of anthracycline uptake and clearance with anthracycline metabolism allowed epirubicin to form minute amounts of doxorubicinolone (~0.5 μM), a potent membrane-permeabilizing agent that favored the release of excess epirubicin (Salvatorelli et al., 2009).

Amrubicin uptake or clearance was reported as “apparent” to denote that it was calculated by measuring myocardial or plasma levels of unmodified amrubicin only. Having previously characterized that amrubicin converted to diffusible deaminated metabolites, we considered that the apparent uptake of amrubicin had to be added with the amount of amrubicin that had been incorporated in the strips and then converted to deaminated metabolites; this amrubicin amount could be calculated by the sum of metabolite in the strips with metabolite in plasma (total metabolite recovery). In a similar manner, the apparent amrubicin clearance had to be added with the amount of amrubicin that was cleared from myocardial strips as deaminated metabolites; this latter amount of amrubicin was calculated by the percentage of metabolite in plasma to total metabolite recovery. During multiple washouts, myocardial strips that had been loaded with 3 or 10 μM doxorubicin or epirubicin released unmodified anthracycline only (Fig. 5, A and B); in contrast, strips that had been loaded with amrubicin released both unmodified amrubicin and 9-deaminoamrubicin and 9-deaminoamrubicinol. In the experiments with 3 μM amrubicin the release of 9-deaminoamrubicin and 9-deaminoamrubicinol was of the same magnitude as that of unmodified amrubicin (Fig. 5C). In the experiments with 10 μM amrubicin, the release of 9-deaminoamrubicin was significantly higher than that of amrubicin; it is noteworthy, however, that 9-deaminoamrubicinol was undetectable in these latter experiments (Fig. 5D). The strips contained only marginal amounts of 9-deaminoamrubicin (0.5 or 0.25 μM at 3 or 10 μM amrubicin, respectively) and lacked measurable 9-deaminoamrubicinol.

Having determined myocardial retention or efflux of amrubicin-deaminated metabolites, we eventually corrected the apparent values of amrubicin uptake or clearance and obtained theoretical values that incorporated the role of such metabolites. In experiments with 3 μM anthracycline, amrubicin theoretical uptake was nearly identical with doxorubicin uptake; in experiments with 10 μM anthracycline amrubicin theoretical uptake still ranked ~56% as doxorubicin.

**Table 3**

<table>
<thead>
<tr>
<th>Amrubicin</th>
<th>Membrane Fraction</th>
<th>Soluble Fraction</th>
<th>Plasma</th>
<th>Total</th>
<th>Membrane Fraction</th>
<th>Soluble Fraction</th>
<th>Plasma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td></td>
<td></td>
<td></td>
<td>μM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 μM</td>
<td>1.4 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>4.7 ± 0.5</td>
<td>N.D.</td>
<td>0.05 ± 0.03</td>
<td>0.34 ± 0.05</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>10 μM</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>9.2 ± 1.1</td>
<td>10.1 ± 1.2*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.47 ± 0.1</td>
<td>0.47 ± 0.1</td>
</tr>
</tbody>
</table>

N.D., not detected.

*Strips + plasma.

*P < 0.001 vs. 3 μM.
Amrubicin Distribution in Human Myocardium

469

rubricin was used for comparative purposes. Verapamil (1 μM) increased the steady-state accumulation of doxorubicin by ~38%, which resulted in a concomitant increase of doxorubicin in membrane and soluble fractions of the strips; under comparable conditions, amrubicin steady-state accumulation was only marginally increased by verapamil (Table 5). It is noteworthy that 10 μM verapamil caused fewer effects on doxorubicin or amrubicin accumulation, presumably because at this concentration level calcium channel blockers induced membrane permeation effects that counteracted inhibition of active efflux (Watts and Handy, 2007; Watts, 2010). Steady-state accumulation and efflux of 9-deaminoamrubicin and 9-deaminoamrubicinol were not appreciably influenced by 1 or 10 μM verapamil; there was a trend toward a decreased efflux of 9-deaminoamrubicinol, but this did not reach a level of statistical significance (Fig. 7).

Discussion

Cardiac anthracycline accumulation and the consequent risk of anthracycline-induced cardiotoxicity correlate with plasma anthracycline $C_{\text{max}}$ (Danesi et al., 2002; Minotti et al., 2004); however, anthracyclines bind extensively to plasma proteins and, hence, only a fraction of these drugs is readily available for diffusing in tissues (Whitaker et al., 2008). We demonstrated that in comparison with doxorubicin or epirubicin amrubicin showed a limited accumulation in human myocardium. This information was obtained by incubating human myocardial strips in plasma to which anthracyclines had been added at concentrations similar to the $C_{\text{max}}$ values determined in patients (3 or 10 μM). These concentration levels were ~15 to ~60 times lower than those adopted to see contractile dysfunction of rabbit isolated atria (Mushlin et al., 1993) or ventricular papillary muscles (Olson et al., 1988) incubated in plasma protein-free media. Moreover, the highest anthracycline concentration adopted in this study was comparable with or many times lower than those required for inducing acute dysfunction of isolated rat heart perfused with plasma protein-free media (Monti et al., 1996; Ramond et al., 2008). Limited amrubicin accumulation could therefore be characterized under clinically relevant, nondamaging conditions that avoided confounding effects due to an anomalous diffusion of suprapharmacological concentrations of anthracyclines in the cardiac tissue. As such, the experimental conditions reproduced pharmacokinetic events that occur at the first chemotherapy cycle and

![Diagram](image_url)

Fig. 4. Formation of 9-deaminoamrubicin or 9-deaminoamrubicinol by isolated membrane or soluble fractions of human myocardial strips. A, isolated membrane or soluble fractions (0.6 mg protein/ml) were incubated with increasing concentrations of amrubicin at 37°C in the absence or presence of 0.25 mM NADPH; where indicated membrane fractions had been heated at 100°C for 30 min before incubations. After 4 h, the incubations were extracted and assayed for 9-deaminoamrubicin. Values are means ± S.E. of three to five experiments; values without S.E. are from duplicate experiments. B, isolated membrane or soluble fractions were supplemented with NADPH (0.25 mM), and incubations were assayed for 9-deaminoamrubicinol; values were taken from two representative experiments.

Table 4

Apparent anthracycline uptake and clearance in human myocardial strips

| Anthracycline | Loading Retention Efflux Apparent Uptake Apparent Clearance |
|---------------|------------------------|------------------------|------------------------|------------------------|------------------------|
|               | μM                     | %                      |                        |                        |                        |
| Doxorubicin   | 3                      | 2.4 ± 0.4              | 2.9 ± 0.4              | 5.4 ± 0.2              | 54.3 ± 6.5             |
| Epirubicin    | 3                      | 4.1 ± 0.1              | 3.5 ± 0.1              | 7.5 ± 0.1*             | 45.9 ± 1.1             |
| Amrubicin     | 3                      | 0.1 ± 0.01             | 1.2 ± 0.09             | 1.3 ± 0.08              | 89.0 ± 1.4†             |
| Doxorubicin   | 10                     | 13.1 ± 0.7             | 6.0 ± 0.2              | 19.1 ± 0.6              | 31.6 ± 1.6             |
| Epirubicin    | 10                     | 17.9 ± 1.8             | 7.1 ± 0.4              | 25.0 ± 1.7*             | 29.3 ± 2.1             |
| Amrubicin     | 10                     | 1.2 ± 0.1              | 3.1 ± 0.1              | 4.3 ± 0.1**             | 72.1 ± 1.1†             |

* Significantly higher ($P < 0.001$) than doxorubicin.
** Significantly lower ($P < 0.001$) than doxorubicin or epirubicin.
† Significantly higher ($P < 0.001$) than doxorubicin or epirubicin.
helped to prospectively identify the risk associated with multiple doses of an anthracycline analog or the other.

Limited amrubicin accumulation was demonstrated under conditions of standard 4-h incubations or multiple washout experiments. Standard 4-h incubations were adopted to allow maximal formation and optimize identification of metabolites that could influence myocardial pharmacokinetics of one anthracycline analog in comparison with another; however, standard 4-h incubations do not allow us to distinguish and quantify anthracycline uptake versus clearance (Salvatorelli et al., 2009). To determine whether the low steady-state accumulation of amrubicin could be caused by its reduced uptake and/or its enhanced clearance, we assessed amrubicin and doxorubicin or epirubicin under conditions of loading and multiple washouts that were more suitable for defining the two processes separately (Salvatorelli et al., 2009). Our results suggest that a pattern of increased clearance contributed to limiting amrubicin accumulation in experiments with anthracycline at 3 and 10 µM; reduced amrubicin uptake was shown in experiments with anthracycline at 10 µM, but this was relatively marginal compared with the near-to-complete clearance that occurred in the experiments. A highly favorable clearance was therefore identified as the main pharmacokinetic determinant of reduced amrubicin accumulation in human myocardial samples.

Although amrubicin could be cleared unmodified, the prevailing pattern of amrubicin clearance was determined by the formation of 9-deaminoamrubicin and 9-deaminoamrubicinol that partitioned in plasma. 9-Deaminoamrubicin formation was documented in some preclinical studies of amrubicin, but whether it reflected amrubicin nonspecific breakdown or authentic biotransformation was not explored (Victoria Sung, Cel-

---

**Fig. 5.** Anthracycline efflux from human myocardial strips. Human myocardial strips were incubated under conditions of loading and multiple washouts as described under Materials and Methods. A and B, the efflux of doxorubicin or epirubicin from strips loaded with 3 µM (A) or 10 µM (B) anthracycline. C and D, the cumulative efflux of amrubicin, 9-deaminoamrubicin, and 9-deaminoamrubicinol, from strips loaded with 3 µM (C) or 10 µM (D) amrubicin. The values are means ± S.E. of three experiments (epirubicin and amrubicin) or six experiments (doxorubicin). Values without vertical bars have their S.E. within symbols.

---

**Fig. 6.** Theoretical uptake and clearance of anthracyclines in human myocardial strips. Human myocardial strips were incubated with anthracyclines at 3 or 10 µM under conditions of loading and multiple washouts. Theoretical uptake (A) or clearance (B) was calculated as described under Materials and Methods. The values are means ± S.E. of three experiments (epirubicin and amrubicin) or six experiments (doxorubicin). Values without vertical bars have their S.E. within symbols. *, eipirubicin uptake significantly higher (P < 0.01) than doxorubicin or amrubicin. #, doxorubicin uptake significantly higher (P < 0.01) than amrubicin uptake. **, amrubicin clearance significantly higher (P < 0.01) than doxorubicin or eipirubicin clearance.
Table 5

Effect of verapamil on anthracycline content and distribution in human myocardial strips after standard 4-h incubations with 10 μM doxorubicin or amrubicin

<table>
<thead>
<tr>
<th>Anthracycline</th>
<th>Verapamil</th>
<th>Membrane Fraction</th>
<th>Soluble fraction</th>
<th>Total</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>1</td>
<td>13.1 ± 0.6</td>
<td>5.0 ± 0.8</td>
<td>18.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>16.7 ± 0.6*</td>
<td>8.0 ± 0.5**</td>
<td>24.7 ± 1.0*</td>
<td></td>
</tr>
<tr>
<td>Amrubicin</td>
<td>1</td>
<td>15.0 ± 0.8</td>
<td>6.7 ± 1.8</td>
<td>21.7 ± 2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.5 ± 0.5</td>
<td>0.32 ± 0.1</td>
<td>1.8 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.01 and ** P < 0.025 for doxorubicin with 1 μM verapamil versus doxorubicin alone.

Fig. 7. Lack of effect of verapamil on 9-deaminoamrubicin (top) or 9-deaminoamrubicinol (bottom) efflux from human myocardial strips. Human myocardial strips were incubated under conditions of standard 4-h incubations with 10 μM amrubicin; where indicated, 1 or 10 μM verapamil was included. The values are means ± S.E. of six experiments.

Gene Corporation, personal communication; Yamaoka et al., 1998). Likewise, there is no information about in vivo, tissue-specific, or whole-body clearance of 9-deaminoamrubicin. Our data suggest that 9-deaminoamrubicin forms through an enzymatic, heat-labile process. The apparent mechanisms of 9-deaminoamrubicin formation (nonhydrolytic, NADPH-independent, nonoxidative, double-bond addition) point to the involvement of one or more members of a heterogenous family of carbon-nitrogen lyases (EC 4.3.1, EC 4.3.2) (Barrell et al., 2009). Identifying the lyases involved in amrubicin deamination will require ad hoc investigation. Here, it is worth noting that in biologic systems the amino group of doxorubicin or epirubicin is liable to derivatization but not to removal (Minotti et al., 2004). Facile removal of the amino group therefore seems to be a unique characteristic of amrubicin.

9-Deaminoamrubicin underwent an NADPH-dependent reduction of its side-chain carbonyl group, yielding its secondary alcohol metabolite, 9-deaminoamrubicinol. Studies of reconstituted metabolism showed that the yield of 9-deaminoamrubicinol first augmented and then diminished with increasing 9-deaminoamrubicin concentration. While possibly indicating substrate and/or product inhibition mechanisms, the concentration-dependent and bell-shaped pattern of 9-deaminoamrubicinol formation could help to explain the following observations: 1) in standard 4-h incubations increasing amrubicin concentration from 3 to 10 μM caused a significant increase of 9-deaminoamrubicin formation but not of 9-deaminoamrubicinol, 2) in the same incubations, some 9-deaminoamrubicinol could be recovered from strips exposed to amrubicin at 3 μM but not 10 μM, which was consistent with a continued formation of 9-deaminoamrubicin in the strips that generated lower amounts of 9-deaminoamrubicin, and 3) in loading and multiple washout experiments, 9-deaminoamrubicin formation occurred at 3 μM but not at 10 μM amrubicin.

Our data also show compartmentalization of 9-deaminoamrubicin or 9-deaminoamrubicinol formation: 9-deaminoamrubicin formation occurred in the membrane fraction only, whereas NADPH-dependent 9-deaminoamrubicinol formation occurred in only the soluble fraction. This latter finding was consistent with cytoplasmic localization of carbonyl reductases or aldo-keto reductases known to catalyze two-equivalent reduction of the side-chain carbonyl group of anthracyclines (Salvatorelli et al., 2006; Menna et al., 2008; Kalabus et al., 2010). Our results therefore characterize that amrubicin clearance occurred through three distinct pathways: 1) elimination of unmodified amrubicin, 2) formation of 9-deaminoamrubicin in the membrane fraction, followed by its diffusion in the soluble fraction and efflux in plasma, and 3) cytoplasmic metabolism of 9-deaminoamrubicin to 9-deaminoamrubicinol and elimination of the latter in plasma (Fig. 8). Depending on the experimental conditions (standard 4-h incubations or loading and multiple washouts, with 3 or 10 μM anthracycline), the three pathways occurred with the following rank of order: 9-deaminoamrubicin ≥ unmodified amrubicin > 9-deaminoamrubicinol.

Anthracyclines equilibrate across extracellular fluids and cancer or cardiac cells by passive diffusion; however, cancer cells have long been known to accelerate anthracycline efflux and develop pharmacological resistance by ejecting anthra-
in the heart (Meissner et al., 2002; Wojnowski et al., 2005). tentatively suggested to diminish anthracycline levels whether these metabolites could be unusually good sub-

The ease with which 9-deaminoamrubicin and 9-deaminoamrubicinol are cleared from the strips. Relative contribution to total amrubicin clearance occurs with the following rank of order: 9-deaminoamrubicin > unmodified amrubicin > 9-deaminoamrubicinol

Cmax or steady-state levels in patients who received tolerable doses of verapamil for inhibiting active efflux of anthracyclines or other chemotherapeutics from tumors (Kuhlmann et al., 1985; Mross et al., 1993).

Anthracycline cardiotoxicity may also depend on anthracycline biotransformation to metabolites that cause more cellular damage than their parent molecules (Gille and Nohl, 1997; Gewirtz, 1999; Menna et al., 2008, 2012). During the course of our studies we observed that both doxorubicin and epirubicin underwent metabolization in human myocardial strips; amrubicin, too, formed metabolites other than 9-deaminoamrubicin or deaminoamrubicinol (Salvatorelli et al., 2012). With each anthracycline, however, the net level of metabolite formation was low enough not to influence the pharmacokinetic parameters of anthracycline accumulation that we determined by measuring unmodified doxorubicin, epirubicin, amrubicin, and 9-deaminoamrubicin or 9-deaminoamrubicinol.

In conclusion, amrubicin shows cardiac pharmacokinetics that are different from those of prototypic anthracyclines, such as doxorubicin or epirubicin. In an ex vivo human myocardial strip model, moderate to normal amrubicin uptake was accompanied by a remarkably sustained clearance that made amrubicin attain lower myocardial levels in comparison with doxorubicin or epirubicin. This pharmacokinetic pattern anticipates an important determinant of the cardiac tolerability of amrubicin in approved or investigational clinical settings. In Salvatorelli et al. (2012) we will characterize how amrubicin compared with doxorubicin or epirubicin in converting to metabolites or by-products possibly involved in cardiotoxicity.

**Authorship Contributions**

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

**Conducted experiments:** Salvatorelli and Menna.

**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


Ishizumi K, Ohashi N, and Tanno N (1987) Stereospecific total synthesis of 9-ami-

---

**Fig. 8.** Pathways of amrubicin clearance from human myocardial strips. The box on the left indicates that in the membrane fraction of the strips amrubicin is metabolized by carbon-nitrogen lyase-type enzymes to give 9-deaminoamrubicin (the solid red circle indicates amino group removal and double-bond addition in the A ring). 9-Deaminoamrubicin diffuses from membranes in cytoplasm and converts to 9-deaminoamrubicinol through the action of NADPH-dependent aldo/keto or carbonyl reductase (the red dotted circle indicates two-equivalent reduction of the side-chain carbonyl group). Both amrubicin and 9-deaminoamrubicin or 9-deaminoamrubicinol are cleared from the strips. Relative contribution to total amrubicin clearance occurs with the following rank of order: 9-deaminoamrubicin > unmodified amrubicin > 9-deaminoamrubicinol (see Discussion for explanations).