Doxorubicin Irreversibly Inactivates Iron Regulatory Proteins 1 and 2 in Cardiomyocytes: Evidence for Distinct Metabolic Pathways and Implications for Iron-mediated Cardiotoxicity of Antitumor Therapy

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ABSTRACT

Changes in iron homeostasis have been implicated in cardiotoxicity induced by the antineoplastic anthracycline doxorubicin (DOX). Certain products of DOX metabolism, like the secondary alcohol doxorubicinol (DOXol) or reactive oxygen species (ROS), may contribute to cardiotoxicity by inactivating iron regulatory proteins (IRP) that modulate the fate of mRNAs for transferrin receptor and ferritin. It is important to know whether DOXol and ROS act by independent or combined mechanisms. Therefore, we monitored IRP activities in H9c2 rat embryo cardiomyocytes exposed to DOXol or to analogues which were selected to achieve a higher formation of secondary alcohol metabolite (daunorubicin), a concomitant increase of alcohol metabolable and decrease of ROS (5-imino daunorubicin), or a defective conversion to alcohol metabolite (mitoxantrone). On the basis of such multiple comparisons, we characterized that DOXol was able to remove iron from the catalytic Fe-S cluster of cytoplasmic aconitase, making this enzyme switch to the cluster-free IRP-1. ROS were not involved in this step, but they converted the IRP-1 produced by DOXol into a null protein which did not bind to mRNA, nor was it able to switch back to aconitase. DOXol was also shown to inactivate IRP-2, which does not assemble or disassemble a Fe-S cluster. Comparisons between DOX and the analogues revealed that IRP-2 was inactivated only by ROS. Thus, DOX can inactivate both IRP through a sequential action of DOXol and ROS on IRP-1 or an independent action of ROS on IRP-2. This information serves guidelines for designing anthracyclines that spare iron homeostasis and induce less severe cardiotoxicity.

INTRODUCTION

Antitumor therapy with the anthracycline DOX is limited by acute and chronic toxicity to the heart. Whereas the acute toxicity is transient and clinically manageable, the chronic toxicity evolves into progressive cardiomyopathy, which limits clinical use of DOX. Several lines of evidence indicate that alterations of iron homeostasis may contribute to both forms of cardiotoxicity (2, 3); therefore, it has been suggested that DOX might alter the function of cytoplasmic aconitase/IRP-1. The role of this protein is to adapt the levels of iron to the metabolic needs of the cell while preventing the accumulation of potentially toxic excess iron. When the cell needs iron, IRP-1 binds to IRE in the mRNA for transferrin receptor and in the mRNA for ferritin, increasing stability of the former while decreasing translation of the latter (4–7). These divergent but coordinate processes make iron uptake prevalent over sequestration and consequently produce a pool of iron that is available for metabolic use. When the cellular levels of iron are too high, IRP-1 assembles a [4Fe-4S] cluster that abolishes IRE-binding capacity but confers an aconitase activity similar to that of the mitochondrial enzyme of the Krebs cycle (5). Under these conditions, iron sequestration prevails over uptake, and the cell is protected from the toxicity of excess iron. Two independent studies have shown that DOX was able to attack aconitase/IRP-1 through the action of its secondary alcohol metabolite DOXol (8) or by producing ROS (9). Whether DOXol and ROS acted by mutually exclusive or combined mechanisms has nonetheless remained an unresolved issue. Because this information would be of value for designing less cardiotoxic analogues, we performed experiments to better define the roles of ROS and DOXol as modifiers of aconitase/IRP-1. We also determined whether DOX was able to target IRP-2, which is similar to IRP-1 in modulating the fate of mRNAs but is not regulated through cluster assembly or disassembly (10) and, hence, may exhibit different responses to ROS or DOXol.

MATERIALS AND METHODS

Drugs and Chemicals. DOX, DNR, and 5-iminoDNR were obtained through the courtesy of Dr. Antonino Sauraro (Department of Chemistry, Pharmacium-Upjohn, Milan, Italy); the corresponding secondary alcohol metabolites were synthesized and purified by us, as described (11). MITOX, cis-acconitase, bovine erythrocyte CuZn SOD (EC 1.15.1.1), and horse heart cytochrome c were from Sigma Chemical Co.-Aldrich (Milan, Italy). cys and FAS were products of Merck (Darmstadt, Germany).

Cell Culture, Treatment, and Preparation of Lysates. We used the embryonic, rat heart-derived cell line H9c2 (American Type Culture Collection-CTRL 1446), which has proven to be useful for characterizing the responses of cardiomyocytes to pathophysiological stimuli (12–14). Cells were grown at 37°C under 5% CO2/air in DMEM adjusted to contain 4 mM glutamine, 18 mM sodium bicarbonate, 25 mM glucose, 1 mM sodium pyruvate, 100 units/ml penicillin, and 0.1 ng/ml streptomycin and supplemented with 10% heat-inactivated FCS. Subconfluent cells (4.5 × 106) were seeded in 75-cm2 flasks and incubated for 16 h with increasing concentrations of DOX, DNR, 5-iminoDNR, or MITOX. At the end of treatment, adherent cells were scraped, washed in PBS, and homogenized in 1 ml of Munro buffer [10 mM HEPES (pH 7.6), 3 mM MgCl2, 40 mM KCl, and 5% glycerol]. Aliquots (800 μl) were extracted with a 4-fold excess of (1:1) CHCl3/CH3OH, and the organic phases were used for drug analysis. The remaining 200 μl were centrifuged at 16,000 × g for 5 min at 4°C, and the supernatants were split in 100-μl aliquots. One aliquot was added with 1 mM DTT and stored frozen until assay for IRP. The other aliquot was used for aconitase assay and was added with the substrate-intermediate cis-acconitase (20 μM) to prevent cluster decay during storage.

RNA-Protein Gel Retardation Assay. The probe for the bandshift assay was transcribed from the linearized pSPT-fer plasmid containing the IRE of human ferritin H chain (15), using T7 RNA polymerase in the presence of α-32P-UTP (Amersham-Pharmacia Biotech, Milan, Italy). Equal amounts of lysates (2 μg of protein) were incubated, in the absence or presence of 1% 2-ME, with...
a molar excess of IRE probe and treated sequentially with RNase T1 and heparin (16). After separation on 6% nondenaturing polyacrylamide gels, complexes between radioactive RNA and IRP-1 or -2 were visualized by autoradiography and quantitated by direct nuclear counting using an Instant Imager (Packard Instruments Co., Milan, Italy).

**Western Blot Analysis.** Aliquots of the lysates used for IRP determination, and containing equal amounts of proteins (80 μg), were electrophoresed in 10% acrylamide–SDS gels, electrobotted to Hybond PVDF membranes (Amersham–Pharmacia Biotech), and incubated with a 1:500 dilution of rabbit antisera against human recombinant IRP-1. IRP-1 was detected by chemiluminescence using an immunodetection kit (ECL Plus; Amersham–Pharmacia Biotech), according to the manufacturer’s instructions.

**Drug Uptake and Assay for Secondary Alcohol Metabolites.** Vacuum-dried organic extracts of cardiomyocytes were suspended in 10–20 μL of CH₃OH and analyzed for DOX, DNR, and 5-iminoDNR or their secondary alcohol metabolites by two-dimensional TLC on 0.25 mm (20 × 20 cm) Silica Gel F₂₅₄₄ Plates (Merck; Ref. 11). Mobile phases (volume for volume) and Rₜ values were: CHCl₃/CH₃OH/CH₃COOH/H₂O (80:20:14:6) for separation of DOX (0.61) and DOXol (0.43); CHCl₃/CH₃OH/CH₃COOH (80:20:20:4) for separation of DNR (0.44) and its alcohol metabolite DNRol (0.28); and CHCl₃/CH₃OH/CH₃COOH (100:20:20) for separation of 5-iminoDNR (0.71) and 5-iminoDNRol (0.59). After identification by cochromatography with authentic anthracelines and secondary alcohol metabolites, DOX(ol), DNR(ol), and 5-iminoDNR(ol) were quantitated fluorometrically against appropriate standard curves. Other metabolites usually were very low or absent; therefore, total drug uptake was calculated as (parent drug + secondary alcohol metabolite).

Recovery was >90% for all anthracelines and their alcohol metabolites; the lowest detection limit was 0.01 nmol/mg protein. MITOX, which was not converted to a secondary alcohol metabolite, was measured by optical spectroscopy by taking advantage of its high absorances at 608 and 658 nm (ε = 19.2 and 20.9 mM⁻¹ cm⁻¹, respectively; Ref. 17). For this purpose, vacuum-dried cell extracts were dissolved in spectroscopy-grade CH₃OH and analyzed in a Hewlett Packard 8453 diode array spectrophotometer equipped with computer-assisted corrections for turbidity and scatter.

Two-dimensional TLC in CHCl₃/CH₃OH/CH₃COOH/H₂O (50:30:35:15) confirmed that cell-extracted MITOX comigrated as a single band (Rₜ = 0.57) with an authentic standard.

**ROS Formation.** Drug-induced ROS formation was determined by measuring O₂⁻ production in cardiac microsomes. The latter were isolated from small samples of human myocardium disposed of during aorto-coronary bypass grafting, using established procedures (18). After isolation, microsomes were solubilized with sodium deoxycholate, at the final ratio of 0.1% detergent:5 mg of protein, to obtain a fraction enriched in NADPH-cytochrome P-450 reductase (specific activity before and after solubilization: 11 versus 29 μM/mg protein). O₂⁻ was measured through the SOD-inhibitable cytochrome c reduction assay in 1-mL systems containing solubilized microsomes (25 μg of protein), NADPH (0.1 mM), drugs (10 μM), and cytochrome c (25 μM) plus or minus SOD (200 units) in 0.3 mM NaCl (pH 7.0), 37°C. Drug-induced O₂⁻ formation was determined as the net increase over a basal rate of 0.6 nmol nmol⁻¹ protein/min. DOX was shown to induce the formation of 0.61 nmol O₂⁻/mg protein/min, whereas DNR, 5-iminoDNR, and MITOX induced the formation of 0.57, 0.1, and 0.31 nmol O₂⁻/mg protein/min, respectively. The expected yield of ROS in cardiomyocytes was determined based on the efficiency of each drug in producing O₂⁻ in vitro and its uptake in H9c2 cells. ROS formation by DOX was assumed to be equal to 100%; ROS formation by the analogues was calculated according to the equation: RSONalogue = [(Uptake × O₂⁻ formation)analogue / (Uptake × O₂⁻ formation)DOX] × 100. Although indirect, these calculations avoided pitfalls in measuring cellular production of ROS with the fluorescent dye 2′,7′-dichlorofluorescein (19).

**Assay for Aconitase.** Aconitase activity was determined spectrophotometrically by monitoring the disappearance of cis-aconitate (ε₉₀₋₁₂₀ = 3.6 mM⁻¹ cm⁻¹; Ref. 20). The incubations (1 mL of final volume) contained lysates (15–50 μg of protein) and 0.1 mM cis-aconitate in 0.3 mM NaCl (pH 7.0), 37°C; one mM was defined as the amount of enzyme which consumed 1 nmol of cis-aconitate/min. Where indicated, lysates were preincubated for 10 min at 37°C with cyst and FAS at the final ratios of 1000 or 50 nmol/mg protein, respectively. These ratios were shown previously to be optimal for reconstituting [4Fe-4S] clusters and aconitase activity (8).

**Other Assays.** Proteins were measured by the Bio-Rad kit assay (Segrate, Milan, Italy). LDH release was measured by the Sigma Chemical Co. kit 228 UV and was expressed as a percentage of total cellular activity. Other conditions are indicated in the legends to figures and Table 1.

**RESULTS**

**Effects of DOX on IRP-1 and -2.** As shown in Fig. 1A, H9c2 cells induced IRP-1 activity by a maximum of 91 ± 21% at 5 μM, but such increase fell to 35 ± 17% at 10 μM (P < 0.001, paired Student’s t test, n = 8); this corresponded to a ~57% loss in IRP-1 activation when DOX was increased from 5 to 10 μM. Treating the cell lysates with 2-ME before the gel shift analysis increased IRP-1 activity in all samples, a finding consistent with the ability of 2-ME to regenerate -SH groups involved in IRP-IRE interactions (21, 22); however, 2-ME did not blunt the concentration-related, bell-shaped response induced by DOX. The effects of DOX were not attributable to modifications in the cellular levels of IRP-1, as Western blot analysis detected similar levels of this protein in both control cells and those exposed to 1–10 μM DOX (Fig. 1B). Because IRP-1 binds to IRE in its cluster-free form, we determined whether DOX acted by posttranslational mechanisms that altered the equilibrium between IRP-1 and its cluster-containing counterpart, aconitase. As shown in Fig. 1C, 1–5 μM DOX caused a significant decrease in aconitase activity, which correlated with the increase in IRP-1 activity observed in the same range of concentrations. Treatment of the cell lysates with cys/FAS, a procedure known to reconstitute [4Fe-4S] clusters, increased aconitase activity and blunted differences between control and 1–5 μM DOX cells. Fig. 1C also shows that 10 μM DOX caused the greatest loss of aconitase activity; however, treatment of the cell lysates with cys/FAS recovered only ~50% of the maximal aconitase activity seen in the other samples. Such a loss in cys/FAS-activatable aconitase was in good agreement with the ~57%, 2-ME-insensitive loss of IRP-1 activation under comparable experimental conditions. Taken as a whole, these findings demonstrated that cardiomyocytes contained a mixed pool of cluster-containing aconitase and cluster-free IRP-1 and that DOX was able to influence the ratio between the two forms.

**Concentrations of DOX** were 1, 10, and 100 μM. For this purpose, lysates were treated with cys, FAS, and DOX, and aconitase activity was measured with a coupled enzyme assay. The activity was measured by calculating the amount of CO₂ formed in the reaction catalyzed by aconitase and measured with an UV spectrophotometer. The activity was expressed as nmol O₂⁻/mg protein and was calculated as the difference between the activity of the control (no DOX) and the activity of the treated samples. The difference was then divided by the activity of the control to obtain the percentage inhibition or stimulation of aconitase activity by DOX.

**Table 1** Uptake and metabolism of DOX, DNR, 5-iminoDNR, and MITOX under conditions of half-maximal concentration

<table>
<thead>
<tr>
<th>Drug</th>
<th>Half-maximal concentration (μM)</th>
<th>Uptake (nmol/mg protein)</th>
<th>Secondary alcohol metabolite (nmol/mg protein)</th>
<th>ROS Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>5.0</td>
<td>0.9 ± 0.08</td>
<td>0.16 ± 0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>DNR</td>
<td>12.5</td>
<td>1.8</td>
<td>0.6</td>
<td>18</td>
</tr>
<tr>
<td>5-iminoDNR</td>
<td>25.0</td>
<td>1.0</td>
<td>0.32</td>
<td>18</td>
</tr>
<tr>
<td>MITOX</td>
<td>5.0</td>
<td>1.4</td>
<td>—</td>
<td>79</td>
</tr>
</tbody>
</table>

* Values are means ± SE of three experiments (DOX) or are taken from duplicate experiments with >90% agreement (DNR, 5-iminoDNR, and MITOX).

Ref. 20. 2-ME, 2-Mercaptoethanol; FAS, farnesyl-AMP synthase.

* Calculated as described under “Materials and Methods.”

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glycosidic bond to the tetracyclic ring. ROS, like $\text{O}_2^-$ and $\text{H}_2\text{O}_2$, are formed after one electron reduction of the quinone moiety by mitochondrial, nuclear, or microsomal NAD(P)H oxidoreductases, like NADPH-cytochrome P450 reductase, producing a semiquinone-free radical that regenerates its parent quinone by oxidizing with oxygen (23). DOXol is formed by cytoplasmic aldo-keto or carbonyl reductases, which add two electrons to the side chain carbonyl group (-CO-CH$_2$OH $\rightarrow$ -CHOH-CH$_2$OH; Refs. 24 and 25). To characterize the role of DOXol and ROS as modifiers of IRP-1 and -2, we compared the effects of DOX to those of the following analogues: DNR, an analogue that contains both quinone and carbonyl moieties and thus retains the ability to form ROS and a secondary alcohol metabolite; 5-iminoDNR, an analogue that forms less ROS because of the replacement of a quinone group with an imino moiety; and

![Fig. 1. Effects of DOX on IRPs in H9c2 cells. A, effects of 1–10 $\mu$M DOX on IRP-1 and -2, measured with or without 2-ME. B, Western blot of IRP-1 in cells treated with 1–10 $\mu$M DOX; rIRP-1 indicates recombinant human IRP-1. C (left), aconitase activity in control and DOX-treated cells. C (right), aconitase activity after lysate preincubation with cys/FAS. Gels were representative of eight experiments; aconitase values were means ±SE of three experiments.](https://www.cancerres.aacrjournals.org)
being that maximal activation occurred at 15 μM DNR, the only difference
between DNR and the other two analogues was the formation of a null protein,
45% of which was recovered with cys/FAS in all samples exposed to ≤15 μM
DNR (Fig. 3A, bottom panel). Increasing DNR from 15 to 25 μM eventually
caused a 2-ME-insensitive, ∼56% loss in IRP-1 activation, which was accom-
panied by a cys/FAS-insensitive loss of ∼45% of aconitase activity. Thus, ap-
proximately half of the exchangeable pool of aconitase/IRP-1 was converted
into a null protein when DNR was increased from 15 to 25 μM, similar to what
was observed when DOX was increased from 5 to 10 μM. Replacing DOX with
DNR, an analogue characterized by a 2-fold increase in the ratio of alcohol
metabolite:ROS, therefore extended the range of anthracycline concentra-
tions within which aconitase switched to IRP-1 before the formation of a null
protein became evident. This suggested that secondary alcohol metabolites
were involved in converting aconitase to IRP-1, whereas ROS probably were
involved in producing a null protein. Support to this interpretation was pro-
vided by experiments with 5-iminoDNR, the analogue characterized by a much
higher ratio of alcohol metabolite:ROS attributable to a concomitant increase
of the former and decrease of the latter. In fact, 5-iminoDNR was able to
switch aconitase to IRP-1 but was unable to induce the formation of a null
protein, as evidenced by persistent elevation of IRP-1 and a complete recovery
of cys/FAS-inducible aconitase activity, even at a concentration of 50 μM
(Fig. 3B). Neither the switch of aconitase to IRP-1 nor the formation of a null
protein was observed in experiments with MITOX, the analogue producing
ROS but not secondary alcohol metabolites (Fig. 3C). This observation con-
firmed that secondary alcohol metabolites were required for switching ac-
conitase to IRP-1 and revealed that ROS produced a null protein by attacking
the IRP-1 produced by secondary alcohol metabolites.

Additional inspection of Fig. 3, A–C shows that DNR and MITOX,
but not the quinone-modified 5-imino DNR, were able to decrease IRP-2. This pattern reproduced the behavior of DOX, indicating that IRP-2 was affected by quinone-derived ROS, regardless of the concomitant formation of secondary alcohol metabolites. While inactivating IRP-2 in a concentration-dependent manner, DOX, DNR, and MITOX also caused an increasing damage to cardiomyocytes, which was evidenced by LDH release in the incubation medium (Fig. 4). This was not observed in the case of 5-iminoDNR, which both spared IRP-2 and failed to induce LDH release. Thus, ROS were important mediators of damage, and IRP-2 was a sensitive indicator of oxidative stress.

**DISCUSSION**

In a previous study, conducted in cytosolic fractions derived from human myocardium, we demonstrated that DOX lacks direct “structural” reactivity with aconitase/IRP-1 (8). However, replacing DOX with DOXol resulted in iron delocalization from aconitase, showing that the anthracycline may gain reactivity toward the Fe-S cluster of this enzyme after metabolic conversion of its side chain carbonyl group into a secondary alcohol moiety. Iron delocalization and consequent loss of aconitase activity were not accompanied by a gain of IRP-1 activity; instead, DOXol produced a null protein which did not recognize IRE nor switch back to aconitase (8). The formation of a null protein was attributed to irreversible modifications of -SH residues mediating both the recognition of IRE and the reassembly of the cluster (e.g., cys437; Refs. 8 and 22). Interestingly, DOXol reactions did not appear to involve ROS, as evidenced by the lack of effect of added scavengers like SOD or catalase (8). This picture has been extended and modified by other investigators, based on their studies in isolated cardiomyocytes derived from rat heart. In these cells, DOX was found to reduce aconitase activity by mechanisms that involved metabolic conversion of the quinone moiety to ROS, evidenced by the protective efficacy of cell-permeable mimetics of SOD or glutathione peroxidase (9). Unfortunately, these latter studies did not elucidate whether the loss in aconitase activity was accompanied by a gain in IRP-1 activity or by formation of a null protein. Moreover, aconitase inactivation was observed after incubation of cardiomyocytes with 20 μM DOX, a concentration exceeding the plasma peaks observed in patients after standard doses of the anthracycline (7–10 μM; Ref. 27). This factor may have produced unusually high levels of DOX in cardiomyocytes, facilitating reactions of O2− and/or H2O2 with aconitase. Similarly, the studies in human cardiac cytosol were performed with a bolus of DOXol (3–4 nmol/mg protein), which exceeded the steady-state levels of this metabolite in intact cardiomyocytes and consequently magnified its ability to target Fe-S clusters. In the present study, H9c2 cardiomyocytes were exposed to concentrations of DOX that never exceeded 10 μM. Moreover, we adopted the strategy of comparing DOX to analogues whose pharmacokinetic and biochemical behavior resulted in a greater formation of alcohol metabolite (DNR), in concomitant increase of alcohol metabolite and decrease of ROS (5-iminoDNR), or in selective deficiency of alcohol metabolite formation (MITOX). Comparing DOX to these analogues provided a mechanism-based tool for dissecting the roles of alcohol metabolites and ROS while also avoiding the need for perturbing the system with ROS scavengers or inhibitors of anthracycline metabolism. These experimental settings have allowed us to demonstrate that DOX modulates aconitase/IRP-1 by mechanisms that convert aconitase to IRP-1 and eventually produce a null protein. Comparisons between DOX and DNR or 5-iminoDNR demonstrate that aconitase is converted to IRP-1 by DOXol, not by ROS. This finding is explained by keeping in mind that aconitase switches to IRP-1 after a complete disassembly of its [4Fe-4S] cluster, an action exhibited by DOXol (8) but not by O2− or H2O2, which, at their best, would remove only the fourth labile iron required for aconitase activity (28, 29). Comparisons between DOX and DNR or 5-iminoDNR show that the role of ROS is to produce a null protein, but the experiments with MITOX clearly demonstrate that such a process requires also the presence of a secondary alcohol metabolite. This latter finding is explained by our previous demonstration that O2− and H2O2 lack sufficient reactivity for inducing irreversible modifications of cys437, even when they are produced at a much greater rate through the xanthine/xanthine oxidase reaction (30). Nonetheless, O2− and H2O2 might gain reactivity if they were converted by iron into more potent oxidants like hydroxyl radicals or iron-peroxo complexes. Secondary alcohol metabolites can facilitate such conversion by inducing cluster disassembly and switching aconitase to IRP-1, a process accompanied by iron release and formation of anthracycline-iron complexes in the closest proximity to cys437 (8). The effects of clinically relevant concentrations of DOX may therefore be more complex than believed previously, as they involve the action of DOXol in converting aconitase to IRP-1 and the action of ROS in converting the newly formed IRP-1 into a null protein.

Unlike IRP-1, IRP-2 lacks the ability to form or disassemble Fe-S motifs. We were unable to detect IRP-2 in our preceding study in human cardiac cytosol (8), nor was IRP-2 characterized in the studies of the effects of DOX in isolated rat cardiomyocytes (9). We therefore exploited our present settings to assess whether IRP-2 was modulated by DOX in a fashion similar to, or different from, that described for IRP-1. Our results demonstrate that DOX induces irreversible inactivation of IRP-2, similar to what was observed for IRP-1; however, only ROS are required for inactivating IRP-2. Such findings are explained by a greater susceptibility of IRP-2 to oxidation, followed by proteasome-mediated degradation (31–33). The observation that DOXol does not attack IRP-2 confirms that its action is targeted to Fe-S motifs and, hence, to aconitase/IRP-1.

Studies in isolated cardiomyocytes provide a good model for a
oxidative damage induced by ROS, such disorders may also misplace modifications in iron availability and develop coordinate adaptations protein, described in our present study, represents a novel example of DOXol, alone or in concert with ROS, has therefore been implicated on the other hand, several lines of evidence indicate that the chronic phase of cardiotoxicity, which develops after multiple doses of DOX, coincides with an accumulation of DOXol inside cardiomyocytes (3, 25, 34, 35). DOXol, alone or in concert with ROS, has therefore been implicated as a potential mediator of the chronic, irreversible cardiomyopathy induced by DOX. The conversion of aconitase/IPR-1 into a null protein, described in our present study, represents a novel example of how DOXol might contribute to chronic cardiotoxicity by interacting with ROS. In fact, the null protein makes cells unable to sense modifications in iron availability and develop coordinate adaptations of the levels of transferrin receptor and ferritin (36). While amplifying oxidative damage induced by ROS, such disorders may also misplace iron at cellular sites that govern the contraction-relaxation cycle of the heart but lose their function after sterical occupation by iron (e.g., the yanode oxidoreductase/calcium release channel of sarcoplasmic reticulum; Ref. 37).

In conclusion, DOXol and ROS irreversibly inactivate IPR-1 and IRP-2 in cardiomyocytes exposed to DOX. The results modify current knowledge in this field, especially with regard to novel evidence for a sequential action of DOXol and ROS in converting aconitase/IPR-1 into a null protein. This information has been obtained in a cardiomyocyte model which, in principle, may be exploited for screening a broad repertoire of anthracyclines. In this study, we chose to focus on selected analogues that have already been assessed for cardiotoxicity; and, hence, may serve for probing relationships between anthracycline metabolism, IRP inactivation, and development of cardiotoxicity; e.g., both 5-iminoDNR and MITOX proved to be more cardiac tolerable than DOX in some preclinical or clinical settings of chronic cardiotoxicity (38, 39), a finding consistent with our demonstration that another drug converted aconitase/IPR-1 into a null protein because of impaired production of ROS or secondary alcohol metabolites, respectively. Results described in this study, therefore, offer a rationale to design other analogues which also spare aconitase/IPR-1 by forming less ROS and/or alcohol metabolites than DOX. Additional guidelines in this setting are provided by the differential roles of anthracycline-derived ROS and alcohol metabolites in cancer cells. Evidence for a possible involvement of ROS in the antitumor activity of anthracyclines has, in fact, been reported (40, 41). In contrast, several lines of evidence demonstrate that secondary alcohol metabolites do not always contribute to, but sometime diminish, the antitumor activity of anthracyclines (42–44). Analogue characterized by a selective impairment in alcohol metabolite formation might therefore prove to be more advantageous for sparing cardiac aconitase/IPR-1 while also retaining equal or improved therapeutic efficacy, serving better alternatives to DOX for use in cancer patients.

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