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Anthracycline secondary alcohol metabolite formation in human or rabbit heart: biochemical aspects and pharmacologic implications

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Abstract

Clinical use of the anticancer anthracyclines doxorubicin (DOX) and daunorubicin (DNR) is limited by development of cardiotoxicity upon chronic administration. Secondary alcohol metabolites, formed after two-equivalent reduction of a carbonyl group in the side chain of DOX or DNR, have been implicated as potential mediators of chronic cardiotoxicity. In the present study we characterized how human heart converted DOX or DNR to their alcohol metabolites DOXol or DNRol. Experiments were carried out using post-mortem myocardial samples obtained by ethically-acceptable procedures, and results showed that DOXol and DNRol were formed by flavin-independent cytoplasmic reductases which shared common features like pH-dependence and requirement for NADPH, but not NADH, as a source of reducing equivalents. However, studies performed with inhibitors exhibiting absolute or mixed specificity toward best known cytoplasmic reductases revealed that DOX and DNR were metabolized to DOXol or DNRol through the action of distinct enzymes. Whereas DOX was converted to DOXol by aldehyde-type reductase(s) belonging to the superfamily of aldo-keto reductases, DNR was converted to DNRol by carbonyl reductase(s) belonging to the superfamily of short-chain dehydrogenase/reductases. This pattern changed in cardiac cytosol derived from rabbit, a laboratory animal often exploited to reproduce cardiotoxicity induced by anthracyclines and to develop protectants for use in cancer patients. In fact, only carbonyl reductases were involved in metabolizing DOX and DNR in rabbit cardiac cytosol, although with different K_m and V_{max}. Collectively, these results demonstrate that human myocardium convert DOX and DNR to DOXol or DNRol by virtue of different reductases, an information which may be of value to prevent alcohol metabolite formation during the course of anthracycline-based anticancer therapy. These results also raise caution on the preclinical value of animal models of anthracycline cardiotoxicity, as they demonstrate that the metabolic routes leading to DOXol in a laboratory animal may not be the same as those occurring in patients.

Keywords: Doxorubicin; Daunorubicin; Aldo-keto reductase; Carbonyl reductase; Anthracycline alcohol metabolite; Cardiotoxicity

1. Introduction

The anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DNR) are highly effective against solid and haematologic malignancies but their use is limited by acute and chronic toxicity to the heart [1]. The acute toxicity develops immediately after initiation of anthracycline treatment and consists of transient and usually manageable arrhythmias and hypotension. In contrast, chronic cardiomyopathy develops any time after completion of cumulative anthracycline regimens and evolves into congestive heart failure, often refractory to inotropic medications. Chronic cardiotoxicity develops in a dose-dependent manner, its incidence increasing sharply at cumulative doses of $\geq$550 mg/m^2 [1,2]. One-electron redox cycling of a quinone moiety in the tetracyclic ring of anthracyclines has been proposed to mediate cardiotoxicity induced by these drugs. In fact, several flavin-centered oxidoreductases (e.g. cytochrome P450 reductase, nitric oxide synthase, NADH dehydrogenase) catalyze one-electron reduction of this

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Abbreviations: DOX, doxorubicin; DNR, daunorubicin; ROS, reactive oxygen species; DOXol, doxorubicinol; DNRol, daunorubicinol; AKR, aldo-keto reductase; SDR, short-chain dehydrogenase/reductase; CB, Cibacron Blue F3G-A; DPI, diphenyleneiodonium chloride; MonoHER, 7-mono-*O*-(β-hydroxyethyl)-7-rutoside.

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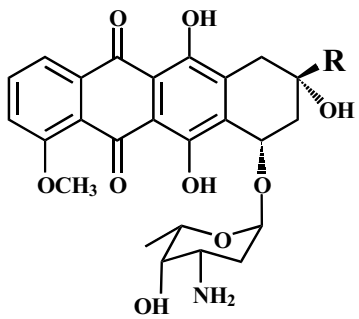
quinone to a semiquinone which readily regenerates its parent compound by reducing molecular oxygen to superoxide anion ($O_2^{\bullet -}$) and its dismutation product hydrogen peroxide (H_2O_2). At the same time the semiquinone reductively releases iron from ferritin by direct or $O_2^{\bullet -}$-mediated electron transfer. All such processes increase the cellular levels of H_2O_2 and Fe(II), and set the stage for formation of $^{\bullet}OH$, ferryl ions and other iron–oxygen complexes able to induce oxidative damage [3–5]. While possibly explaining several aspects of the acute phase of cardiotoxicity, these mechanisms may not always explain the development of chronic cardiomyopathy. In fact, studies in laboratory animals have shown that chronic cardiomyopathy not always is accompanied by biochemical indices of oxidative damage (reviewed in Ref. [6]). Moreover, antioxidants have proven useful in delaying or preventing chronic cardiotoxicity in rodents [7] but not in dogs [8] or patients [9,10]. In the light of these uncertainties, some investigators have proposed that chronic cardiomyopathy develops after conversion of DOX and DNR to the corresponding secondary alcohol metabolites doxorubicinol (DOXol) and daunorubicinol (DNRol) [6,11,12]. These metabolites are formed after two-electron reduction of a side chain C-13 carbonyl group (Fig. 1), mediated by poorly characterized cytoplasmic oxidoreductases sharing similarities with the aldo-keto reductase (AKR) and/or short-chain dehydrogenase/reductase (SDR) multigene superfamilies [13,14]. The involvement of secondary alcohol metabolites is suggested by the following pharmacokinetic evidence: (i) the cardiac function of anthracycline-treated rodents usually declines when alcohol metabolites reach their maximum levels in the heart [6]; (ii) transgenic mice bearing cardiac-restricted overexpression of human carbonyl reductase exhibit an accelerated course of development of cardiomyopathy [12]; (iii) investigational anthracyclines with inherent resistance to carbonyl reduction induce less severe chronic cardiotoxicity in the rat [15]. From a mechanistic view point secondary alcohol metabolites are significantly less effective than their parent drugs at producing oxygen radicals, presumably because they exhibit reduced affinity for one-electron quinone reductases [16]. However, secondary alcohol metabolites are several times more potent at inactivating membrane ATPases [11,17] and cytoplasmic aconitase/iron regulatory protein 1 [18,19].



Anthracycline	R
Doxorubicin	$-C(=O)-CH_2OH$
Doxorubicinol	$-CH(OH)-CH_2OH$
Daunorubicin	$-C(=O)-CH_3$
Daunorubicinol	$-CH(OH)-CH_3$

Fig. 1. Chemical structure of DOX, DNR, DOXol and DNRol.

The possible role of secondary alcohol metabolites as biochemical determinants of chronic cardiomyopathy anticipates that the clinical use of anthracyclines could be improved by minimizing their conversion to such metabolites. As already mentioned, one approach might be to develop new anthracyclines that form less alcohol metabolites than DOX or DNR. An alternative approach might be to develop inhibitors of the reductases converting DOX and DNR to DOXol or DNRol. This latter approach clearly requires improved identification and knowledge of the function and substrate specificity of anthracycline C-13 reductases and an appraisal of possible differences between the reductases of humans and those of laboratory animals in which the protective efficacy of the inhibitors would be tested. We therefore designed experiments in which the activity and specificity of anthracycline C-13 reductases were assessed in cytosolic fractions derived from human myocardium and, for comparative purposes, in rabbit cardiac cytosol.

2. Materials and methods

2.1. Chemicals

Doxorubicin, DOXol, DNR and DNRol were kindly provided by Pharmacia-Upjohn; stock solutions were prepared in double-distilled deionized water and shown to be stable for at least 1 month if stored at 4° in the dark. Quercetin dihydrate, kaempferol, Cibacron Blue F3G-A (CB), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) and 85% *ortho*-phosphoric acid were obtained from Fluka AG; NADPH (tetrasodium salt), diphenyleneiodonium chloride (DPI), *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), ammonium sulfate, EDTA (disodium salt), rutin hydrate, diethylstilbestrol, 2-[(2,6-dichloro-3-methyl-phenyl)amino]benzoic acid sodium salt (meclofenamic acid), 4′,5,7-trihydroxyisoflavone (genistein) and betamethasone, were purchased

from Sigma Chemical Co; ethyl-1-benzyl-3-hydroxy-2(5*H*)-oxopyrrole-4-carboxylate (EBPC) was from Tocris; Tolrestat was from Wyeth; spectrophotometry-grade absolute ethyl alcohol was from Carlo Erba; HPLC-grade acetonitrile and chloroform and di-sodium hydrogen phosphate 12-hydrate ($Na_2HPO_4 \cdot 12H_2O$) were from Merck; 1-heptanol was from BDH; AL1576 was from Alcon Laboratories; 7-mono-*O*-(β-hydroxyethyl)-7-rutoside (MonoHER) was from Novartis, and Sorbinil and Zopolrestat were from Pfizer. All other chemicals were of the highest commercially available grade.

2.2. Preparation of cytosolic fractions

Human heart left ventricle 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 to Institutional Ethical Guidelines for the use of human tissues for teaching and research purposes. Samples derived from 25- to 40-year male (N = 2) or female (N = 1) individuals with morphologically normal myocardium and no clinical history of acute myocardial infarction, severe cardiosclerosis or other cardiomyopathies. All samples were collected 24 hr after death and stored at −80° until use. Cytosolic fractions were prepared by sequential homogenization, 20 min centrifugation at 8500 and 23,000 *g*, and 90 min ultracentrifugation at 140,000 *g*, all in 0.3 M NaCl–10 mM HEPES, pH 7.4. Next, 140,000 *g* supernatants were stirred overnight with 65% ammonium sulfate and centrifuged at 10,000 *g* for 20 min. Protein precipitates were resuspended in 5–6 mL of homogenization buffer, dialyzed against three 1-L changes of the same buffer added with 1 mM EDTA (to remove adventitious iron) and then against three 1-L changes of EDTA-free buffer (to remove EDTA and EDTA–iron complexes). After low speed centrifugation to remove any insoluble material cytosolic proteins were assayed by the bicinchoninic acid method [20] and stored in aliquots at −80° until used. No apparent loss of activity was observed after storage. Rabbit heart cytosol was prepared by the same procedures.

2.3. Anthracycline reductase assay

Unless otherwise indicated, anthracycline metabolism was assayed by reconstituting 50 μM DOX or DNR with 1 mg cytosolic protein/mL in 0.3 M NaCl–10 mM HEPES, pH 7.4, 37°. After 5 min preincubation, the reaction was started by adding 250 μM NADPH. Where indicated, incubations were carried out in the presence of known inhibitors of aldo-keto or carbonyl reductases. Assays were performed by preincubating appropriate concentrations of each inhibitor with cytosol for 5 min. After this period, anthracyclines were added and the reaction was started with NADPH.

2.4. Secondary alcohol metabolite assays

At specified times 0.5 mL of the incubation mixtures were mixed with an equal volume of 0.2 M Na_2HPO_4, pH 8.4, and extracted with 4 mL of a 9:1 (v/v) chloroform/1-heptanol mixture. After 15 min vigorous shaking, samples were centrifuged for 10 min at 20° to separate an upper aqueous phase and a lower organic phase. The latter was re-extracted with 0.25 mL of 0.1 M *ortho*-phosphoric acid and vortexed vigorously for 1 min at room temperature to obtain an upper aqueous layer from which 50 μL were eventually removed and used for HPLC analysis as described by Fogli *et al*. [21]. The chromatographic apparatus consisted of a Hewlett-Packard 1100 system (Hewlett-Packard Co) equipped with diode array and fluorescence detectors. Reversed-phase chromatography was performed with a Hewlett-Packard ZORBAX CN column (250 mm × 4.6 mm, 5 μm) protected by a ZORBAX CN analytical guard column (12.5 mm × 4.6 mm, 5 μm). Isocratic elution was performed with a freshly-prepared mobile phase consisting of a 75:25 (v/v) mixture of 50 mM sodium dihydrogen phosphate:acetonitrile, adjusted to pH 4.0 with *ortho*-phosphoric acid and filtered through a 0.22 μm membrane (Millipore). The flow rate was 1 mL/min. C-13 alcohol metabolites were detected fluorimetrically with excitation at 480 and 560 nm. Retention times were as follows: DOX 10 min, DOXol 6 min, DNR 18 min, DNRol 9 min.

All data are expressed as the arithmetic mean ± SEM, and analyzed by Student's *t*-test. Results are considered statistically significant when $P < 0.05$.

Other experimental conditions are given in the legends to figures and tables.

3. Results

3.1. Properties of anthracycline reductase activity of human heart

Reconstitution of human cardiac cytosol with NADPH and DOX or DNR resulted in formation of DOXol or DNRol, whose production increased linearly with the incubation time (Fig. 2A). DNRol formation always exceeded that of DOXol, the DNRol/DOXol ratio ranging from 10 to 14. Under the same experimental conditions, neither microsomal nor mitochondrial fractions converted DOX or DNR to DOXol or DNRol (not shown). Heat denaturation of cytosolic fractions, omission of NADPH, or replacement of NADPH with NADH abolished formation of alcohol metabolites, regardless of whether the samples were incubated with DOX or DNR. Moreover, DOXol or DNRol formation was suppressed by ∼85% upon replacing NADPH with Cibacron Blue, a structural analogue widely used as dead-end type inhibitor of NADPH-dependent oxidoreductases (Fig. 2B). Conver-

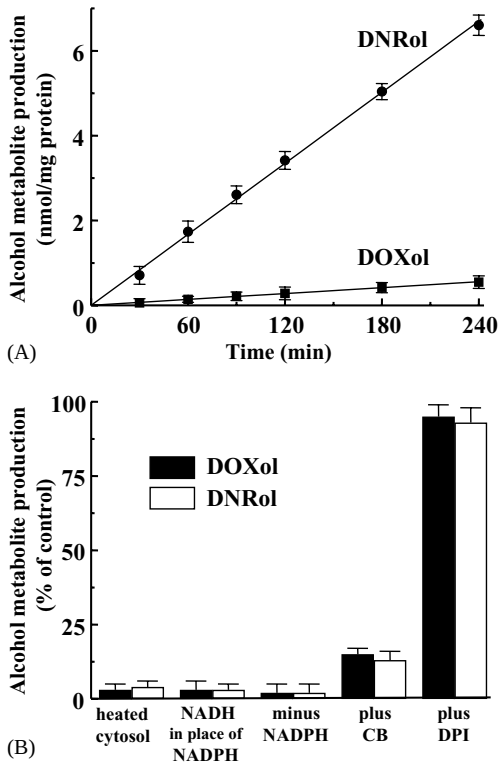


Fig. 2. Anthracycline alcohol metabolite formation by human heart cytosolic reductases. (A) DOXol or DNRol formation was measured by incubating 50 μM DOX or DNR with cytosolic fractions (1 mg protein/mL) in 10 mM HEPES–300 mM NaCl, pH 7.4, 37°. Reactions were started by adding NADPH (250 μM), and alcohol metabolites were measured at regular times as described in Section 2. (B) Experiments were replicated by replacing NADPH with equimolar NADH or by including 50 μM inhibitors like Cibacron Blue (CB) or DPI (plus DPI). Where indicated cytosol had been denatured by 2 hr at 100°. Alcohol metabolites were measured at 4 hr. Values are means ± SE of at least three separate experiments performed in triplicate.

sely, DPI, a selective inhibitor of flavoenzymes, had no effect at impairing anthracycline reductive metabolism (see also Fig. 2B). These results showed that in human heart anthracycline carbonyl reduction occurred through an enzymatic process which involved flavin-independent enzymes and required NADPH as a specific cofactor. These enzymes exhibited a single optimum pH at 9.0 for both DOX and DNR (not shown).

3.2. Effects of inhibitors

Currently, human cytosolic reductases acting on carbonyl residues are grouped into two distinct superfamilies: (i) AKR, including aldehyde reductases (AKR1A1), aldose reductases (AKR1B1) and the less well characterized hydroxysteroid dehydrogenases (AKR1C1–AKR1C4) [13]; (ii) SDR, a heterogenous superfamily that includes carbonyl reductases, retinol/retinal dehydrogenases and sepiapterin reductases as best known members [14]. We evaluated the effects of AKR or SDR inhibitors on the conversion of DOX and DNR to DOXol or DNRol in human cardiac cytosol; this was done by incubating cytosol with concentrations of inhibitors always in excess of IC_{50} previously determined in presence of natural or model substrates [22–27]. As shown in Fig. 3, DOXol formation was not inhibited by EPBC, the most potent and selective inhibitor of aldose reductases (AKR1B1) [22], nor was it inhibited by Sorbinil, another popular albeit less potent and specific inhibitor of this class of reductases [23]. In contrast, DOXol formation decreased by approx 50% when cytosol was incubated with AL1576 (the most potent and specific inhibitor of aldehyde reductases/AKR1A1) [24], and either Tolrestat or Zopolrestat (previously reported as mixed inhibitors of aldehyde/AKR1A1 and aldose reduc-

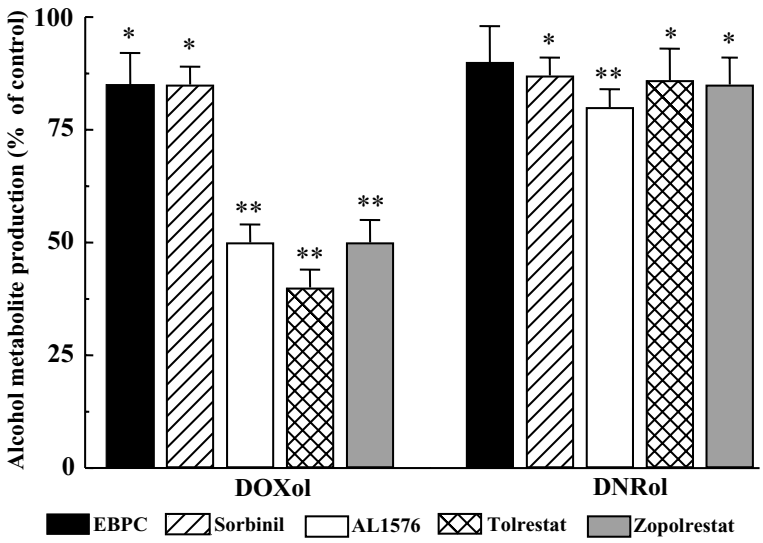


Fig. 3. Effects of aldo-keto reductase inhibitors on DOXol or DNRol formation. Incubations were prepared as in Fig. 2, in the presence of the following inhibitors (50 μM): EBPC (black bars), Sorbinil (hatched bars), AL-1576 (white bars), Tolrestat (cross-hatched bars) and Zopolrestat (grey bars). Alcohol metabolites were measured at 4 hr. Values are means ± SE of at least three separate experiments performed in triplicate; $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 1
Effect of hydroxysteroid dehydrogenase inhibitors on anthracycline reductase activity of human heart

Inhibitor group	Inhibitor	Percent of inhibition	
		DOXol	DNRol
Non-steroidal anti-inflammatory drug	Meclofenamic acid	4 ± 3	9 ± 4
Steroidal anti-inflammatory drug	Betamethasone	3 ± 2	5 ± 4
Synthetic estrogen	Diethylstilbestrol	13 ± 4	17 ± 5
Phytoestrogen	Genistein	4 ± 3	41 ± 6

Assays were performed by pre-incubating cytosolic fractions (1 mg protein/mL) in 10 mM HEPES–300 mM NaCl, pH 7.4, with each inhibitor (50 μM) added 5 min before DOX or DNR (50 μM). Reactions were started by adding NADPH (250 μM) and carried out at 37°. Alcohol metabolites were assayed at 4 hr following the procedures described in Section 2. Values are means ± SE of three separate determinations in triplicate.

tases/AKR1B1) [25]. Reportedly specific inhibitors of 20α-hydroxysteroid dehydrogenases/AKR1C1 and/or 3α-hydroxysteroid dehydrogenases/AKR1C3, like meclofenamic acid or betamethasone or diethylstilbestrol [26], had no significant effect on the conversion of DOX to DOXol (Table 1). Under comparable conditions, none of these compounds proved effective at inhibiting conversion of DNR to DNRol (see again Fig. 3 and Table 1). These results anticipated that (i) DOXol formation was mediated by AKR-type reductases, most probably belonging to aldehyde reductases/AKR1A1; (ii) AKR-type reductases were not involved in DNR to DNRol conversion. Based upon these findings, we evaluated the effects of flavonoids (quercetin, rutin, kaempferol, MonoHER) currently referred to as more specific inhibitors of SDR-type reductases [27]. As shown in Fig. 4, these inhibitors did not impair but actually increased the conversion of DOX to DOXol. Possible clues for explaining these effects will be given in Section 4. In contrast, all flavonoids significantly inhibited the formation of DNRol, quercetin ranking the most effective (see also Fig. 4). At this time, we thought it was important to evaluate also the effects of genistein. This phytoestrogen has been shown to potently inhibit 3α-hydroxysteroid dehydrogenases/AKR1C3 [26], but the presence of a flavone backbone in its structure led us to wonder whether it could also inhibit DNRol formation as it was observed with typical flavonoids. As also shown in Table 1, genistein had no effect on DOXol formation, as one would expect if this metabolite was formed by aldehyde reductases/AKR1A1 rather than 3α-hydroxysteroid dehydrogenases/AKR1C3; however, genistein did inhibit DNRol formation by approximately 40%, consistent with our hypothesis that its flavone moiety could have contributed to inhibiting SDR-type reductase with activity on DNR. Collectively, experiments with flavonoids and genistein formed the basis to conclude that DNR to DNRol conversion was mediated by SDR-type reductases.

3.3. Comparisons between human and rabbit anthracycline reductases

Having characterized the anthracycline reductases forming alcohol metabolites in the human heart we performed comparative experiments using cytosolic fractions from

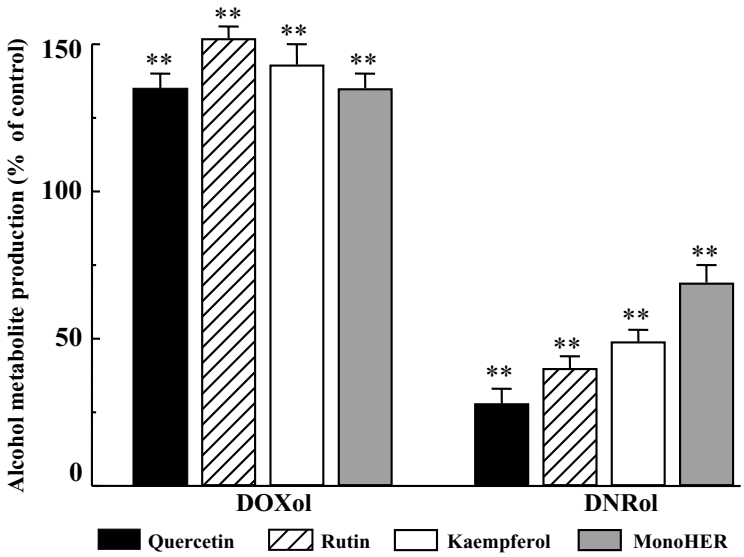


Fig. 4. Effects of flavonoids on DOXol or DNRol formation. Incubations were prepared as in Fig. 2, in the presence of the following inhibitors (50 μM): quercetin (black bars), rutin (hatched bars), kaempferol (white bars), and MonoHER (grey bars). Alcohol metabolites were measured at 4 hr. Values are means ± SE of at least three separate experiments performed in triplicate; $^{**}P < 0.01$.

rabbit heart. The rationale was offered by the fact that the rabbit serves a widely used animal model for inducing anthracycline-dependent chronic cardiotoxicity [28]; hence, it was important to see whether such metabolites were formed in the rabbit by the same reductases characterized in the human heart. Rabbit cardiac cytosol was highly effective in converting DOX and DNR to DOXol or DNRol, respectively. Under the same standard conditions as those used in the human heart (50 μM anthracycline, 0.25 mM NADPH and 1 mg protein/mL), DOXol formation averaged 2.7 ± 0.5 nmol/mg protein/4 hr, whereas DNRol formation averaged 41.9 ± 3.9 nmol/mg protein/4 hr. In either case alcohol metabolite formation largely exceeded that measured in the human heart (0.55 ± 0.05 nmol/mg protein/4 hr or 6.6 ± 0.6 nmol/mg protein/4 hr for DOXol or DNRol, respectively), but the ratio of DNRol to DOXol was essentially the same as determined in human heart (12–16 vs. 10–14, respectively). Rabbit heart cytosol was similar to the human counterpart also in exhibiting a single optimum pH at 9.0. After these preliminary characterizations, we determined whether the anthracycline reductases of rabbit heart exhibited the same responses to inhibitors characterized in the human heart. Table 2 summarizes the effects of inhibitors with specific activity on aldose reductases (EBPC) or exhibiting mixed activity on aldose and aldehyde reductases (Sorbinil, Tolrestat, AL1576, Zopolrestat). The data clearly indicate that EBPC had marginal or no effect on the formation of DOXol or DNRol in rabbit cardiac cytosol, as already seen in the human heart; however, important differences were noticed when examining the metabolism of DOX or DNR in presence of mixed inhibitors. Whereas DNRol formation was insensitive to Tolrestat or AL1576 or Zopolrestat in either human or rabbit heart, DOXol formation was suppressed by such inhibitors to much greater extent in human heart than in rabbit heart (usually ≥50% vs. ≤20%). These results indicated that human and rabbit heart were similar in metabolizing DNR through AKR-independent pathways, but differed in the mechanisms through which they metabolized DOX. Whereas human heart seemed to rely on AKR-type reductases, rabbit heart seemed to form DOXol by other reductases which were insensitive to AKR inhibitors. Based upon these findings we compared rabbit and human heart in regard to their responsiveness to SRD-reductase inhibitors (quercetin, rutin, kaempferol and MonoHER). Table 2 shows that such inhibitors were much similar at diminishing DNRol formation in rabbit or human heart but different effects were seen when examining DOXol formation. In fact, both quercetin and rutin and kaempferol were confirmed to stimulate DOXol formation by approximately 35–50% in human heart but all of them proved able to inhibit DOXol formation by ≥50% in rabbit heart. Collectively, these results showed that (i) in human heart carbonyl reductases metabolized DNR, whereas AKR-type reductases (most probably aldehyde reductases) metabolized DOX; (ii) in rabbit heart carbonyl reductases metabolized both DOX and DNR. Further evidence for the involvement of distinct anthracycline reductases in human heart but not in rabbit heart was obtained by performing

Table 2
Effect of AKR and SDR inhibitors on DOXol and DNRol production by human and rabbit heart

Inhibitor	Human		Rabbit	
	DOXol (%)	DNRol (%)	DOXol (%)	DNRol (%)
EBPC	85 ± 7	90 ± 7	90 ± 6	98 ± 4
Sorbinil	85 ± 4	87 ± 4	94 ± 7	95 ± 5
AL1576	50 ± 3	80 ± 4	82 ± 8	90 ± 7
Tolrestat	40 ± 4	86 ± 7	80 ± 4	92 ± 5
Zopolrestat	50 ± 5	85 ± 6	85 ± 7	96 ± 3
Quercetin	135 ± 5	28 ± 3	47 ± 5	35 ± 6
Rutin	152 ± 4	40 ± 5	49 ± 4	41 ± 3
Kaempferol	143 ± 7	49 ± 4	54 ± 4	41 ± 3
MonoHER	135 ± 5	69 ± 6	50 ± 5	51 ± 5

Assays were performed by pre-incubating cytosolic fractions (1 mg protein/mL) in 10 mM HEPES–300 mM NaCl, pH 7.4, with each inhibitor (50 μM) added 5 min before DOX or DNR (50 μM). Reactions were started by adding NADPH (250 μM) and carried out at 37°. Alcohol metabolites were assayed at 4 hr following the procedures described in Section 2. Values are means ± SE of three separate determinations in triplicate.

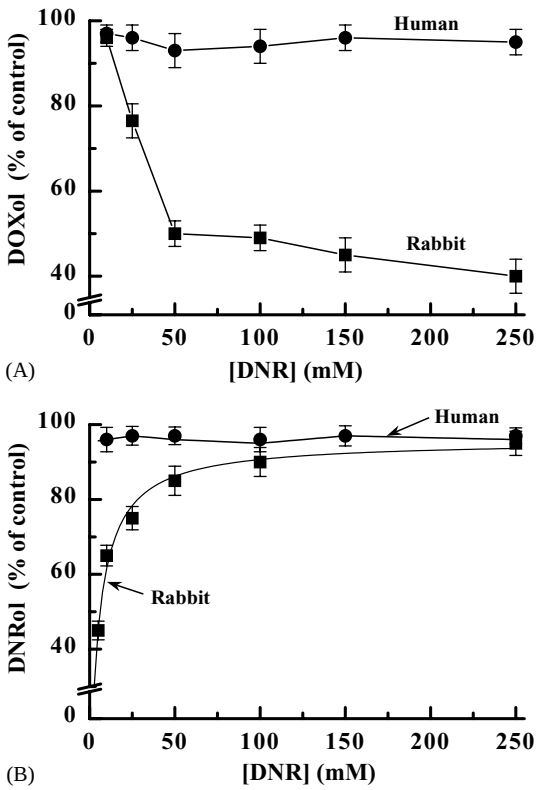


Fig. 5. Competition between DOX and DNR for human and rabbit heart cytosolic reductases. Incubations were prepared as described in Fig. 2, except that DOX was held constant at 50 μM and DNR was included at increasing concentrations. In panel (A) incubations were assayed for DOXol; in panel (B) incubations were assayed for DNRol. All values were determined at 4 hr and were means ± SE of at least three separate experiments performed in triplicate.

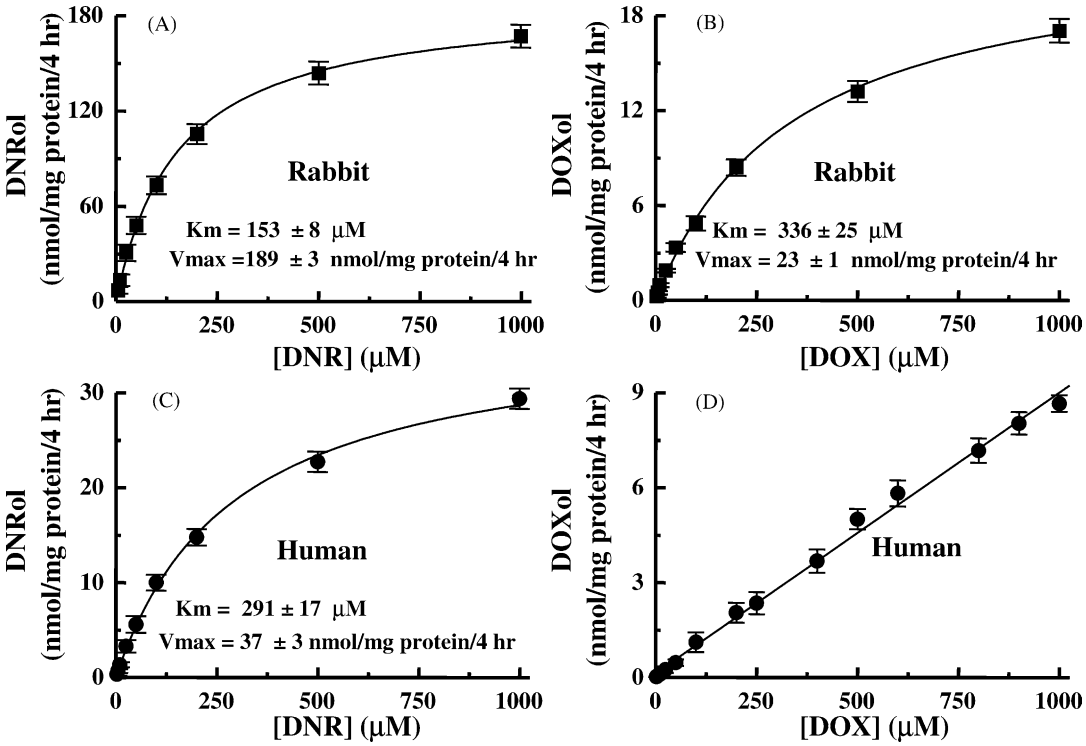


Fig. 6. Effect of DNR and DOX concentration on anthracycline carbonyl reduction in human and rabbit heart. Human or rabbit cytosolic fractions (1 mg protein/mL) were incubated with increasing concentrations of DNR or DOX at 37° in 10 mM HEPES–300 mM NaCl, pH 7.4, 37°. Reactions were started by adding NADPH (250 μM), and incubations were assayed for DNRol (A, C) or DOXol (B, D) at 4 hr. Values are means ± SE of at least three separate experiments performed in triplicate.

experiments in which the formation of DOXol from 50 μM DOX was measured in the absence or presence of increasing concentrations of DNR. As shown in Fig. 5A, 10–250 μM DNR did not interfere with DOXol formation in human heart, as one would expect if DOX were metabolized by AKR-type reductases having no activity on DNR; however, DNR concentration-dependently suppressed DOXol formation in the rabbit heart, with half-maximal efficacy at ∼25 μM. This latter finding indicated that in rabbit heart DNR and DOX competed for the same type of reductases. Competition or lack of competition between DOX and DNR in rabbit or human cytosol was confirmed by measuring concomitant conversion of DNR to DNRol. As shown in Fig. 5B, incubation of 10–250 μM DNR with 50 μM DOX in the human cytosol gave essentially the same levels of formation of DNRol as those observed in the absence of DOX, providing a good correlate to the fact that DNR did not suppress DOXol formation under the same experimental conditions. In contrast, co-incubation of 10–250 μM DNR with 50 μM DOX in rabbit heart cytosol revealed partial reduction in the level of formation of DNRol from 10 to 25 μM DNR, that is the range of concentrations at which this anthracycline was in competition with 50 μM DOX. Concentrations of DNR above 50 μM eventually outweighed DOX and converted to DNRol at essentially the same level observed in the absence of DOX (see also Fig. 5B). The observation that ∼25 μM DNR suppressed the metabolism of ∼50 μM DOX by approximately 50% anticipated that DNR was a better substrate than DOX for the carbonyl reductases of rabbit heart. Accordingly, kinetic measurements showed that the apparent K_m of rabbit cytosol for DNR was significantly lower than that for DOX (153 ± 8 μM vs. 336 ± 25 μM), whereas its V_{max} was significantly higher (189 ± 3 nmol/mg protein/4 hr vs. 23 ± 1 nmol/mg protein/4 hr) (Fig. 6A and B). It is worth noting that similar determinations could only in part be replicated in the human cytosol. In the case of DNR distinct apparent K_m and V_{max} could be determined (291 ± 17 μM and 37 ± 1 nmol/mg protein/4 hr, respectively) (Fig. 6C). These values showed that human heart cytosol had reduced affinity for DNR and lower activity in DNRol formation, consistent with the fact that the levels of DNR to DNRol conversion were significantly lower as compared to rabbit heart. In the case of DOX, however, the formation of DOXol never reached saturation kinetics and the apparent K_m and V_{max} for this anthracycline in human heart cytosol could not be determined (Fig. 6D).

4. Discussion

Anthracycline-induced cardiotoxicity is a multifactorial process in which distinct drug metabolites or byproducts may be involved in mediating the acute and chronic phases of myocardial dysfunction [29]. As already mentioned,

anthracyclines generate free radicals and the fact that cardiac-restricted catalase overexpression protects transgenic mice against anthracycline-induced cardiotoxicity supports the importance of an oxidative stress in these settings [30,31], as does the therapeutic benefit of treating laboratory animals or patients with the cell permeable iron chelator dexrazoxane [8,32]. However, antioxidants do not prevent or delay cardiomyopathy in patients [9,10]. The last few years have witnessed growing interest in the possibility that also secondary alcohol metabolites may be involved in inducing chronic cardiomyopathy. In the present study, we characterized how anthracyclines might be converted to secondary alcohol metabolites in the human heart. Experiments were carried out in cytosolic fractions derived from post-mortem myocardial samples, which we have previously shown to give essentially the same results as those obtained with *ex vivo* samples [33]. Our data show that the reductases converting DOX to DOXol or DNR to DNRol in human heart share common features, for example, pH-dependence and specific requirement for NADPH over NADH; however, the reductases acting on DOX belong to AKR1A1-type aldehyde reductases of the aldo-keto reductase superfamily, whereas the reductases acting on DNR belong to carbonyl reductases of the short chain dehydrogenase superfamily. Further supportive evidence has been offered by competition experiments in which DNR was shown not to interfere with the metabolism of DOX (Cf Fig. 5). During the course of these studies we also observed that flavonoids with prominent activity on SDR-type reductases (quercetin, rutin, kaempferol, MonoHER) not only failed to inhibit DOX to DOXol conversion, as one would expect if DOX were metabolized by flavonoid-insensitive aldo-keto reductases, but actually afforded 30–50% stimulation of DOXol formation (Cf Fig. 4 and Table 2). While resembling the results obtained by others when titrating the substrate-binding sites of several NAD(P)H oxido-reductases with appropriate concentrations of flavonoids [34,35], these findings suggest that aldo-keto reductases are liable to allosteric-type stimulation and anticipate that potentially toxic formation of DOXol in the heart might be enhanced by drugs administered in combination with DOX. This might be the case of paclitaxel, a tubulin-active taxane which we have previously shown to increase DOXol formation when reconstituted with NADPH and DOX in human cardiac cytosol [36] and consistently accelerates the development of cardiomyopathy when combined with DOX for improved treatment of metastatic breast cancer [37].

An intriguing feature of anthracyclines is that minor changes in their chemical structure may cause major modifications of their biochemical and biological behaviour. The only difference between DOX and DNR is that the side chain of DOX terminates with a primary alcohol whereas that of DNR terminates with a methyl group (Cf Fig. 1). Despite such a minor structural difference, DOX exhibits prominent activity against solid tumors and lymphomas, whereas DNR is most active against leukemias [1]. Our findings that DOX and DNR are metabolized by different reductases of human cardiac cytosol indicate that the side chain terminus may influence also an enzymatic reduction of the juxtaposed carbonyl group. Interactions of carbonyl reductases with DNR seem to be considerably more efficient than interactions of aldo-keto reductases with DOX. This is indicated by the fact that DNRol formation always exceeded that of DOXol several-fold (Cf Fig. 2); it is indicated also by the fact that DNR exhibited a defined apparent K_m of 291 ± 17 μM for human cardiac cytosol (Cf Fig. 6C) whereas the dependence of DOXol formation on DOX concentration never approached saturation kinetics which could be used to approximate an apparent K_m (Cf Fig. 6D). The remarkable differences between DOX and DNR do not rule out that the latter could be metabolized also by aldo-keto reductases under defined conditions. For example, we have previously shown that human cardiac cytosol can convert DNR to DNRol even after calcium hydroxyapatite chromatography under low ionic strength conditions, a procedure enriching samples in aldo-keto reductases [33]; however, the yield of DNRol was somewhat lower than that of DOXol, consistent with our finding that DNR would not be a particularly good substrate for aldo-keto reductases.

Laboratory animals, and rodents in particular, offer established models for evaluating the efficacy of investigational protectants against anthracycline-induced cardiotoxicity [28]. In this context it is worth noting that flavonoids antagonize negative-inotropism induced by acute [38] and chronic [39] administration of DOX. Some flavonoids probably protect by exerting direct positive inotropism [40], but the prevailing belief is that they act by scavenging free radicals generated by DOX [38]. Results described in the present study offer an opportunity to reappraise this interpretation; in fact, inhibitor and competition experiments show that in rabbit heart cytosol not only DNR but also DOX was metabolized by flavonoid-inhibitable carbonyl reductases. These findings suggest that in rodents flavonoids might prevent DOX-induced cardiotoxicity not only by scavenging free radicals but also by inhibiting its conversion to the potentially toxic DOXol. More in general, these results demonstrate that laboratory animals may not always help to predict the clinical efficacy of potential protectants due to the different mechanisms of alcohol metabolite formation in human vs. animal heart, as described in our present study.

There are of course potential arguments against the hypothesis that secondary alcohol metabolites mediate chronic cardiomyopathy. Some investigators reported that cardiac dysfunction induced in rabbits after systemic administration of DRN correlated with cardiac concentration of DNRol but not with that of DNR [41]. This would be consistent with the alcohol metabolite hypothesis. However, others have shown that systemic administration of DNRol to rats did not induce cardiotoxicity whereas

administration of DNR, giving comparable cardiac levels of DNRol, caused severe cardiac impairment [42]. This suggested that cardiotoxicity was mediated by DNR rather than DNRol. An additional caveat is introduced by the fact that aldo-keto and/or carbonyl reductases are present in most tissues, making it difficult to understand how secondary alcohol metabolite formation would be toxic in the heart but not in other tissues. Finally, the fact that human cardiac cytosol forms much more DNRol than DOXol does not always correlates with clinical evidence of increased cardiac toxicity of DNR compared to DOX [43]. Such divergences might be reconciled by keeping in mind critical factors like the animal species used in different experiments, the different intracellular distribution of exogenously administered vs. endogenously formed alcohol metabolites, as well as possible differences between various anthracyclines in their intracardiac distribution (e.g. endothelial cells vs. cardiomyocytes) [44,45]. An additional consideration pertains the metabolic behaviour of cardiomyocytes in respect to other cell types. For example, the greater toxicity of alcohol metabolites compared to parent anthracyclines against iron regulatory proteins might have more serious consequences in cardiomyocytes due to their accelerated iron metabolism and continuous demand for metabolically available iron [5]. Overall, the multifactorial nature of cardiotoxicity implies that alcohol metabolites are important but certainly not the unique determinants of cardiac damage.

In conclusion, we have characterized anthracycline carbonyl reduction in human cardiac cytosol and, for comparative purposes, in rabbit cardiac cytosol. Our data demonstrate that in human heart DOX and DNR are metabolized by distinct aldo-keto- or carbonyl-reductases, whereas in rabbit heart only carbonyl reductases are involved in metabolizing either anthracycline. Because inhibitors of carbonyl reducing enzymes have been considered as potential protectants against anthracycline-induced cardiotoxicity, this information may be of both methodological and conceptual value. In particular, these results encourage identification and development of specific and safe inhibitors of the aldo-keto reductases which convert DOX to DOXol in human heart. Previous limited studies have shown that barbiturates may serve as models for such inhibitors, as phenobarbital reduced DOXol formation and diminished DOX cardiotoxicity in the rat by inhibiting what appeared to be an aldo-keto reductase [46]. Whereas the clinical value of barbiturates as inhibitors of anthracycline metabolism would be limited by obvious problems of safety and tolerability, we note that in our study barbiturates never inhibited DOXol formation in either human or rabbit cardiac cytosol (not shown). This offers further evidence of the complex behavior of such reductases, which may be expressed unevenly not only among humans and rodents but also among different rodent species or strains [47]. Further work is therefore needed to characterize the molecular human aldo-keto and/or short-chain reductases and design structure- and mechanism-based inhibitors of anthracycline metabolism.

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