Paclitaxel and Docetaxel Stimulation of Doxorubicinol Formation in the Human Heart: Implications for Cardiotoxicity of Doxorubicin-Taxane Chemotherapies

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ABSTRACT

Antitumor therapy with the anthracycline doxorubicin is limited by a dose-related cardiotoxicity that is aggravated by a concomitant administration of the taxane paclitaxel. Previous limited studies with isolated human heart cytosol showed that paclitaxel was able to stimulate an NADPH-dependent reduction of doxorubicin to its toxic secondary alcohol metabolite doxorubicinol. Here we characterized that 0.25 to 2.5 μM paclitaxel caused allosteric effects that increased doxorubicinol formation, whereas high concentrations decreased it. Doxorubicinol formation reached its maximum on adding plasma with 6 μM paclitaxel or docetaxel; this corresponded to the partitioning of 1.5 to 2.5 μM taxanes in the cytosol of the strips. Taxane-stimulated doxorubicinol formation was not mediated by vehicles, nor was it caused by increased doxorubicin uptake or de novo protein synthesis; however, doxorubicinol formation was blunted by AL1576. These results show that allosteric interactions with cytoplasmic aldehyde reductases enable paclitaxel or docetaxel to stimulate doxorubicinol formation in human heart. This information serves metabolic insights into the risk of cardiotoxicity induced by doxorubicin-taxane therapies.

The anthracycline doxorubicin and the taxane paclitaxel are both highly active in breast cancer. Doxorubicin and paclitaxel exhibit different mechanisms of action (DNA intercalation and topoisomerase II inhibition versus microtubule stabilization), nonoverlapping toxicities (cardiomyopathy versus neuropathy), and incomplete cross-resistance; therefore, combining doxorubicin and paclitaxel came as a natural step toward an improved treatment of breast cancer (reviewed by Valero and Hortobagyi, 2003). Pivotal trials showed that bolus doxorubicin followed by paclitaxel infusion with a 15-min interval induced excellent response rates in women with metastatic breast cancer but also caused congestive heart failure at a cumulative dose of 420 to 480 mg of doxorubicin/m², which was below the safety limit usually set at 500 to 550 mg of doxorubicin/m² (Gianni et al., 1995). Subsequent pharmacokinetic studies (Gianni et al., 1997) showed that paclitaxel increased the plasma area under the curve (AUC) of doxorubicin by ~30% and more than doubled the AUC of doxorubicinol, a secondary alcohol metabolite that had been shown to mediate cardiomyopathy in laboratory animals (Olson and Mushlin, 1990). Therefore, pharmacokinetic interactions that increased the plasma exposure to

ABBREVIATIONS: AUC, plasma area under the curve; EBPC, ethyl-1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate; AL1576, 2,7-difluorospirofluorene-9,5'-imidazolidine-2',4'-dione; HPLC, high-performance liquid chromatography.

paclitaxel or docetaxel and their clinical vehicles Cremophor EL or polysorbate 80. Low concentrations of taxanes stimulated doxorubicinol formation, whereas high concentrations decreased it. Doxorubicinol formation reached its maximum on adding plasma with 6 μM paclitaxel or docetaxel; this corresponded to the partitioning of 1.5 to 2.5 μM taxanes in the cytosol of the strips. Taxane-stimulated doxorubicinol formation was not mediated by vehicles, nor was it caused by increased doxorubicin uptake or de novo protein synthesis; however, doxorubicinol formation was blunted by AL1576. These results show that allosteric interactions with cytoplasmic aldehyde reductases enable paclitaxel or docetaxel to stimulate doxorubicinol formation in human heart. This information serves metabolic insights into the risk of cardiotoxicity induced by doxorubicin-taxane therapies.

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doxorubicinol were implicated to explain the expected incidence of cardiac events induced by doxorubicin–paclitaxel treatments.

Pharmacokinetic interactions between paclitaxel and doxorubicin or doxorubicinol were attributed to the formulation of paclitaxel in Cremophor EL, a nonionic surfactant that inhibits gp170 and related mechanisms of anthracycline elimination through the bile (Gianni et al., 1997). Therefore, limiting the cumulative dose of doxorubicin to 360 mg/m² and separating doxorubicin and paclitaxel by longer than 4 h were identified as possible measures for preventing excess cardiac toxicity induced by doxorubicin–paclitaxel schedules (Perotti et al., 2003). Replacing paclitaxel with its closely related analog docetaxel was also considered a valuable option; in fact, docetaxel neither interfered with anthracycline elimination (D’Incalci et al., 1998) nor caused more cardiotoxicity when administered 1 h after bolus doxorubicin (Nabholtz et al., 2003), likely because of its formulation in polysorbate 80 instead of Cremophor EL.

Previously, we reported that doxorubicin and taxanes also might interact on metabolic grounds. We showed that both paclitaxel and docetaxel stimulated an NADPH-dependent reduction of doxorubicin to doxorubicinol in isolated human cardiac cytosol (Minotti et al., 2001b), a translational model that obviated potential pitfalls caused by the different enzymology of anthracycline secondary alcohol metabolite formation in laboratory animals versus humans (Maessen et al., 1987; Behnia and Boroujerdi, 1999; Minotti et al., 2004; Salvatorelli et al., 2006). The effect of paclitaxel or docetaxel did not require Cremophor EL or polysorbate 80, nor was it observed on replacing the taxanes with the structurally unrelated tubulin-active Vinca alkaloid vinorelbine; this showed that stimulation of doxorubicinol formation was inherently related to the taxoid backbone of paclitaxel or docetaxel (Minotti et al., 2001b).

These results changed the mechanistic appraisal of the safety or toxicity of doxorubicin-taxane therapies. On the one hand, the ability of paclitaxel to stimulate doxorubicinol formation in human heart cytosol was considered more relevant to cardiotoxicity than was its ability to increase doxorubicinol levels in plasma; in fact, a polar metabolite like doxorubicinol would take too long to partition from plasma and reach cardiac levels high enough to induce toxicity (Olson and Mushlin, 1990; Minotti et al., 2001b). On the other hand, the observation that docetaxel also increased doxorubicinol formation raised the possibility that the cardiac safety of doxorubicin–docetaxel therapies could have been determined primarily by dose-related factors, e.g., a median cumulative dose of only 378 mg of doxorubicin/m² (Nabholtz et al., 2003).

Interestingly, low concentrations of paclitaxel or docetaxel stimulated doxorubicinol formation, but higher concentrations decreased it back to control levels (Minotti et al., 2001b). This pattern of modulation was suggestive of allosteric interactions of paclitaxel or docetaxel with the reductases that converted doxorubicin to doxorubicinol, but limitations in myocardial sample availability did not allow further characterization of this putative mechanism. Moreover, studies with isolated cytosol could not explore other important determinants of the metabolic interactions between doxorubicin and taxanes, e.g., the effects of plasma proteins and vehicles on the partitioning of these drugs in the heart.

Therefore, we extended our studies with human heart cytosol and exploited also a novel model in which whole human myocardium was incubated in plasma and exposed to doxorubicin, taxanes, and their vehicles.

### Materials and Methods

**Chemicals.** We used doxorubicin [7-(3-amino-2,3,6-trideoxy-L-lyxo-hexopyranosyl)doxorubicinone] and doxorubicinol [7-(3-amino-2,3,6-trideoxy-L-lyxo-hexopyranosyl)-13-dihydroxydoxorubicinone] (Nerviano Medical Sciences, Milan, Italy); lypoilized paclitaxel [5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one, 4,10-diacetate, 2-benzoate, 13-ester, with (2R,3S)-N-benzyol-3-phenylisoserine] (Bristol Myers Squibb, Wallingford, CT); lypoilized docetaxel [(2R,3S)-N-carboxy-3-phenyliso-serine, N-tert-buty1 ester, 13-ester, with 5β-20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one, 4-acetate, 2-benzoate, trihydrate] (Rhône-Poulenc Rorer, Vitry-sur-Seine Cedex, France); ethyl-1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylic acid (EBPC) (Tocris Biosciences, Bristol, UK); tolrestat (N-f6-methoxy-5-trifluoromethyl-1-naphthyl(thiocarbonyl))-N-methylglycine) (Wyeth Ayerst Research, Princeton, NJ); and 2,7-difluoroisoprinofluorene-9,5′-imidazolidin-2′,4′-dione (AL1576) (Alcon Laboratories, Fort Worth, TX). Ethanol (formal concentration 17.1 M) was a product of Carlo Erba (Milan, Italy). Cremophor EL (polyoxyethylenelecoglycerol triricinoleate 35), polysorbate 80 (polyoxyethyleneorbitan-20-mono-oleate), quercetin (3,3′,4′,5,7-pentahydroxyflavone), and all other chemicals were obtained from Sigma Aldrich (Milan, Italy).

**Human Myocardial Samples.** Small myocardial samples were obtained from 67 male and female patients (62 ± 4 years) undergoing aortocoronary bypass grafting. All the specimens were derived from the lateral aspect of excluded right atrium and were routinely disposed of by the surgeons during cannulation procedures for cardiopulmonary bypass; therefore, the patients were not subjected to any unjustified loss of tissue. Sampling procedures were in compliance with guidelines of the Institutional Ethical Committee, and written informed consent was obtained from all the patients.

**Reconstitution of Doxorubicin Metabolism in Human Heart Cytosol.** Pools of 10 to 15 myocardial specimens were processed for cytosol preparation by homogenization, ultracentrifugation, and 65% ammonium sulfate precipitation of 105,000g supernatants (Minotti et al., 2001b). Next, cytosolic proteins were treated with 100 mM DTT, pH 8.9, and gel-filtered on homemade Sepharose 6B minicolumns to induce disassembly of cytoplasmic Fe-S clusters that otherwise oxidized doxorubicin back to doxorubicin and limited metabolite accumulation and kinetic measurements (Minotti et al., 2001b; Salvatorelli et al., 2006). Doxorubicin metabolism was reconstituted for 4 h in 0.25-mL incubations that contained cytosol (0.15 mg protein), NADPH (0.25 mM), and 50 μM doxorubicin in 0.3 M NaCl, pH 7.0, 37°C. Ethanol-dissolved taxanes or inhibitors were included as appropriate, and control incubations contained an equivalent volume of ethanol (5 μL, formally corresponding to 0.34 mM ethanol); under such defined conditions ethanol never precipitated cytosolic proteins.

**Kinetics of Basal or Taxane-Modulated Doxorubicinol Formation.** In the experiments for determination of Vmax doxorubicinol was measured over the linear phase of the reaction (usually 30–60 min); in the experiments for determination of Km, doxorubicinol was used over a 10 to 500 μM range, and the reaction was stopped after 4 h. The experimental data were analyzed according to the following reactions:

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_{cat}} E + P \\
1 \to K_1 \xrightarrow{1} K_2 \\
1 + E + S \xrightarrow{k_1} ES \xrightarrow{k_{cat}} E + P
\]
day coefficients of variation were 2 and 7%, respectively. Moreover, control experiments showed that taxanes or inhibitors did not interfere with the extraction and HPLC assay of doxorubicin or doxorubicinol.

Other Assays and Conditions. Proteins were measured by the bicinchoninic acid method. Myocardial release of myoglobin, tropolin T, and creatinine kinase isoenzyme MB was determined by an electrochemiluminescence immunoassay with an Elecsys 2010 Analyzer (Roche Diagnostics, Mannheim, Germany), according to manufacturer’s instructions. Unless otherwise indicated, all the values were means ± S.E. of at least three experiments. Data were analyzed by one-way analysis of variance, followed by Bonferroni’s test for multiple comparisons; where indicated, unpaired Student’s t test was also applied. Differences were considered significant when p < 0.05. Other details are given under Results and in figure legends and tables.

Results

Doxorubicinol Formation in Human Heart Cytosol. Doxorubicin is composed of a tetracyclic ring with adjacent quinone-hydroquinone moieties, a short side chain with a carbonyl group at C-13, and an aminosugar called daunosamine. Doxorubicinol is formed through a two-electron reduction of the side chain carbonyl group (Fig. 1A). In this study, the reconstitution of doxorubicin and NADPH in human cardiac cytosol caused the formation of 1.5 ± 0.2 nmol of doxorubicinol/mg protein/4 h (range 1.1–2.4, n = 8).

Purified human recombinant carbonyl reductase, a member of the superfamily of short chain dehydrogenases/reductases, was shown to catalyze the conversion of anthracyclines to secondary alcohol metabolites (Slupe et al., 2005); moreover, mice with a cardiac-specific overexpression of human carbonyl reductase were shown to exhibit an increased conversion of doxorubicin to doxorubicinol and an accelerated course of development of cardiomyopathy (Forrest et al., 2000), whereas mice bearing a null allele of the carbonyl reductase gene exhibited lower levels of doxorubicinol formation and less severe cardiac toxicity (Olson et al., 2003). These reports highlighted the possible role of carbonyl reductases in doxorubicinol formation and cardiotoxicity; therefore, we characterized whether quercetin, a known inhibitor of carbonyl reductases (Forrest and Gonzalez, 2000), diminished doxorubicinol formation in human heart cytosol. As shown in Fig. 1B, quercetin diminished doxorubicinol formation, but this occurred only at high concentrations of the inhibitor (IC50 ~ 0.1 mM). We next assessed the roles of aldose and aldehyde reductases, members of a superfamily of aldoketo reductases that were also considered to form anthracycline secondary alcohol metabolites (Felsted et al., 1974; Behnia and Boroujerdi, 1999). The role of these enzymes was probed with EBPC (specific inhibitor of aldose reductases) (Mylari et al., 1991), tolrestat (mixed inhibitor of aldose and aldehyde reductases) (Barski et al., 1996), and AL1576 (specific inhibitor of aldehyde reductases) (Barski et al., 1995, 1996). EBPC exhibited a very high IC50 value (~1 mM), whereas tolrestat and AL1576 exhibited IC50 values of 30 μM or 5 μM, respectively (see also Fig. 1B). These results identified aldehyde reductases as important catalysts of doxorubicinol formation in human heart cytosol, but the possible contribution from carbonyl reductases should not be ruled a priori.

Taxane Modulation of Doxorubicinol Formation in Human Heart Cytosol. Both paclitaxel and docetaxel influ-
enced doxorubicinol formation in a bell-shaped concentration-dependent manner, i.e., low concentrations of paclitaxel or docetaxel increased doxorubicinol formation, whereas higher concentrations decreased it back to control levels. The only difference between paclitaxel and docetaxel pertained to the range of concentrations within which they could stimulate doxorubicinol formation; paclitaxel began increasing doxorubicinol at 0.25 M and caused a significant stimulation at 0.5 to 2.5 M, whereas docetaxel increased doxorubicinol and caused a significant stimulation at 1 to 2.5 M (Fig. 2, A and B). Paclitaxel and docetaxel were probed in cytosol samples that exhibited slightly different basal levels of doxorubicinol formation (nmol/mg protein/4 h: 1.4 ± 0.2 in the experiments with paclitaxel versus 1.8 ± 0.2 in the experiments with docetaxel, n = 3, overall range 1.1–2.1) (see also Fig. 2, A and B). It follows that the absolute levels of taxane-stimulated doxorubicinol formation were also different, but normalizing them to basal doxorubicinol showed that both paclitaxel and docetaxel caused a 54 to 59% maximum stimulation at 1 M.

We determined the effects of enzyme inhibitors on taxane-stimulated doxorubicinol formation; paclitaxel and docetaxel were used 1 M, whereas inhibitors were used 5 M. Under such defined conditions, only AL1576 caused a significant inhibition of doxorubicinol formation, which decreased to 50% of its level of formation in taxane-free incubations (Fig. 3A); moreover, the efficacy with which AL1576 inhibited doxorubicinol formation did not change on varying paclitaxel or docetaxel over a 0.25 to 10 M range (Fig. 3B). These results showed that aldehyde reductases catalyzed both basal and taxane-stimulated doxorubicinol formation and that AL1576 potently abrogated the effects of taxanes.

The effects of taxanes on the kinetics of doxorubicinol formation were examined (Table 1). One micromolar paclitaxel or docetaxel decreased the $K_m$ value by 44 or 37% while also causing a more remarkable 3- to 2.5-fold increase of the $V_{\text{max}}$ value; therefore, 1 M taxanes caused a ~4- to 5-fold improvement of the apparent catalytic efficiency ($V_{\text{max}}/K_m$) with which the human heart cytosol converted doxorubicin to doxorubicinol. Ten micromolar paclitaxel or docetaxel only marginally influenced the $V_{\text{max}}$ value but increased the $K_m$ value by 108 or 67% as compared with taxane-free controls; therefore, 10 M taxanes caused a ~15 to 30% reduction of the catalytic efficiency with which the cytosol formed doxorubicinol.

These results showed that low concentrations of paclitaxel
of the experimental results, but a virtual fitting procedure could be adopted (see equations 1–3 under Materials and Methods). By fixing the $V_{\text{max}}/K_m$ value measured experimentally in the absence of taxanes ($R^0$), and by allowing the equilibrium dissociation constant of the taxane binding to the regulatory site ($^{2}K$) to float between 0.2 and 1.5 $\mu M$ taxanes, we could approximate the $V_{\text{max}}/K_m$ value for the reductase(s) ligated by taxanes at the regulatory site ($^{2}R$) and the equilibrium dissociation constant of the taxane binding to the catalytic site ($^{2}K$). In the case of paclitaxel, the fitting procedure determined that $^{2}K$ was $\sim$5-fold $^{2}K$ (6.5 versus 1.2 $\mu M$), whereas $^{2}R$ was $\sim$40-fold $R^0$ and $\sim$8-fold the $V_{\text{max}}/K_m$ value measured experimentally with 1 $\mu M$ paclitaxel ($82 \times 10^{-4}$ versus $1.8 \times 10^{-4}$ or $9.5 \times 10^{-4}$, respectively) [ml/(mg protein/min)]. By fitting the experimental data to $R^0$, $^{2}K$, and $^{2}R$, the $V_{\text{max}}/K_m$ values over 0.25 to 10 $\mu M$ paclitaxel were eventually derived. As shown in Fig. 4, the fitting followed the usual bell-shaped concentration-dependent pattern, with an apparent peak of the $V_{\text{max}}/K_m$ value at 0.5 $\mu M$ paclitaxel. These analyses suggested that low concentrations of taxanes increased the catalytic competence of doxorubicin reductases by binding with high affinity to a regulatory/allosteric site; however, taxanes also decreased the catalytic competence of the reductases by binding with low affinity to the catalytic site, eventually competing with doxorubicin. The kinetic model, and the marked difference between $^{2}R$ and $V_{\text{max}}/K_m$ value determined in incubations with 1 $\mu M$ paclitaxel, outlined that $^{2}R$ could not be measured experimentally because a parallel binding of the taxane to the catalytic site impeded the achievement of the reductases fully ligated at the regulatory site.

**Doxorubicinol Formation in Human Myocardial Strips.** We assessed anthracycline distribution and metabolism in human myocardial strips incubated in plasma and exposed to 1 or 10 $\mu M$ doxorubicin for 4 h. Under such defined conditions, the strips did not release myoglobin, troponin T, or creatine kinase-MB isozyme (not shown). This showed that the cardiac tissue had not been exposed to an acute

or docetaxel stimulated doxorubicinol formation mainly by increasing the $V_{\text{max}}$ value, whereas higher concentrations inhibited doxorubicinol formation mainly by increasing the $K_m$ value. One such pattern could only be explained by assuming that taxanes bound with different affinities to at least two sites on cytoplasmic reductases (e.g., a regulatory/allosteric site and another site that was identical with the catalytic site or part of it). Because of limitations in sample availability, the effects of paclitaxel or docetaxel on the Michaelis constants of doxorubicin-reductase reactions were measured only under the experimental conditions reported in Table 1 (0, 1, and 10 $\mu M$ taxane). This approach and the limited pool of available data did not allow an iterative fitting
damage that could have influenced the mechanisms and levels of formation of anthracycline metabolites (Gewirtz, 1999; Salvatorelli et al., 2006). Doxorubicin distributed to the soluble or membrane fractions of the strips in a concentration-dependent manner (Fig. 5A). The total levels of doxorubicin recovered in the strips (soluble + membrane fraction) formally exceeded those added in plasma (1.8 ± 0.2 or 14.4 ± 3 μM at 1 or 10 μM doxorubicin, respectively, n = 7–12). This finding was consistent with the known ability of the cardiac tissue to concentrate anthracyclines, but the accumulation factors were significantly lower than those determined by tissue to concentrate anthracyclines, but the accumulation factors were significantly lower than those determined by others when studying isolated rabbit heart in common laboratory buffers (factors ≥3) (Olson et al., 1988). This denoted the ability of plasma proteins to bind doxorubicin and limit its tissue partitioning. Doxorubicinol was found in the soluble fraction of myocardial strips exposed to 10 μM doxorubicin (Fig. 5B), and its levels correlated with those of doxorubicin in that fraction (r² = 0.48, p = 0.018). The apparent lack of doxorubicinol formation in the membrane fraction of the strips could not be attributed to experimental artifacts, e.g., an inadequate extraction of doxorubicinol from that fraction; in fact, titrating membrane fractions from control strips with known amounts of doxorubicin or doxorubicinol showed that the extraction method adopted in this study (4-fold volume of 1:1 CHCl₃/CH₃OH) gave a near-to-complete recovery of either anthracycline molecule (97 ± 1 versus 95 ± 2%, n = 6–9). Thus, doxorubicinol was formed primarily by cytoplasmic reductases.

**Taxane Modulation of Doxorubicinol Formation in Human Myocardial Strips.** We characterized the effects of taxanes and their vehicles on doxorubicinol formation in human myocardial strips exposed to 10 μM doxorubicin. Paclitaxel was used 2 and 6 or 15 μM, whereas Cremophor EL was used 10 μM/ml. Ethanol (10 μM/ml) was also included to reproduce its 1:1 ratio to Cremophor EL in the complete cosolvent system of the clinical formulation of paclitaxel. Docetaxel was similarly used 2 and 6 or 15 μM, along with a clinically relevant concentration of 1 μl of polysorbate 80/ml and an equivalent volume of ethanol (Loos et al., 2003). Under such defined conditions, paclitaxel and docetaxel distributed to the soluble fraction of the strips in a concentration-dependent manner (Table 2). Plotting the levels of taxanes in the soluble fraction versus those of doxorubicin or doxorubicinol.

### TABLE 2

<table>
<thead>
<tr>
<th>Taxane in Plasma</th>
<th>Taxane in the Soluble Fraction</th>
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<tbody>
<tr>
<td></td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>2.5 ± 0.5*</td>
</tr>
<tr>
<td>15</td>
<td>6.4 ± 0.9*</td>
</tr>
</tbody>
</table>

*a p < 0.05 versus 2 μM paclitaxel (p > 0.05 by unpaired Student’s t test).

*b p < 0.01 versus 2 or 6 μM paclitaxel.

*c p < 0.05 versus 2 μM docetaxel (p < 0.01 by unpaired Student’s t test).

*d p < 0.01 versus 2 or 6 μM docetaxel.

**Fig. 5.** Doxorubicin distribution and doxorubicinol formation in human myocardial strips. Human myocardial strips were incubated in plasma that contained 1 or 10 μM doxorubicin. After 4 h, the strips were assayed for doxorubicin or doxorubicinol in soluble or membrane fractions as described under Materials and Methods.

**Fig. 6.** Effects of increasing concentrations of paclitaxel or docetaxel on the levels of doxorubicin or doxorubicinol in the soluble fraction of human myocardial strips. Human myocardial strips were incubated in plasma that contained 10 μM doxorubicin and 2, 6, or 15 μM paclitaxel or docetaxel formulated in cosolvent systems (10 μl of ethanol/ml + 10 μl of Cremophor EL/ml, or 1 μl of ethanol/ml + 1 μl of polysorbate 80/ml, respectively). After 4 h, the strips were assayed for taxanes and doxorubicin or doxorubicinol in the soluble fraction. A, the relation between the individual levels of taxanes and those of doxorubicin. Values were expressed as percentages of doxorubicin content in strips exposed to ethanol/Cremophor EL without paclitaxel (μM: 4.2 ± 0.9, range 0.9–10, n = 12) or ethanol/poly sorbate without docetaxel (μM: 3.8 ± 0.7, range 1–8, n = 9). B, the relation between the individual levels of taxanes and doxorubicinol. Values were expressed as percentages of doxorubicinol content in strips exposed to ethanol/Cremophor EL without paclitaxel (μM: 0.031 ± 0.05, range 0.01–0.06, n = 12) or ethanol/poly sorbate without docetaxel (μM: 0.037 ± 0.01, range 0.01–0.08, n = 9).
doxorubicinol showed that paclitaxel and docetaxel did not modify the steady-state levels of doxorubicin (Fig. 6A) but modulated its conversion to doxorubicinol in a concentration-dependent bell-shaped manner (Fig. 6B). Maximal stimulation of doxorubicinol formation (56 ± 6 or 74 ± 22%) occurred on adding plasma with 6 μM paclitaxel or docetaxel, respectively; under the latter condition, the levels of paclitaxel or docetaxel in the soluble fraction of the strips averaged 2.4 ± 0.5 or 1.5 ± 0.3 μM, respectively (n = 3–6, p > 0.05 for paclitaxel versus docetaxel), similar to the taxane levels that stimulated doxorubicinol formation in the isolated human heart cytosol (cf. Fig. 2, A and B). Further experiments showed that 1) neither ethanol nor ethanol/Cremophor EL or ethanol-polysorbate 80 increased doxorubicinol formation; 2) an 100 μM bolus of cycloheximide, potent inhibitor of protein synthesis (Engel et al., 1999), did not prevent the stimulation of doxorubicinol formation induced by 6 μM paclitaxel or docetaxel formulated in their cosolvent systems; and 3) the levels of paclitaxel or docetaxel in the soluble fraction of the strips always proved similar to those that stimulated doxorubicinol formation in the isolated cytosol (Tables 3 and 4). Adding plasma with 2 to 15 μM paclitaxel or docetaxel caused a concentration-dependent distribution of either taxane also to the membrane fraction of the strips, but this was not accompanied by the formation of measurable doxorubicinol in that fraction (not shown).

We next determined whether AL1576 inhibited doxorubicinol formation in myocardial strips exposed to 6 μM taxanes and their cosolvent systems. AL1576 distributed to the soluble fraction of these strips in a concentration-dependent manner (Table 5), thus diminishing and eventually abolishing doxorubicinol formation (Fig. 7, A and B). The effect of AL1576 could not be attributed to an impaired partitioning of taxanes or doxorubicin in the soluble fraction of the strips; AL1576 tended to increase the levels of doxorubicin in that fraction, likely because its high affinity for plasma proteins caused the displacement of some doxorubicin and increased the fraction of unbound anthracy-

### Table 3

Doxorubicin distribution and doxorubicinol formation in the soluble fraction of human myocardial strips exposed to ethanol and Cremophor EL, with or without paclitaxel.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Paclitaxel</th>
<th>Doxorubicin</th>
<th>Doxorubicinol</th>
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<tr>
<td>μM</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Ethanol</td>
<td>6 ± 1</td>
<td>0.06 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>+ Ethanol</td>
<td>7.1 ± 2</td>
<td>0.06 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>+ Cremophor EL</td>
<td>6.5 ± 1</td>
<td>0.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>+ Ethanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Cremophor EL</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>+ Paclitaxel</td>
<td>1.9 ± 0.2</td>
<td>5.9 ± 1.5</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>+ Ethanol</td>
<td>+ Cremophor EL</td>
<td>+ Paclitaxel</td>
<td>+ Cycloheximide</td>
</tr>
</tbody>
</table>

* 10 μM/mL.
* 10 μM/mL.
* 6 μM.
* 100 μM.

| p < 0.05 versus control, ethanol, or ethanol/Cremophor EL.

Collectively, these experiments showed that neither vehicles nor modifications of doxorubicin distribution contributed to stimulating doxorubicinol formation in myocardial strips exposed to taxanes. Inhibition of doxorubicinol formation by AL1576 but not cycloheximide pointed to direct interactions of paclitaxel or docetaxel with cytoplasmic aldehyde reductases.
Taxanes, Doxorubicin Metabolism, and Cardiotoxicity

Fig. 7. Concentration-dependent effects of AL1576 on the levels of taxanes, doxorubicin, and doxorubicinol in the soluble fraction of human myocardial strips. Human myocardial strips were incubated in plasma that contained 10 μM doxorubicin and 6 μM paclitaxel or docetaxel formulated in ethanol/Cremophor EL or ethanol/polysorbate 80, as described in the legends to Tables 3 and 4. AL1576 was also added at 1, 10, and 100 or 500 μM. After 4 h, the strips were assayed for taxanes and anthracyclines in the soluble fraction. A, the effects of AL1576 on the levels of paclitaxel and doxorubicin or doxorubicinol. Values were expressed as percentages of the corresponding levels in the soluble fractions of strips exposed to ethanol/Cremophor EL/paclitaxel without AL1576

Discussion

Doxorubicin-induced cardiotoxicity is determined by the cardiac levels and reactivity of doxorubicin and its metabolites. In this regard, several lines of evidence indicate that doxorubicinol is ~20 to 40 times more potent than doxorubicin at inhibiting calcium-handling proteins and related contractile events (Olson et al., 1988; Olson and Mushlin, 1990; Minotti et al., 2001a), shows a higher potency in suppressing the expression of the ryanodine receptor 2 (Gambriel et al., 2002), exhibits a unique reactivity toward the Fe-S cluster of cytoplasmic aconitase, and disrupts its functions in iron and energy metabolism (Minotti et al., 2004). Post-mortem studies of patients deceased long after anthracycline administration also showed that the cardiac levels of doxorubicinol equaled those of doxorubicin and exceeded those of anthracycline aglycones; this showed that a polar metabolite like doxorubicinol exhibited a reduced cardiac clearance as compared with doxorubicin or apolar metabolites (Stewart et al., 1993). Therefore, several factors allow doxorubicinol to accumulate in the heart and play an important role in cardiotoxicity, possibly amplifying the damage induced by other doxorubicin-derived species (e.g., free radicals). Thus, anomalous increases of doxorubicinol formation in the heart were expected to accelerate the progression of cardiotoxicity and cause the development of heart failure at lower than expected cumulative doses of doxorubicin (Minotti et al., 2004). We extended our previous work with isolated human heart cytosol and showed that the bell-shaped concentration-dependent effects of paclitaxel on doxorubicinol formation were accompanied by consistent modifications of the $V_{\text{max}}/K_{\text{m}}$ values with which the cytosol metabolized doxorubicin, showing that paclitaxel acted as an allosteric modulator of anthracycline reductases. These changes were not accompanied by formation of paclitaxel metabolites (not shown), indicating that paclitaxel acted through direct interactions with cytoplasmic reductases. Virtual fitting of the experimental data also suggested that low concentrations of paclitaxel stimulated DOXOL formation by binding with high affinity to a regulatory site, whereas higher concentrations inhibited doxorubicinol formation by binding with low affinity to the catalytic site, eventually competing with the substrate. Here it is worth noting that doxorubicinol was formed primarily by AL1576-inhibitable aldehyde reductases, a subfamily of aldo-keto reductases whose structural and functional features may well fit in the aforesaid mechanisms of modulation. For example, the C-terminal loop of these reductases accommodates the cofactor binding site, participates in the formation of the active site, and contributes to determining substrate specificity, with all such domains and functions being exposed to potential modulators (Barski et al., 1995, 1996). We also showed that the efficacy with which AL1576 inhibited doxorubicinol formation was independent of the absence or presence of increasing concentrations of paclitaxel (cf. Fig. 3). This latter finding would be consistent with the mode of action of AL1576, which inhibits aldehyde reductases by tightly binding to and blocking dissociation of an enzyme-NADP$^+$ complex (Barski et al., 1995). Therefore, AL1576 should displace paclitaxel from the reductases or induce conformational changes that prevented its association with regulatory or catalytic sites, eventually nullifying the effects of paclitaxel on doxorubicinol formation.

Plasma proteins bind doxorubicin and paclitaxel, limiting their diffusion in tissues (Finlay and Baguley, 2000; ten Tije et al., 2003); moreover, Cremophor EL forms micelles that entrap doxorubicin or paclitaxel and may further attenuate their diffusion in tissues (Kessel, 1992; Ng et al., 2004). Therefore, a major effort was paid to measuring doxorubici-
nol in human myocardial strips incubated in plasma and exposed to doxorubicin in the absence or presence of paclitaxel and its ethanol-Cremophor EL cosolvent system. Under such defined conditions, doxorubicin still diffused in myocardial strips and converted to doxorubicinol. The basal levels of doxorubicinol formation were ~3- to ~15-fold lower than the median or highest concentration of doxorubicinol in post-mortem cardiac samples (~0.06 versus ~0.17 or ~0.9 μM, respectively) (cf. Table 3 and work by Stewart et al., 1993). This marked difference may be explained by considering that post-mortem samples derived from patients exposed to cumulative doses of doxorubicin, whereas our experimental model characterizes doxorubicinol formation that occurs in response to a single dose of doxorubicin. It is noteworthy that paclitaxel also diffused in myocardial strips and modulated doxorubicinol formation in a concentration-dependent bell-shaped manner that was not mediated by its vehicle nor by modifications of doxorubicin uptake or de novo protein synthesis. Furthermore, AL1576 inhibited paclitaxel-stimulated doxorubicinol formation without affecting doxorubicin or paclitaxel uptake, and the concentration of AL1576 that decreased doxorubicinol to ~50% of its unstimulated level was the same as that characterized in isolated cytosol samples.

A pharmacologically relevant issue pertains to the plasma levels of doxorubicin and paclitaxel formulations that caused an increased formation of doxorubicinol in myocardial strips. Doxorubicin and paclitaxel were 10 and 6 μM, similar to their respective C max in patients receiving a 5-min bolus of 60 mg of doxorubicin/m 2 followed 15 min later by a 3-h infusion of 200 mg of paclitaxel/m 2 (Gianni et al., 1997); moreover, Cremophor EL was 10 μl/ml, as was its C max in patients receiving a similar paclitaxel infusion (Sparreboom et al., 1998). Therefore, paclitaxel-induced doxorubicinol formation occurred at clinically relevant drug levels, especially if one appreciates that cardiotoxicity also correlates with the C max of doxorubicin (Danesi et al., 2002). A related important finding was that adding plasma with 6 μM paclitaxel caused the formation of myocardial cytoplasmic levels of paclitaxel similar to those that stimulated doxorubicinol formation in the isolated cytosol. These results support the concept that an almost concomitant administration of doxorubicin and paclitaxel would be accompanied by an increased formation of doxorubicinol in the heart, thus explaining how this treatment induced cardiomyopathy at lower than expected cumulative doses of doxorubicin.

Docetaxel did not aggravate doxorubicin-related cardiotoxicity in women with metastatic breast cancer, but our studies with human heart cytosol confirm that it modulates doxorubicinol formation through the same mechanisms described with paclitaxel. Docetaxel proved similar to paclitaxel also when delivered to myocardial strips incubated in plasma in the presence of its vehicle polysorbate 80, which was included to account for its possible interferences with the ratio of free to protein-bound docetaxel (Loos et al., 2003). These observations support the concept that clinically relevant metabolic interactions between doxorubicin and docetaxel might have been precluded by a cautionary reduction of the cumulative dose of doxorubicin, resulting in a limited formation of doxorubicinol in the heart. Other dose-related factors also may be considered. Stimulation of doxorubicinol formation occurred on adding plasma with 10 μM doxorubicin and 6 μM docetaxel, similar to the experiments with doxorubicin and paclitaxel. In patients treated with doxorubicin followed by docetaxel, the C max of doxorubicin might nonetheless be lower than ~10 μM because doxorubicin was used 50 mg/m 2 over 15 min instead of 60 mg/m 2 over 5 min (Nabholtz et al., 2003). Likewise, in patients receiving doxorubicin—docetaxel treatments, docetaxel was used 75 mg/m 2 over 1-h infusion (Nabholtz et al., 2003), a schedule that per se would give a C max of only ~2 μM (Baker et al., 2004). Therefore, one might conclude that the cardiac safety of doxorubicin—docetaxel was determined not only by the lower cumulative dose of doxorubicin administered to the patients but also by the lower C max attained by doxorubicin and docetaxel at each doxorubicin—docetaxel cycle. Further complexity is introduced by possible pharmacokinetic interactions between doxorubicin and docetaxel. We mentioned that in women with metastatic breast cancer doxorubicin did not interfere with doxorubicin elimination, but the same studies showed that the AUC of docetaxel was almost doubled by the previous infusion of doxorubicin (D’Incalci et al., 1998); in such a case, the cardiac exposure to docetaxel might be higher than generally assumed, and cardiotoxicity would be limited primarily by the pharmacokinetics and cumulative doses of doxorubicin. In assessing the relative importance of all such factors, it may be worth considering recent studies in which four cycles of doxorubicin—paclitaxel or six cycles of doxorubicin—docetaxel were used as primary or adjuvant treatments of operable breast cancer. In these studies, the cumulative dose of doxorubicin was higher with docetaxel than paclitaxel (300 versus 240 mg/m 2 in >90% of evaluable patients), and comparisons with taxane-free anthracycline regimens showed that only doxorubicin—docetaxel caused a trend toward more cardiotoxicity (Nabholtz et al., 2002; Gianni et al., 2005; Martin et al., 2005). Thus, the safety or toxicity of doxorubicin—docetaxel versus doxorubicin—paclitaxel seems to be determined primarily by the cumulative dose of doxorubicin associated with the taxane.

In summary, both paclitaxel and docetaxel act as allosteric modulators of cytoplasmic reductases and stimulate doxorubicinol formation in human myocardium. These observations offer novel insights into the cardiotoxicity induced by doxorubicin—paclitaxel schedules, and raise caution against combining docetaxel with cumulative doses of doxorubicin higher than those adopted in available clinical studies. The translational models of human heart described in this study will prove useful in screening the safety of any new anthracycline-taxane regimen.

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References


