Inhibition of Anthracycline Alcohol Metabolite Formation in Human Heart Cytosol: A Potential Role for Several Promising Drugs

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ABSTRACT

The clinical efficacy of anthracyclines (e.g., doxorubicin and daunorubicin) in cancer therapy is limited by their severe cardiotoxicity, the etiology of which is still not fully understood. The development of anthracycline-induced cardiomyopathy has been found to correlate with myocardial formation and accumulation of anthracycline secondary alcohol metabolites (e.g., doxorubicinol and daunorubicinol) that are produced by distinct cytosolic NADPH-dependent reductases. The aim of the current study is to identify chemical compounds capable of inhibiting myocardial reductases implied in anthracycline reductive metabolism in an attempt to decrease the production of cardiotoxic C-13 alcohol metabolites. Among the variety of tested compounds (metal chelators, radical scavengers, antioxidants, β-blockers, nitrone spin traps, and lipid-lowering drugs), ebselen, cyclopentenone prostaglandins, nitric oxide donors, and short-chain coenzyme Q analogs resulted in being effective inhibitors of both doxorubicinol and daunorubicinol formation. In particular, ebselen (as well as ebselen diselenide, its storage form in the cells) was the most potent inhibitor of cardiotoxic anthracycline alcohol metabolites with 50% inhibition of doxorubicinol formation at 0.2 mol Eq of ebselen with respect to doxorubicin concentration. The high efficacy, together with its favorable pharmacological profile (low toxicity, lack of adverse effects, and metabolic stability) portends ebselen as a promising cardioprotective agent against anthracycline-induced cardiotoxicity.

Introduction

The anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DNR) are among the most potent anticancer drugs ever developed, and despite half a century of clinical use they continue to play, either individually or in combination with other chemotherapeutics, an undisputed role in the treatment of a variety of hematologic neoplasms and solid tumors (Minotti et al., 2004; Gianni et al., 2008; Blanco et al., 2012). Moreover, anthracyclines are an essential component of childhood cancer therapy, as evidenced by their incorporation into more than 50% of frontline therapeutic regimens (Blanco et al., 2012). Unfortunately, the clinical utility of anthracyclines is severely limited by their selective toxicity for myocardial tissue, leading to a progressive cardiomyopathy that irreversibly evolves to congestive heart failure (Minotti et al., 2004; Gianni et al., 2008; Menna et al., 2012). Although the etiopathogenesis of anthracycline-related cardiomyopathy has not yet been completely elucidated (Minotti et al., 2004; Gianni et al., 2008; Mordente et al., 2009; Menna et al., 2012), the cardiotoxicity is thought to be due to a multifactorial process that correlates with myocardial accumulation of anthracyclines, their byproducts, and/or their metabolites (Minotti et al., 2004; Salvatorelli et al., 2006; Gianni et al., 2008; Menna et al., 2008; Mordente et al., 2009; Menna et al., 2012; Octavia et al., 2012). Current thinking is that anthracyclines are cardiotoxic per se, but develop further toxicity after intracellular activation by reductive metabolism (Salvatorelli et al., 2006; Menna et al., 2008; Mordente et al., 2009; Menna et al., 2012). One-electron reduction of the quinone moiety of anthracyclines and the subsequent semiquinone redox cycling result in reactive oxygen species overproduction that causes oxidative stress and energy depletion in cardiomyocytes (Minotti et al., 2004; Tokarska-Schlattner et al., 2006; Gianni et al., 2008; Mordente et al., 2009; Octavia et al., 2012). Alternatively, two-electron reduction of the side chain C-13 carbonyl group converts anthracyclines to their secondary alcohol metabolites, doxorubicinol (DOXol) or daunorubicinol (DNRol), which are much less effective in killing cancer cells (Heibei et al., 2012) but remarkably more potent than their parent compounds at impairing myocardial cell calcium (Menna et al., 2012; Octavia et al., 2012) and iron homeostasis (Minotti et al., 1998, 2004). Oxidative stress (Minotti et al., 2004; Salvatorelli et al., 2006; Gianni et al., 2008; Menna et al., 2012; Octavia et al., 2012), mitochondrial dysfunction (Tokarska-Schlattner et al., 2006; Mordente et al., 2012), energy depletion (Minotti et al., 2004; Tokarska-Schlattner et al., 2006), ion dysregulation (Minotti et al., 2004; Menna et al., 2012), and concomitant alterations of the cardiосpecific signaling pathways (Mordente et al., 2012) can also be assumed to be part of the multifactorial process that eventually leads to cardiomyopathy.

The involvement of secondary alcohol metabolites in anthracycline-induced cardiomyopathy is indicated by the evidence presented in

ABBREVIATIONS: AKR, aldo-keto reductase; CBR1, carbonyl reductase 1; CoQ, coenzyme Q; cyPG, cyclopentenone prostaglandin; DEANO, diethylamine NONOate; DNR, daunorubicin; DNRol, daunorubicinol; DOX, doxorubicin; DOXol, doxorubicinol; DTT, dithiothreitol; GSH, glutathione; GSNO, S-nitrosoglutathione; GSSG, glutathione disulfide; IDB, idebenone; NO, nitric oxide; PG, prostaglandin; SNAP, (+)-S-nitroso-N-acetylpenicillamine.
several biochemical, pharmacokinetic, and genetic studies (Minotti et al., 2004; Salvatorelli et al., 2006; Gianni et al., 2008; Mordente et al., 2009; Ferguson et al., 2015). Anthracycline alcohol metabolite formation is catalyzed mainly by distinct cytosolic NADPH-dependent oxidoreductases (Mordente et al., 2003; Jin and Penning, 2007; Oppermann, 2007; Bains et al., 2010; Malátková et al., 2010; Blanco et al., 2012) that metabolize a broad range of endogenous and exogenous carbonyl-containing compounds, including steroids, eicosanoids, cofactors, neurotransmitters, and polyols (Jin and Penning, 2007; Oppermann, 2007; Bains et al., 2010; Malátková et al., 2010). Moreover, the conversion of C-13 carbonyl moieties into an alcohol group renders anthracycline secondary alcohol metabolites appreciably more polar than the parent drugs (Salvatorelli et al., 2007; Gianni et al., 2008; Menna et al., 2008). Accordingly, anthracycline alcohol metabolites, due to their lowered clearances, tend to accumulate in cardiomyocytes forming a long-lived drug reservoir that eventually represents the only or prevailing remnant of an anthracycline treatment (Gianni et al., 2008; Menna et al., 2008). Therefore, the unique pharmacokinetic characteristics of secondary alcohol metabolites might explain how anthracycline regimens foreshadow a lifelong risk of cardiotoxicity (Gianni et al., 2008; Menna et al., 2008).

The aforementioned reasoning indicates that inhibitors of myocardial reductases might be useful in mitigating cardiotoxicity and improving the therapeutic index of these anticancer drugs. Although this attractive strategy has been repeatedly prompted, the data are still scarce and often disappointing (Tanaka et al., 2005; Silvestrini et al., 2006). Therefore, in the present study we have characterized several compounds capable of inhibiting human heart cytosolic reductases involved in anthracycline carbonyl reduction in order to decrease the production of toxic C-13 alcohol metabolites responsible for anthracycline-induced cardiomyopathy.

Materials and Methods

Chemicals. DOX, DOXol, DNR, and DN Rol were kindly provided by Nerviano Medical Sciences (Milan, Italy). Anthracycline stock solutions were prepared in 18.2 MΩ-cm double-distilled deionized water (Milli-Q, Millipore, Bedford, MA) and shown to be stable for at least 1 month if stored at 4°C in the dark. Melatonin, 2-cyclopenten-1-one, cyclopentenone, 1-octen-3-ol, oenanthic acid, sodium dihydrogen phosphate monohydrate (NaH2PO4·H2O), 85% orthophosphoric acid, NADPH (tetrasodium salt), HEPES, ammonium sulfate, 2-phenyl-1,2-benziselenazol-3(2H)-one (ebelsen), sodium bovine serum albumin, EDTA (disodium salt), dicumarol, para-hydroxymercaptoc benzoic acid, N-ethylmaleimide, dimethyl sulfoxide, all-trans-retinal (vitamin A aldehyde), N-tet-butyl-a-phenylnitrite, dithiothreitol (DTT), 2-(3-aminopropyl)aminomethylphosphorothioate (amifostine), DL-α-Tocopherol, (−)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), mevinolin (Lovastatin), mevastatin; α-(4-pyridyl, 1-oxide)-N-tet-butylthiophosphorothioate and adenosine, 4-hydroxy-2-nonenal, ethacrynic acid, acrolein, decylubiquinone, and glutathione (GSH) were purchased from Sigma-Aldrich Co. (St. Louis, MO); L-α-lysophosphatidylethanolamine (PNACtate) (DEANO, prostaglandin (PG) A1 (PGA1), A2 (PG A2), B1 (PG B1), B2 (PG B2), D1 (PG D1), D2 (PG D2), E1 (PG E1), E2 (PG E2), J1 (PG J1), 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) and 9,11-dihydro-15-deoxy-Δ12,14-PGJ2, and Δ12,15-PGJ2 were obtained from Cayman Chemicals (Ann Arbor, MI); S-nitrosothioglutathione (GSNO) and (−)-S-nitroso-N-acetylpenicillamine (SNAP) were obtained from Calbiochem (Darmstadt, Germany); high-performance liquid chromatography-grade acetonitrile and chloroform and di-sodium hydrogen phosphate 12-hydrate (Na2HPO4· 12 H2O) were obtained from Merck (Darmstadt, Germany); ethyl alcohol absolute for spectrophotometry was obtained from Carlo Erba (Milan, Italy); 1-heptanol was obtained from BDH (Poole, United Kingdom); bicinchoninic acid protein assay reagent kit was purchased from Pierce (Rockford, IL), 2,3-dimethoxy-5-methyl-6-(10-hydroxycycl-1)-4-benzoxquinone, idebenone (IDB) was kindly provided by Takeda Pharmaceutical Co. (Tokyo). Coenzyme Q (CoQ) (CoQ0 and short chain CoQ analogs: CoQ1, CoQ2, CoQ3, and CoQ4) and Carvedilol was a generous gift from Hoffmann-La Roche (Basel, Switzerland), and Dextrazoxane (Cardioxane) was a generous gift from Chiron (Milan, Italy). Ebelsen diselenide was a kind gift from Arne Holmgren, (Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden). The reduced form of CoQ was prepared as described by Mordente et al. (1994).

Preparation of Cytosolic Fractions. Human heart ventricular samples (10–20 g) were obtained during authorized autopsies at the Department of Forensic Medicine of the Catholic University School of Medicine. Tissue removal and examination were in accordance with the ethical guidelines for the use of human tissues for teaching and research purposes of the Catholic University School of Medicine. The study was carried out in accordance with the Declaration of Helsinki (World Medical Association, 2013). Samples were derived from 20 to 40 year old male (n = 3) or female (n = 2) individuals with morphologically normal myocardium and no clinical history of acute myocardial infarction, severe cardioclesion, or other cardiomyopathies. All samples were collected 24 hours after death and stored at −80°C until use. Heart samples were carefully rinsed in ice-cold saline and homogenized in 4 volumes of ice-cold 10 mM HEPES buffer (pH 7.4), containing 0.3 M NaCl and 0.5 mM EDTA, using an Ultra Turrax (Ika, Staufen, Germany) and a Glass/Teflon Potter-Elvehjem Homogenizer (Corning, Corning, NY). Cytosolic fractions were prepared by sequential centrifugation, 20 minutes at 8500 and 23,000×g and 90 minutes ultracentrifugation at 140,000×g, all in 0.3 M NaCl/10 mM HEPES, pH 7.4 (standard buffer). Next, the 140,000×g supernatants were stirred overnight with 65% ammonium sulfate and centrifuged at 10,000×g for 20 minutes. Protein precipitates were suspended in 5 to 6 ml of homogenization buffer, dialyzed against three 1 liter changes of the same buffer, in which 1 mM EDTA was added (to remove adventitious iron), and then dialyzed against three 1 liter changes of EDTA-free buffer (to remove EDTA and EDTA-iron complexes) (Mordente et al., 2003). After low-speed centrifugation to remove any insoluble material, cytosolic proteins were assayed by the bicinchoninic acid method against bovine serum albumin standard curves and stored in aliquots at −80°C until use.

Effect of the Tested Compounds on Anthracycline Secondary Alcohol Metabolite Forming. The effect of xenobiotics on anthracycline metabolism was characterized in incubation mixtures that contained human heart cytosol (1.0 mg protein/ml) and xenobiotic (or vehicle) in standard buffer at 37°C. The incubation time and the concentration of each tested compound are specified in the respective figure legends and tables. At the indicated time intervals, 50 μM (final concentration) DOX or DNR was added into the incubation mixture and the reaction was started by adding 250 μM (final concentration) NADPH. After 240 minutes at 37°C, aliquots (500 μl) were withdrawn from the incubation mixture and assayed for anthracycline secondary alcohol metabolites, as described subsequently. Where indicated, the IC50 values (the concentration of the inhibitor required to produce 50% inhibition of anthracycline alcohol metabolite formation) were determined by nonlinear regression analysis of the dose-inhibition curves. Each curve was obtained using at least eight concentrations of xenobiotic. All values are the mean ± S.E. of three separate experiments performed in triplicate.

To identify the inhibition mechanism of ebelsen and ebelsen diselenide and to calculate the inhibition constants, the formation of anthracycline alcohol metabolites was measured in the absence or presence of different concentrations of drug by varying the DOX concentration (25–500 μM) at a fixed concentration of NADPH (250 μM). The results are presented as double-reciprocal Lineweaver-Burk plots.

Effect of Nitric Oxide (NO) Donors on Anthracycline Secondary Alcohol Metabolite Formation. Human heart cytosolic fractions (1.0 mg protein/ml) were incubated at 37°C in the absence (vehicle) or presence of varying concentrations of NO donors. Aliquots were withdrawn from the incubation mixture and the NO donor was removed by 3 cycles of concentration/dilution into standard buffer using a microcentrifugal concentration device with a 10 kDa cutoff membrane (Centricron, Millipore, Bedford, MA). After readjusting the protein concentration (1 mg/ml, final concentration), 50 μM (final concentration) of either DOX or DNR was added into the mixture and the reaction was started by adding 250 μM (final concentration) NADPH. After 240 minutes at 37°C, aliquots (500 μl) were withdrawn from the incubation mixture and assayed for anthracycline secondary alcohol metabolites, as described subsequently. Where indicated, the IC50 values (the concentration of the inhibitor required to produce 50% inhibition of anthracycline alcohol metabolite formation) were determined by nonlinear regression analysis of the dose-inhibition curves. Each curve was obtained using at least eight concentrations of xenobiotic. All values are the mean ± S.E. of three separate experiments performed in triplicate.

Effect of Nitric Oxide (NO) Donors on Anthracycline Secondary Alcohol Metabolite Formation. Human heart cytosolic fractions (1.0 mg protein/ml) were incubated at 37°C in the absence (vehicle) or presence of varying concentrations of DOX by varying the DOX concentration (25–500 μM) at a fixed concentration of NADPH (250 μM). The results are presented as double-reciprocal Lineweaver-Burk plots.
The concentrations of GSNO and SNAP were determined by absorption using $\varepsilon_{330\text{nm}} = 767$ and 717 M$^{-1}$ cm$^{-1}$, respectively (Tao and English, 2004). Fresh GSNO solutions were prepared in MilliQ water just before being used and kept on ice and always protected from light. Decomposed GSNO was prepared by storing aqueous solutions at room temperature in the dark for 72 hours (72-hour decomposed GSNO) (Tao and English, 2004).

**High-Performance Liquid Chromatography Assay for Anthracycline Secondary Alcohol Metabolites.** Unless otherwise indicated, the reaction mixture (500 $\mu$L) was stopped by adding an equal volume of 0.2 M Na$_2$HPO$_4$, pH 8.4, and samples were extracted with 4 ml of a 9:1 (v/v) chloroform/1-heptanol mixture. After vigorous shaking (15 minutes), samples were centrifuged at 4000 rpm for 10 minutes at 20°C to separate an upper aqueous phase and a lower organic phase. The aqueous phase was recovered and spectrophotometrically assayed in order to determine the NADPH content. The organic phase was re-extracted with 250 $\mu$L of 0.1 M orthophosphoric acid and vortexed vigorously for 1 minute at room temperature to obtain an upper aqueous layer, from which 50 $\mu$L were eventually removed and used for high-performance liquid chromatography analysis as previously described (Mordente et al., 2003; Silvestrini et al., 2006). The chromatographic apparatus consisted of an Agilent 1200 system (Agilent Technologies, Santa Clara, CA) equipped with diode array and fluorescence detectors. Reverse-phase chromatography was performed with an Agilent ZORBAX CN column (250 $\times$ 4.6 mm, 5 $\mu$m) with a ZORBAX CN analytical guard column (12.5 $\times$ 4.6 mm, 5 $\mu$m). Isocratic elution was performed at 1 ml/min with a daily prepared mobile phase consisting of a 75:25 (v/v) mixture of 50 mM sodium dihydrogen phosphate:acetonitrile, adjusted to pH 4.0 with orthophosphoric acid and filtered through a 0.22 $\mu$m membrane (Millipore). Isocratic elution was performed in triplicate. The data were analyzed by an unpaired Student’s t test, and statistical probability (P) is expressed as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

**Results**

**Effect of Various Drugs on the Formation of Anthracycline Secondary Alcohol Metabolites.** We initially screened a variety of drugs (metal chelators, radical scavengers, antioxidants, $\beta$-blockers, nitro spin traps, and lipid-lowering drugs) that are known to mitigate anthracycline-induced cardiotoxicity in animal models and/or in humans (Minotti et al., 2004; van Dalen et al., 2011; Cardinale et al., 2013). However, studies in this area are still few, often reporting conflicting results between preclinical models and clinical settings (Minotti et al., 2004; van Dalen et al., 2011; Menna et al., 2012; Cardinale et al., 2013). Except for all-trans-retinal, which slightly inhibited DNRol but not DOXol formation, none of the compounds reported in Table 1 significantly affected anthracycline secondary alcohol metabolite formation in our experimental model. Conversely, ebselen, cyclopentenone PGs (cPGs), NO donors, and short-chain CoQ analogs resulted in being efficient inhibitors of both DOXol and DNRol formation (presented subsequently).

**Effect of Ebselen.** Ebselen is a synthetic organo-selenium compound (see the structure in Fig. 1) that exhibits antioxidant, anti-inflammatory, and cytoprotective properties in a variety of animal models (Sakurai et al., 2006; Sarma and Mugesh, 2008). Moreover, unlike other selenium compounds, ebselen displays very low toxicity due to its great structural stability that does not allow the selenium moiety to be released during drug transformation and does not impair selenium metabolism (Sies and Masumoto, 1997; Zhao and Holmgren, 2002). Accordingly, ebselen is well tolerated in humans and has been successfully used in phase III clinical trials for the treatment of patients with acute ischemic stroke (Yamaguchi et al., 1998) or delayed neurologic deficits after aneurismal subarachnoid hemorrhage (Saito et al., 1998).

Interestingly, ebselen significantly ameliorated anthracycline-induced cardiomyopathy in animal models; however, the molecular mechanism of this cardioprotective effect is still unclear (Pritsis et al., 1992; Saad et al., 2006).

As shown in Fig. 2A, ebselen inhibited anthracycline alcohol metabolite formation in a dose-dependent manner, displaying higher efficacy on DOXol (IC$_{50}$ = 9.8 ± 0.7 $\mu$M) than on DNRol (IC$_{50}$ = 32.7 ± 5.6 $\mu$M) production. In our experimental conditions (i.e., 50 $\mu$M anthracycline), about 50% inhibition of DOXol formation was achieved with 0.2 $\mu$M of ebselen with respect to DOX concentration. Accordingly, since during chemotherapy DOX concentration reaches an intracellular level of approximately 1 $\mu$M, speculatively, 0.2 $\mu$M ebselen might thus be enough to inhibit significantly DOXol formation in vivo.

The ebselen-induced inhibition occurred quickly and the extent of inhibition did not depend on the duration of the preincubation period (from 5 to 60 minutes) or the presence of anthracycline (see the subsequent paragraphs and the discussion section), NADPH, or both in the reaction mixture (data not shown). Accordingly, a double-reciprocal Lineweaver-Burk plot (Fig. 3A) showed that ebselen inhibited DOXol formation by a noncompetitive mechanism (K$_{i}$ = 5.586 ± 0.254 $\mu$M). The same inhibition behavior has previously been observed for other enzymes treated with ebselen (Schewe et al., 1994; Semianrio-Vidal et al., 2010). Moreover, the inhibition was irreversible because ultrafiltration or long-term dialysis of ebselen-treated heart cytosolic fractions failed to restore anthracycline reductase activity (activity recovery was less than 5%). In addition, when the ebselen-inactivated cytosolic fractions were treated with 5 $\mu$M DTT or 5 $\mu$M GSH for 4 hours at 37°C, only partial recovery of the original anthracycline reductase activity was observed (28% ± 6% with DOX and 23% ± 5% with DNR after DTT treatment; 25% ± 3% with DOX and 24% ± 5% with DNR after GSH treatment).

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percentage of Inhibition</th>
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<tr>
<td></td>
<td>DOXol Formation</td>
</tr>
<tr>
<td>Dextrazole</td>
<td>4 ± 5</td>
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<tr>
<td>Melatonin</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>$\alpha$-tocopherol</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Trollox</td>
<td>4 ± 2</td>
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<tr>
<td>Amifostine</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>Adenosine</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>PBN</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>POBN</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>Mevastatin</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>All-trans-retinal</td>
<td>2 ± 4</td>
</tr>
</tbody>
</table>

PBN, N,N’-di-n-butyl-1,4-phenylnitroso; PBN, N-(4-pyridyl, 1-oxide)-N,N’-di-n-butyl-1,4-phenylnitroso;
The chemistry of ebselen is considered complex and still controversial (see the viewpoints presented in Sakurai et al., 2006; Sarma and Mugesh, 2008). In fact, although, most of the biologic activities of ebselen seem to be related to its ability to quickly react with protein and nonprotein thiol groups, GSH included (Sakurai et al., 2006; Sarma and Mugesh, 2008), the details of these reactions remain obscure, mainly because of the lack of undisputed identification of the reaction intermediates. According to the revised catalytic mechanism, ebselen rapidly reacts with GSH to produce the corresponding selenenyl sulfide derivative that undergoes a disproportionation reaction to produce ebselen diselenide, which is now considered the storage form of ebselen in the cells (Sarma and Mugesh, 2008). Therefore, we investigated the effect of ebselen diselenide on anthracycline alcohol metabolite formation by human heart cytosolic fractions. As shown in Fig. 2B, ebselen diselenide (which due to its low water solubility cannot be used at concentrations higher than 10 μM) is a powerful inhibitor of both DOXol (IC50 = 5.3 ± 1.1 μM) and DNRol (IC50 = 11.6 ± 4.1 μM) formation with higher efficiency (about double) than that of ebselen. Similarly to ebselen, ebselen diselenide behaves as a noncompetitive inhibitor (Ki = 3.782 ± 0.189 μM) of DOX reductase activity (Fig. 3B).

To confirm the importance of cysteine residues in the catalytic activity of the reductases involved in anthracycline metabolism, human heart cytosolic fractions were treated with the thiol alkylating agent N-ethylmaleimide and with the thiol-specific blocker para-hydroxymercuric benzoic acid. Both N-ethylmaleimide (50 μM) and para-hydroxymercuric benzoic acid (50 μM) strongly inhibited DOXol formation (90% ± 6% and 88% ± 5%, respectively) and DNRol formation (91% ± 5% and 87% ± 6%, respectively).

**Effect of cyPGs.** PGs are a family of biologically active eicosanoids that are involved in the regulation of numerous physiopathological processes, including inflammation, cellular growth, and differentiation (Sánchez-Gómez et al., 2010; Garzón et al., 2011; Díez-Dacal and Pérez-Sala, 2012). Within the PG family, the A and J series, known as cyPGs, possess an α,β-unsaturated carbonyl group in the cyclopentene ring, which confers high reactivity toward nucleophiles, such as thiol groups, and can lead to the formation of covalent adducts by Michael addition reactions (Sánchez-Gómez et al., 2010; Garzón et al., 2011; Díez-Dacal and Pérez-Sala, 2012). Post-translational modification of proteins by cyPGs (referred to as prostanoylation or eicosanoylation) affects the function of specific transcription factors, tumor suppressors, and antioxidant enzymes, and therefore seems to be responsible for
separate experiments performed in triplicate.

The inhibitory effects of cyPGs on DOXol formation were dose dependent, showing saturation properties of the NO derivatives and on the reaction conditions alike. Leading to either activation or inhibition, depending on the chemical properties of the NO derivatives and on the reaction conditions alike (SRivastava et al., 2001; Baba et al., 2009). Furthermore, GSNO, the

three chemical compounds containing different 5-member ring moieties but all lacking aliphatic side chains typical of the eicosanoid structure. Again, cytosolic fractions were also treated with 1-octen-3-ol and oenanthic acid, which have a chemical structure resembling that of the two aliphatic side chains of PGA1, 2-cyclopenten-1-one actually inhibited both DOXol (IC50 = 8.8 ± 0.45 mM) and DNRol (IC50 = 9.1 ± 0.44 mM) formation but at much higher concentrations (about 150-fold) than PGA1 or PGA2. Similar results were obtained by Straus et al. (2000), who observed that 100–200-fold higher concentrations of 2-cyclopenten-1-one in comparison with 15d-PGJ2 were needed to inhibit NF-κB activation. Conversely, neither cyclopentanone (a compound similar to 2-cyclopenten-1-one, but containing a saturated 5-member ring) nor cyclopentene (a 5-member ring system containing a double bond, but not a carbonyl group) affected anthracycline alcohol metabolite formation. Similarly, neither 1-octen-3-ol nor oenanthic acid were able to decrease DOXol or DNRol production (less than 3% inhibition at 250 μM, final concentration).

**Effect of NO Donors.** NO is an endogenous cell-signaling molecule essential for the integrity of the cardiovascular system, and decreased production and/or bioavailability of NO lead to the development of cardiovascular diseases and heart failure (Zhu et al., 2011). Moreover, NO sensitizes tumor cells to ionizing radiation and photodynamic therapy and increases anticancer activity of several chemotherapeutic agents, including anthracyclines (Matthews et al., 2001; Frederiksen et al., 2003). Accordingly, NO donors could be promising therapeutic agents against anthracycline-induced cardiotoxicity (Zhu et al., 2011) and multidrug resistance in tumor cells (de Luca et al., 2011).

In addition to the classic cGMP-dependent pathway, NO also regulates cell function through protein S-nitrosylation (also referred to as S-nitrosation), a reversible, redox-dependent, post-translational protein modification that involves attachment of a NO group to a nucleophilic protein sulfhydryl group (Tao and English, 2004; Hartmanová et al., 2013).
major intracellular storage and transport form of NO in vivo, has recently been identified as a carbonyl reductase 1 (CBR1) substrate (Bateman et al., 2008; Staab et al., 2011), whereas at higher concentrations, GSNO inactivates human CBR1 by covalent modification of cysteine residues (Staab et al., 2011). Therefore, we have studied the effect of two biologically important nitrosothiols, GSNO and SNAP, and a non-thiol-based NO donor, DEANO, on anthracycline alcohol metabolite formation.

Incubation of human heart cytosolic fractions at 37°C with different concentrations of GSNO, SNAP, or DEANO led to time-dependent (data not shown) and dose-dependent inhibition of DOXol and DNRol formation (Fig. 7). GSNO, SNAP, and DEANO, unlike ebselen and cyPGs, were more effective in inhibiting DNRol than DOXol formation (compare Fig. 7A with Fig. 7B). In fact, the inhibition of DOXol formation remains incomplete even upon increasing GSNO, SNAP, or DEANO concentration and/or the incubation time, with \( I_{\text{max}} \) values of about 76% for GSNO and SNAP and about 65% for DEANO (Fig. 7A). As evidenced by the IC\(_{50}\) values (Fig. 7, A and B), the inhibitory potency of GSNO and SNAP was practically similar, whereas DEANO, whose action can be attributed solely to the released NO group and its subsequent chemistry (Yang et al., 2002), was considerably less effective in inhibiting anthracycline alcohol metabolite formation.

As suggested by Tao and English (2004), GSNO is capable of both S-nitrosylation and S-glutathiolation of reactive cysteines, and the degree of S-nitrosylation/S-glutathiolation depends upon both the protein structure and the chemistry of GSNO. Freshly prepared GSNO was more effective in S-nitrosylation of proteins through transnitrosylation reactions, whereas decomposed GSNO was more effective in S-glutathiolation of proteins (Tao and English, 2004). Therefore, we compared the effects of fresh and 72-hour decomposed GSNO on anthracycline alcohol metabolite formation by human heart cytosol. As shown in Fig. 8, decomposed GSNO was much less effective than fresh GSNO in inhibiting both DOXol and DNRol formation. Conversely, glutathione disulfide (GSSG), the main GSNO dismutation product (the stoichiometry of GSSG formation by GSNO is 1:2, i.e., 1 mol of GSSG per 2 mol of GSNO), only slightly inhibited DOXol and DNRol formation except for the highest concentrations employed (again, see Fig. 8); however, that should be ruled out in our experimental conditions. Indeed, it is noteworthy that in fresh GSNO solutions, the amount of GSSG is negligible and its concentration increases minimally during the incubation period.

Also supporting the S-nitrosylation mechanism, SNAP, which is known to modify protein thiols exclusively by S-nitrosylation because of a S-nitroso group more sterically hindered than GSNO (Konorev et al., 2000), inhibited DOXol and DNRol formation with an efficiency practically similar to that of GSNO (see Fig. 7). Moreover, very remarkably, the inhibitory effect of S-nitrosothiols on anthracycline alcohol metabolite formation was reversible. When the GSNO- or SNAP-inactivated heart cytosolic fractions were treated with DTT (10 mM) for 60 minutes at 37°C, most of anthracycline reductase activity was recovered (80% ± 6% and 82% ± 5% using DOX and DNR as the substrate, respectively).

**Effect of Short-Chain CoQ Analogs.** CoQ\(_{10}\) or ubiquinone is an essential component of the mitochondrial electron transport chain, which plays a key role in cellular energy production (Genova and Lenaz, 2011;
Orsucci et al., 2011). In its reduced hydroquinone form, CoQ_{10} is a powerful antioxidant protecting cells both directly, by preventing membrane lipid peroxidation, and/or indirectly, by regenerating other antioxidants such as ascorbate and α-tocopherol (Genova and Lenaz, 2011; Orsucci et al., 2011). Preclinical and clinical studies (Conklin, 2005; Hintzpeter et al., 2015), we initially evaluated the effects on anthracycline reductive metabolism of a variety of compounds (metal chelators, radical scavengers, antioxidants, β-blockers, nitrone spin traps, and lipid-lowering drugs) employed as protective agents against anthracycline-induced cardiotoxicity. Among the compounds examined here, many failed to significantly inhibit anthracycline alcohol metabolite formation (see Table 1), thus confirming that their effectiveness might be due to their interference with anthracycline metabolism without interfering with anthracycline anticancer activity. Many mechanisms can be evoked for the cardioprotective action of CoQ_{10}; however, the current hypothesis suggests that CoQ_{10} may limit anthracycline semiquinone formation and then reactive oxygen species overproduction by competing with anthracyclines for the active site of mitochondrial NADH:CoQ oxidoreductase (Complex I) (Conklin, 2005). Interestingly, one of the subunits of mitochondrial NADH:CoQ oxidoreductase displays a strong homology with several members of short-chain dehydrogenase/reductase, a cytosolic reductase superfamily involved in anthracycline metabolism (Baker et al., 1999).

The extreme hydrophobicity of CoQ_{10}, which precludes its use in our experimental conditions, and the potentiality of short-chain CoQ_{10} analogs to act as substrates and/or inhibitors of mitochondrial NADH: CoQ oxidoreductase (King et al., 2009) and as substrates for several different cytosolic NADPH oxidoreductases (Jin and Penning, 2007; Malátková et al., 2010) motivated us to investigate the eventual effect of several water soluble CoQ_{10} analogs on anthracycline alcohol metabolite formation. As shown in Fig. 9, CoQ_{0}, CoQ_{1}, CoQ_{2}, decylubiquinone, and IDB significantly inhibited DOXol and even more DNRol formation, with CoQ_{0} displaying the highest efficiency, whereas CoQ_{4}, and CoQ_{6} were almost totally ineffective. The IC_{50} values for short-chain CoQ_{10} analogs are reported in Table 2.

Unlike the quinone form, the hydroquinone form of any short-chain CoQ_{10} analog was almost totally ineffective in inhibiting (less than 5%) either DOXol or DNRol formation. Moreover, the addition of 20 μM dicumarol into the reaction mixture together with IDB significantly enhanced quinone-dependent inhibition of both DOXol and DNRol formation (Fig. 10, A and B; Table 2). Indeed, dicumarol is the most potent inhibitor of NADPH-quinate oxidoreductase 1, a cytosolic flavoenzyme that catalyzes the obligatory two-electron reduction of various quinones to their hydroquinone forms (Haefeli et al., 2011; Erb et al., 2012). Instead, dicumarol alone very slightly affected anthracycline alcohol metabolite formation (less than 5% of inhibition).

**Discussion**

A pharmacological strategy aimed at inhibiting the conversion of anthracyclines into their cardiotoxic metabolites could provide a 2-fold advantage by thwarting anthracycline-induced cardiomyopathy as well as by overcoming tumor resistance toward these anticancer drugs. Because the evidence in this regard is still scarce and unsettled (Tanaka et al., 2005; Hintzpeter et al., 2015), we initially evaluated the effects on anthracycline reductive metabolism of a variety of compounds (metal chelators, radical scavengers, antioxidants, β-blockers, nitrone spin traps, and lipid-lowering drugs) employed as protective agents against anthracycline-induced cardiotoxicity. Among the compounds examined here, many failed to significantly inhibit anthracycline alcohol metabolite formation (see Table 1), thus confirming that their cardioprotective effects against anthracycline-induced cardiotoxicity should be ascribed to their antioxidant and/or antiapoptotic properties (Gianni et al., 2008; van Dalen et al., 2011; Octavia et al., 2012; Cardinale et al., 2013). Other drugs (i.e., ebselen, cyPGs, NO donors, and short-chain CoQ_{10} analogs) are characterized here for the first time.
two different cytosolic NADPH-dependent oxidoreductases, namely CBR1 (Zembowicz et al., 1993; Terentis et al., 2010). In human myocardium, substrate complex. reductases by binding equally well to the free enzyme or to the enzyme-

that the two compounds are capable of inhibiting NADPH-dependent (belonging to the short-chain dehydrogenase/reductase superfamily) as well as that of ebselen diselenide is noncompetitive (Hintzpeter et al., 2015). The inhibition pattern of ebselen and even more so that of ebselen diselenide, is comparable to that

DNRol formation. Interestingly, the inhibitory potency of ebselen, ebselen diselenide are the most potent inhibitors of both DOXol and

metabolites were assayed after 240 minutes at 37°C as described in Materials and Methods. Values are the mean ± S.E. of at least three separate experiments performed in triplicate. Statistical probability (P) is expressed as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

as effective inhibitors of the enzymatic conversion of anthracyclines into their cardiotoxic metabolites.

As can be evinced from the IC50 values, ebselen and its derivative ebselen diselenide are the most potent inhibitors of both DOXol and DNRol formation. Interestingly, the inhibitory potency of ebselen, and even more so that of ebselen diselenide, is comparable to that of curcumin, which is one of the more potent tight-binding inhibitors of human CBR1 (Hintzpeter et al., 2015). The inhibition pattern of ebselen as well as that of ebselen diselenide is noncompetitive (K values of 5.856 ± 0.254 and 3.782 ± 0.189 μM, respectively), suggesting that the two compounds are capable of inhibiting NADPH-dependent reductases by binding equally well to the free enzyme or to the enzyme-substrate complex.

Ebselen exhibits strong electrophilic activity, and is therefore capable of forming selenyl-sulfide bonds with cysteines of a variety of proteins (Zembowicz et al., 1993; Terentis et al., 2010). In human myocardium, two different cytosolic NADPH-dependent oxidoreductases, namely CBR1 (belonging to the short-chain dehydrogenase/reductase superfamily) and aldehyde reductase (AKR1A1, belonging to the AKR superfamily)

are by far the most potent carbonyl reducing enzymes involved in anthracycline metabolism (Mordente et al., 2003; Salvatorelli et al., 2007; Barski et al., 2008; Bateman et al., 2008; Kassner et al., 2008). CBR1 primarily reduces DNR to DNRol, whereas AKR1A1 prevalently converts DOX into DOXol (Mordente et al., 2003; Salvatorelli et al., 2007; Kassner et al., 2008). Human CBR1 contains five cysteines located in, or close to, the active site, and among them Cys227 has been identified as the residue involved in the binding of both substrate and GSH (Tinguely and Wermuth, 1999; Hartmanová et al., 2013). AKR1A1 contains six cysteines, none of which appears to be implicated in the catalytic mechanism of the enzyme (Barski et al., 2008).

It can be then inferred that ebselen is capable of inhibiting human heart anthracycline reductases by forming covalent adducts with catalytically essential (in CBR1) or nonessential cysteine residues (in AKR1A1, i.e., Cys4 and Cys259), either by increasing local hydrophobicity or steric hindrance with its bulky, hydrophobic aromatic groups, and thereby leading to dose-dependent alterations in the protein native structure with perturbation of the anthracycline binding site and inhibition of enzyme activity. Moreover, the ebselen-protein adduct looks stable, being well sheltered at or near the enzyme active site, such that DTT or GSH are practically unable to rescue the native anthracycline reductase activity. The high efficiency of ebselen (and of its intracellular storage form ebselen diselenide) in decreasing toxic anthracycline reductive metabolism, together with its unique biochemical properties and favorable pharmacological profile (low toxicity, lack of adverse effects, and metabolic stability), makes ebselen a most promising cardioprotective agent against anthracycline-induced cardiotoxicity (see Fig. 1).

At pharmacological concentrations (Straus and Glass, 2001), cyPGs have also been found to be potent inhibitors of anthracycline alcohol metabolite formation in isolated human heart cytosol. Structure-activity studies have evidenced that the eicosanoid structure is not a prerequisite for cyPG-induced inhibition of anthracycline alcohol metabolite formation. Conversely, the presence of a reactive α,β-unsaturated carbonyl group in the cyclopentenone ring moiety (endocyclic α,β-unsaturated ketone) is an absolute requirement for PG inhibitory activity. Indeed, PGs containing an endocyclic α,β-unsaturated ketone (i.e., PGA1, PGA2, PGJ2, 15d-PGJ2, and 15Δ2-PGJ2) were effective inhibitors of anthracycline alcohol metabolite formation, whereas PGs either lacking (i.e., PGD1, PGD2, PGE1 and PGE2 as well as 9,10-dihydro-15d-PGJ2) or containing a sterically hindered α,β-unsaturated carbonyl group (i.e., PGB1, and PGB2) were ineffective. Moreover, the presence of a second, potentially reactive, β-carbon located on one of the two aliphatic side chains (exocyclic α,β-unsaturated ketone) does not actually increase, but instead

**TABLE 2**

Inhibitory parameters of short-chain CoQ analogs on DOXol and DNRol formation by human heart cytosolic fractions

Experiments were performed by incubating cytosolic fractions (1 mg protein/ml) in standard buffer at 37°C with or without (vehicle) 50 μM of each short-chain CoQ analog added 5 minutes before 50 μM DOX (A) or DNR (B). Reactions were started by adding 250 μM NADPH. Alcohol metabolites were assayed after 240 minutes at 37°C as described in Materials and Methods. Values are the mean ± S.E. of at least three separate experiments performed in triplicate. Statistical probability (P) is expressed as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>lmax (%)</th>
<th>IC50 (μM)</th>
<th>lmax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoQ0</td>
<td>11.5 ± 0.6</td>
<td>103.6 ± 1.7</td>
<td>10.1 ± 1.2</td>
<td>100.9 ± 0.6</td>
</tr>
<tr>
<td>CoQ1</td>
<td>151.5 ± 9.3</td>
<td>98.7 ± 3.8</td>
<td>77.8 ± 5.1</td>
<td>102.1 ± 3.0</td>
</tr>
<tr>
<td>CoQ3</td>
<td>N.D.</td>
<td>N.D.</td>
<td>93.5 ± 7.4</td>
<td>103.0 ± 4.1</td>
</tr>
<tr>
<td>DB</td>
<td>198.7 ± 8.6</td>
<td>102.3 ± 5.4</td>
<td>150.7 ± 9.0</td>
<td>101.1 ± 4.2</td>
</tr>
<tr>
<td>IDB</td>
<td>94.3 ± 6.7</td>
<td>99.4 ± 4.1</td>
<td>58.5 ± 4.4</td>
<td>98.3 ± 2.8</td>
</tr>
<tr>
<td>IDB plus Dicumarol (20 μM)</td>
<td>27.0 ± 1.7</td>
<td>98.7 ± 3.3</td>
<td>21.6 ± 3.2</td>
<td>101.7 ± 0.9</td>
</tr>
</tbody>
</table>

DB, decylubiquinone; N.D., not determinable.
Anthracycline Reductive Metabolism in Human Heart

In conclusion, this study identifies novel compounds able to target for PGA1 modification. PGA1 inhibited AKR1B10 and increased the accumulation of DOX in lung cancer cells, thus potentiating anthracycline anticancer effects and helping in counteracting multidrug chemoresistance.

Also, NO donors are capable of regulating myocardial reductive metabolism of anthracyclines. GSNO, SNAP, and albeit less efficiently DEANO, significantly inhibited both DOXol and DNRol formation. Experiments with DTT, fresh or decomposed GSNO, and GSSG indicate that S-nitrosylation of functionally important cysteine residues of cytosolic reductases might be the prevalent mechanism accounting for nitrosothiol-dependent inhibition of cytosolic reductases. Moreover, a recent study shows that GSNO-dependent S-glutathiolation of cysteines of CBR1, along with the formation of a disulfide bridge between Cys226 and Cys227 (Hartmanová et al., 2013) may be also an important mechanism of enzyme regulation. Apart from the precise molecular mechanism (S-nitrosylation and/or S-glutathiolation) underlying their inhibitory effects on human heart cytosolic reductases, NO donors prevent the development of hypoxia-induced drug resistance (Matthews et al., 2001; Frederiksen et al., 2003).

Finally, the present study shows, for the first time, that the quinone form of short-chain CoQ10 analogs is an effective inhibitor of anthracycline alcohol metabolite formation, whereas the respective hydroquinone form is completely ineffective. CoQ0, IDB, and CoQ1 are the most potent inhibitors of both DOXol and DNRol formation, whereas quinones with a slightly longer isoprenoid side chain were much less effective (see CoQ2) or almost completely ineffective (see CoQ4 and CoQ5). It is noteworthy that all the short-chain CoQ10 analogs are more powerful in inhibiting DNRol than DOXol formation, which probably reflects the greater affinity of quinones for CBR1 than for AKR1A. The experiments with dicumarol confirmed the following: 1) the quinone form of the short-chain CoQ10 analogs is the functionally active form; 2) IDB is a good substrate for NADPH-quinone oxidoreductase 1; and 3) NADPH-quinone oxidoreductase 1 is not involved in anthracycline reductive metabolism in human heart cytosol.

Mechanistically, as already suggested for mitochondrial NADH, CoQ oxidoreductase, short-chain quinones might decrease anthracycline alcohol metabolite formation by competing with the anticancer drug for the active site of NADPH-dependent cytosolic reductases. Some short-chain quinones were investigated as potential therapeutic molecules in many mitochondrial diseases (Becker et al., 2010; Erb et al., 2012; Koopman et al., 2012); however, because of their severe cytotoxic effects, extreme caution must be warranted in therapeutic applications of these quinones (Haefeli et al., 2011). Instead, clinical trials have evidenced that IDB is safe (only mild adverse effects were observed) and well tolerated in a single oral dose and in repeated daily doses (Kutz et al., 2009; Becker et al., 2010). IDB supplementation consistently improved cardiomyopathy commonly associated with Friedrich ataxia (Kearney et al., 2012) and, although a positive phase III study is still lacking, IDB therapy is nonetheless temporarily authorized for treating cardiomyopathy in these patients (Becker et al., 2010). Therefore, like ebselen, IDB also might be considered putatively beneficial for the treatment of anthracycline-induced cardiotoxicity.

In conclusion, this study identifies novel compounds able to significantly inhibit reductive anthracycline metabolism in reconstituted human heart cytosolic fractions as potential candidates in association therapy for the prevention or attenuation of anthracycline-induced cardiomyopathy. With careful attention paid to the results reported herein, we hope that this study will open the way to the clinical testing of these novel compounds.

Fig. 10. Effect of CoQ0 and IDB on anthracycline secondary alcohol metabolite formation. Experiments were performed by incubating human heart cytosolic fractions (1 mg protein/ml) in standard buffer at 37°C with or without (vehicle) different concentrations of CoQ0(○), IDB(□), or IDB plus 20 μM dicumarol (□) added 5 minutes before 50 μM DOX (A) or DNR (B). Reactions were started by adding 250 μM NADPH. Alcohol metabolites were assayed after 240 minutes at 37°C as described in Materials and Methods. Values are the mean ± S.E. of at least three separate experiments performed in triplicate.
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Conducted experiments: Mordente, Silverstrini, Meucci.
Contributed new reagents or analytic tools: Mordente, Tavaran, Maruccia, Meucci.
Performed data analysis: Mordente, Tavaran.
Wrote or contributed to the writing of the manuscript: Mordente, Silverstrini, Maruccia, Meucci.

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