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Review

Anthracycline-induced cardiotoxicity: Overview of studies examining the roles of oxidative stress and free cellular iron

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Abstract:
The risk of cardiotoxicity is the most serious drawback to the clinical usefulness of anthracycline antineoplastic antibiotics, which include doxorubicin (adriamycin), daunorubicin or epirubicin. Nevertheless, these compounds remain among the most widely used anticancer drugs. The molecular pathogenesis of anthracycline cardiotoxicity remains highly controversial, although the oxidative stress-based hypothesis involving intramyocardial production of reactive oxygen species (ROS) has gained the widest acceptance. Anthracyclines may promote the formation of ROS through redox cycling of their aglycones as well as their anthracycline-iron complexes. This proposed mechanism has become particularly popular in light of the high cardioprotective efficacy of dexrazoxane (ICRF-187). The mechanism of action of this drug has been attributed to its hydrolytic transformation into the iron-chelating metabolite ADR-925, which may act by displacing iron from anthracycline-iron complexes or by chelating free or loosely bound cellular iron, thus preventing site-specific iron-catalyzed ROS damage. However, during the last decade, calls for the critical reassessment of this "ROS and iron" hypothesis have emerged. Numerous antioxidants, although efficient in cellular or acute animal experiments, have failed to alleviate anthracycline cardiotoxicity in clinically relevant chronic animal models or clinical trials. In addition, studies with chelators that are stronger and more selective for iron than ADR-925 have also yielded negative or, at best, mixed outcomes. Hence, several lines of evidence suggest that mechanisms other than the traditionally emphasized "ROS and iron" hypothesis are involved in anthracycline-induced cardiotoxicity and that these alternative mechanisms may be better bases for designing approaches to achieve efficient and safe cardioprotection.

Key words: anthracycline cardiotoxicity, doxorubicin (adriamycin), daunorubicin (daunomycin), epirubicin, dexrazoxane (ICRF-187), oxidative stress, cellular iron metabolism

Abbreviations: ANT – anthracycline, CHF – congestive heart failure, DAU – daunorubicin, DFO – deferoxamine, DMPO – 5,5-dimethylpyrroline-N-oxide, DOX – doxorubicin, DXZ – dexrazoxane, EDTA – ethylenediaminetetraacetic acid, eNOS – endothelial nitric oxide synthase, Fe – iron, GSH/GSSG – reduced/oxidized glutathione, H_2O_2 – hydrogen peroxide, iNOS – inducible nitric oxide synthase, IRP – iron regulatory protein, MDA – malondialdehyde, monoHER – 7-monohydroxyethylrutoside, NAC – N-acetylcysteine, NAD(P) – nicotinamide adenine dinucleotide (phosphate), NO – nitric oxide, *o*-108 – *ortho*-chlorbenzoyl isonicotinoyl hydrazone, $O_2^{\bullet-}$ – superoxide radical, $OH^{\bullet}$ – hydroxyl radical, PIH – pyridoxal isonicotinoyl hydrazone, RNS – reactive nitrogen species, ROS – reactive oxygen species, SIH – salicylaldehyde isonicotinoyl hydrazone, SOD – superoxide dismutase

Introduction – the anthracyclines

The introduction of anthracycline (ANT) antineoplastic antibiotics to the chemotherapy of malignant neoplasms has been one of the major successes of cancer medicine. This is particularly evident in pediatric oncology, where the 5-year survival rate for childhood cancer has increased from ≈30% in the 1960s to 70–80% today [32, 57], while more than 50% of childhood cancer survivors have received ANTs [74].

The first two ANTs, daunorubicin (DAU, also known as daunomycin and rubidomycin) and doxorubicin (DOX, also known as adriamycin), were isolated in the 1960s from *Streptomyces peucetius*, a species of actinobacteria [5, 141] (Fig. 1). While DAU has been shown to be highly effective against acute lymphoblastic and myeloblastic leukemias, DOX has been found to have a much broader anticancer spectrum, which includes numerous solid tumors in addition to hematological malignancies. Although more than 40 years old, ANTs are still frequently used in clinical practice, and DOX in particular remains an important component of many current chemotherapy protocols for treating breast cancer, sarcomas, childhood solid tumors (e.g., Wilms' tumor), leukemias, Hodgkin's disease, non-Hodgkin's lymphomas, and many other cancers [75]. Interestingly, rather than being replaced with novel progressive "targeted" agents, current clinical practice tends to combine them with the novel therapeutics to maximize the therapeutic response [112].

Despite their extensive use, the mechanism of ANT antineoplastic action is still a subject of debate and apparently it is a combination of several different mechanisms, which accounts for the high efficiency of this class of anticancer drugs [34, 95]. After their discovery, the anticancer effect of ANTs was attributed to their intercalation between base pairs of the DNA strands, which prevents replication of rapidly-growing cancer cells [129]. However, more recent studies have shown that at clinically relevant ANT concentrations, intercalation is unlikely to play a major role. Today, topoisomerase II is generally recognized to be the main cellular target of ANTs [9]. ANTs are called "topoisomerase poisons" because they act by stabilizing a reaction intermediate in which DNA strands are cut and covalently linked to tyrosine residues of topoisomerase II, which blocks subsequent DNA resealing. Failure to relax the supercoiled DNA blocks DNA replication and transcription. Furthermore, DNA strand breaks may trigger apoptosis of cancer cells, apparently *via* the p53-dependent pathway [114]. Although some older studies have also suggested that formation of reactive oxygen species (ROS) and lipid peroxidation participate in the anticancer effects of ANTs [99], several more recent studies have shown the opposite [104,

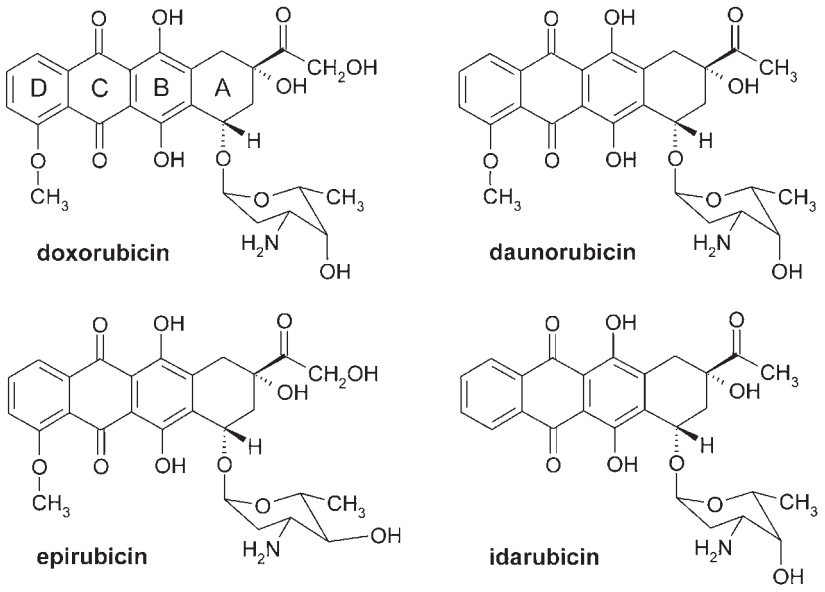

Fig. 1. Chemical structures of four main anthracyclines

156], and there now appears to be general agreement that oxidative stress is unlikely to be a significant contributor to the antitumor activity of ANTs [34, 95].

As with all traditional cytostatic drugs, ANT administration is accompanied by adverse drug reactions arising from the limited selectivity of their anticancer action. Particularly common are bone marrow suppression, resulting in leukopenia; mucositis; and gastrointestinal disturbances, such as nausea and vomiting [75]. However, introduction of highly effective oncological supportive therapy including modern antiemetic agents and colony-stimulating factors has made both the nausea and myelosuppression largely manageable. This has made even aggressive ANT regimens more bearable for patients.

Anthracycline-induced cardiotoxicity

While undetected during preclinical animal studies [46], cardiac toxic effects were first documented during early clinical evaluations of DAU [141] as well as DOX [10, 80]. In the late 1970s, the first retrospective clinical studies were published and they showed convincingly that the observed cardiac disturbances are directly attributable to repeated DAU or DOX administration. At the same time, these studies established the cumulative ANT dose received as the main risk factor of cardiotoxicity [149, 150].

Despite the thousands of chemical ANT structures synthesized and tested, very few compounds have entered stages of advanced development, and only a few agents have been approved for clinical use. For some indications, these approved drugs, especially epirubicin and idarubicin (Fig. 1), have become valuable alternatives to DOX and DAU, respectively [75]. However, none of the newer analogs has stronger antitumor efficacy than their forerunners, and none of them has fulfilled drug developers' expectations of substantially improved cardiac safety [115, 152].

Types, incidence and risk factors of ANT cardiotoxicity

Four types of ANT cardiotoxicity can be recognized [28, 53, 59, 155]. (1) "Acute" cardiotoxicity occurs during ANT administration or immediately afterwards. It occurs mainly with the drug administered as a bolus or rapid intravenous infusion, and it typically involves vasodilatation, hypotension and transient cardiac rhythm disturbances [28]. (2) "Subchronic" cardiotoxicity is extremely uncommon. It manifests itself as a pericarditis-myocarditis syndrome within 1–3 days after the ANT treatment and was more frequently seen in early trials using very high doses of ANTs [12, 39]. (3) "Early chronic" ANT cardiotoxicity develops later in the treatment course, or weeks to months after the completion of chemotherapy. It is characterized by dilated (less often restrictive) cardiomyopathy, with subsequent development of left ventricular contractile dysfunction and congestive heart failure (CHF). Histopathological changes are quite unique and consist of distension of the sarcoplasmic reticulum of myocytes, cytoplasmic vacuolization, swelling of mitochondria and myofibrillar disarray and loss [28]. (4) The last type is "delayed" cardiotoxicity, also called "late-onset chronic". This type was described at the start of the 1990s among survivors of childhood cancers [37, 87, 133], and it is now well established that ANT cardiotoxicity may manifest even decades after the completion of anticancer treatment [120].

Whereas acute cardiotoxicity does not constitute a major clinical problem and it usually resolves shortly after the end of an infusion, the types of chronic toxicity are serious and clinically significant, substantially affecting overall morbidity and mortality and requiring long-term therapy. The incidence of chronic ANT-induced cardiotoxicity and CHF ranges between 1% and 16% weeks to months after the ANT-containing chemotherapy, and it further increases with the length of the follow-up [120]. The "classical" study by von Hoff et al. in 1979 estimated that 7% of patients developed DOX-related CHF after a cumulative dose of 550 mg/m^2 [149], and this dose was considered for many subsequent years to be the highest recommended for DOX and DAU. However, the meta-analysis by Swain et al. published in 2003 estimated a much higher cardiotoxicity incidence of ≈26% of patients at risk of DOX-related CHF for a cumulative dose of 550 mg/m^2 [138]. A retrospective analysis in 2006 revealed that compared to expected values, 30-year childhood cancer survivors had a 15-fold higher rate of heart failure, a 10-fold higher rate of other cardiovascular diseases, and a nine-fold higher rate of stroke [107].

In addition to the total cumulative ANT dose, the other main risk factors for development of ANT car-

diotoxicity include: low or high age at treatment, concomitant (or previous) chemotherapy containing drugs with cardiotoxic potential, mediastinal irradiation and cardiac disease in anamnesis [7, 163]. The interindividual variation in susceptibility to ANT cardiotoxicity has been attributed to different genetic backgrounds [22].

Treatment and prevention of ANT cardiotoxicity

Chronic ANT-induced cardiotoxicity is associated with a bad prognosis for the affected patients, and their survival seems to be worse than that of patients with ischemic cardiomyopathy [27]. At present, there is no specific evidence-based treatment of ANT-induced cardiotoxicity. Rather than actual cure, clinicians aim to prevent existing cardiomyopathy from worsening [82]. For patients with end-stage heart failure, heart transplantation remains the last option [143].

Given the difficulty or even impossibility of effective treatment, prevention of ANT cardiotoxicity is highly rational and of crucial importance. The first and apparently most effective method is to limit the cumulative ANT dose [138, 155]. For each ANT there are now recommended total cumulative doses that should not be exceeded or should be exceeded only with very careful cardiac monitoring [1] and/or pharmacological cardioprotection. These dose-limitation strategies have reduced the incidence of ANT-related cardiac events, but unfortunately they have not completely eliminated the risk of cardiotoxicity [35]. In addition, a lower ANT dose may lead to a lower cancer response rate. Several reports have suggested that continuous infusion, which limits peak ANT levels, is associated with a smaller risk of cardiotoxicity than is administering ANT as a bolus [81, 121], but other studies have failed to find any relationship between a particular type of ANT administration and the risk of cardiotoxicity [83, 88, 132]. Advanced pharmaceutical formulations providing targeted distribution of the drug to tumors have shown promise for reducing cardiotoxicity in clinical trials [3]. The greatest experience is with the liposomal forms of ANTs and with their polyethyleneglycol-coated ("PEG-ylated") variants. However, definitive data on the cardiac safety of these formulations require a longer follow-up of both cardiotoxic effects and anticancer response.

Finally, a very important method for ANT cardiotoxicity prevention is pharmacological cardioprotection. Despite decades of research and testing of thousands of potentially protective agents, only one drug has been approved for use in clinical practice: dexrazoxane (DXZ, ICRF-187). Interestingly, DXZ is not an outcome of any sophisticated and rational drug design; rather, its cardioprotective effects were discovered accidentally during its preclinical testing as a potential anticancer drug in combination with DAU [44, 47]. Later, DXZ has been repeatedly demonstrated to efficiently protect myocardium against all ANTs in numerous animal species [45] as well as in humans [139, 154]. Analyses of clinical trials revealed that cardioprotection from DXZ did not compromise the antitumor action of ANTs [137, 147]. Thus, DXZ today serves as the reference drug for preventing ANT cardiotoxicity.

Molecular pathophysiology of ANT cardiotoxicity

As described earlier, cardiotoxicity may be viewed as an effect of the entire class of ANTs, which may indicate that it is inseparable from their antitumor effect. However, numerous experimental reports, together with clinical observations that DXZ can prevent ANT cardiotoxicity while preserving their anticancer effect, strongly suggest that the cardiotoxicity of ANTs is distinct from their anticancer action.

The mechanisms of ANT cardiotoxicity have been the subject of considerable controversy, and dozens of various potential pathways have been proposed and studied [1, 7, 17, 35, 53, 59, 93, 95, 110, 120, 140, 163] (Fig. 2). Nevertheless, the iron-mediated formation of ROS and promotion of myocardial oxidative stress remain by far the most frequently proposed mechanism. This "ROS and iron hypothesis" will be critically reviewed below.

Other suggested cardiotoxicity mechanisms include: metabolism of ANT into more hydrophilic and cardiotoxic substances, which subsequently accumulate in cardiomyocytes [92]; impaired expression of various important cardiac proteins [11]; disruption of cellular and mitochondrial Ca^{2+} homeostasis [131]; induction of mitochondrial DNA lesions [79] and disruption of mitochondrial bioenergetics [144]; degradation of myofilamental and cytoskeletal proteins, including titin [85] and dystrophin [18]; and interference with various pro-survival kinases [110]. These few examples of a much larger set of proposed cardiotoxic pathways are not mutually exclusive: they may each contribute to cardiac cell damage, ultimately re-

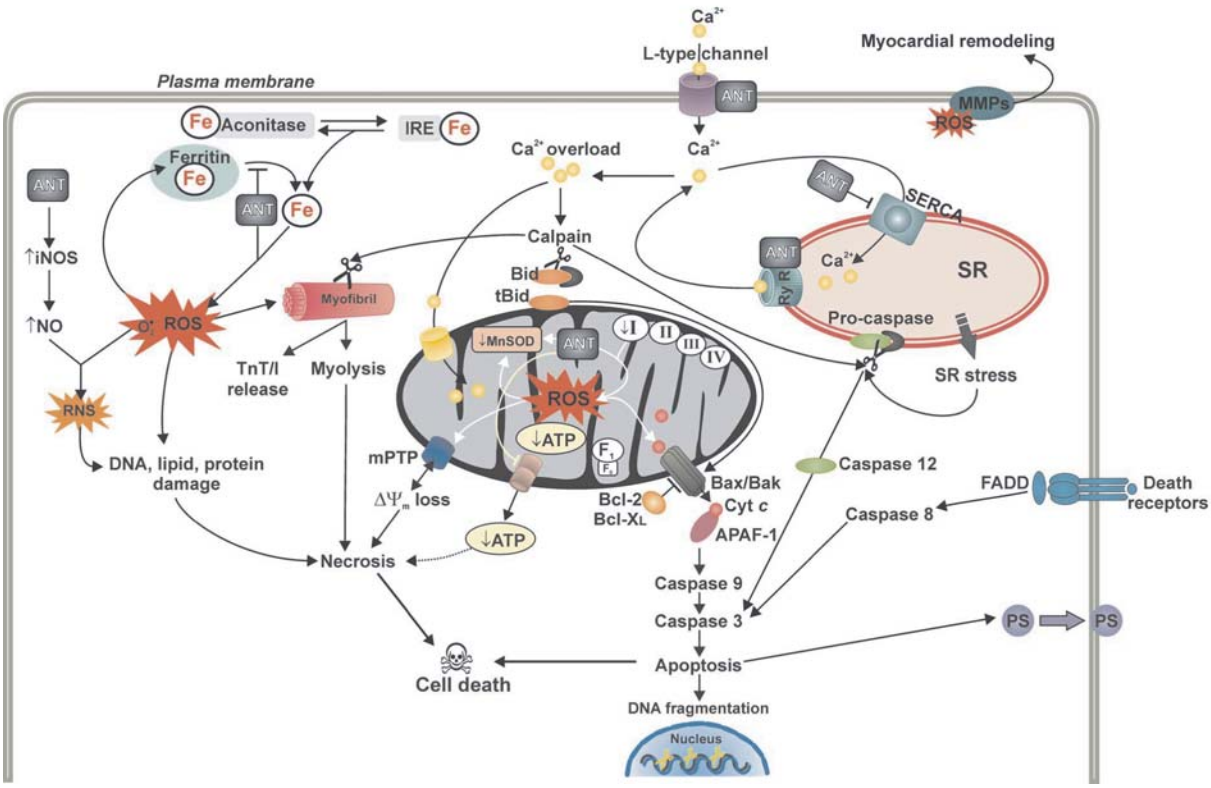

Fig. 2. Schematic overview of the pathways proposed to explain chronic anthracycline-induced cardiotoxicity. ANT – anthracycline, FADD – Fas-associated death domain protein, iNOS – inducible nitric oxide synthase, MMP – matrix metalloproteinase, MnSOD – manganese (mitochondrial) superoxide dismutase, mPTP – mitochondrial permeability transition pore, PS – phosphatidylserine, ROS – reactive oxygen species, RNS – reactive nitrogen species, NO – nitric oxide, RyR – ryanodine receptor, SR – sarcoplasmic reticulum, TnT/I – troponin T/I

sulting in myocyte death, either by the long-recognized pathway of necrosis or the more recently described pathway of apoptosis [119] (Fig. 2).

An important issue, which has to be considered when evaluating numerous ANT cardiotoxicity papers, is the use of different experimental conditions. Obviously, the best and the most relevant data should be obtained from patients suffering from cardiotoxicity. However, human myocardial biopsy or necropsy samples are hard to obtain. In addition, gathering meaningful information from these samples is difficult since the majority of cancer patients are treated with multidrug regimens and their clinical pictures are very heterogeneous.

Therefore, the use of experimental models is necessary in order to obtain better insight into the mechanisms of anthracycline cardiotoxicity and to study the cardioprotective potential of novel agents. Apart from cell-free *in vitro* experiments such as those involving microsomes, the simplest and most straightforward approach is the use of cellular models using isolated cardiac myocytes, especially primary neonatal rat cardiomyocytes [6, 43, 52, 77] and less often adult cardiomyocytes [66, 119]. Recently, studies with cardiomyocyte-derived cell lines have appeared, particularly studies involving H9c2 rat embryonic cardiomyoblasts [68, 78, 96]. *Ex vivo* studies often use isolated atria (even though these are not the main target for anthracycline toxicity), papillary muscles or whole heart preparations perfused according to Langendorff [113]. For *in vivo* studies, mouse, rat, rabbit, pig and dog are the most commonly used animals [46]. These whole-animal experiments enable repeated administration of ANTs, which can lead to chronic cardiotoxicity, similar to that seen in clinical practice [33, 46, 124]. However, numerous studies have used regimens in which animals were treated with only a single and/or an extremely high ANT dose. The outcomes from these studies must therefore be treated cautiously. There is a risk not only of oversimplification but also of inappropriately mixing acute and chronic cardiotoxic effects, which do not appear to share much in common.

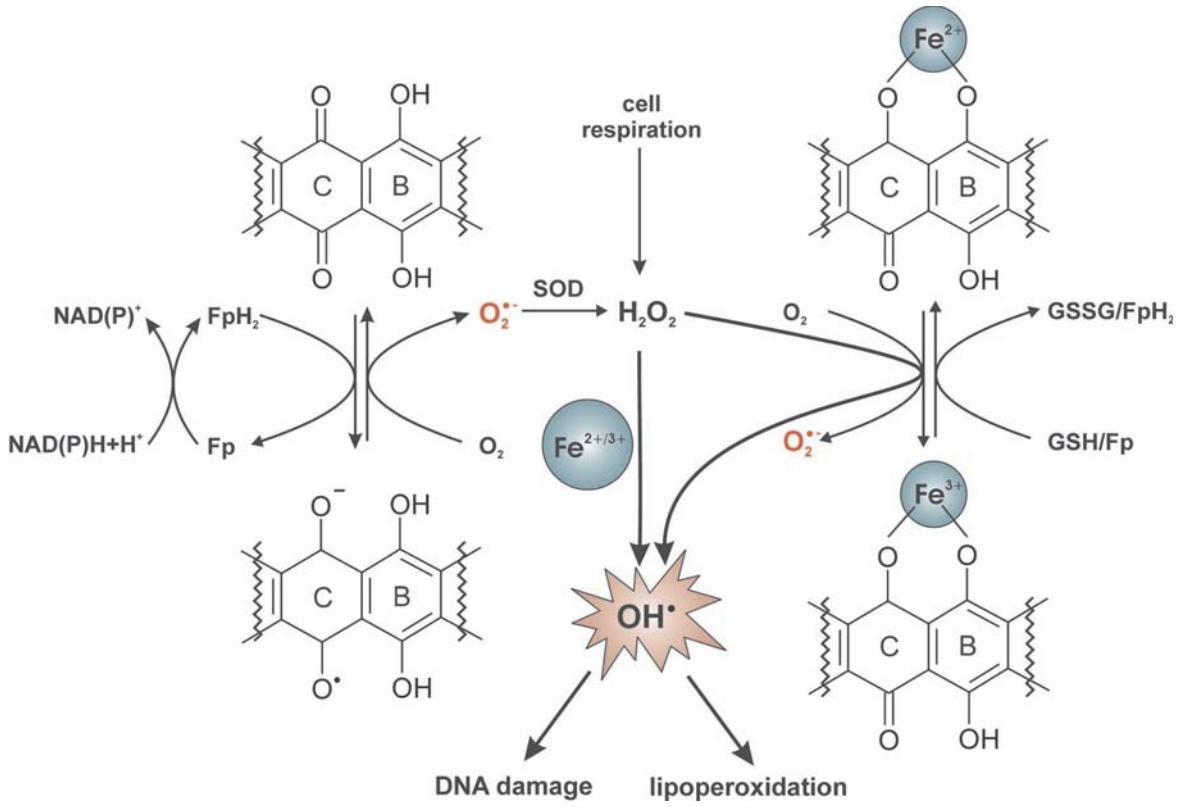

Fig. 3. Proposed mechanisms of ROS formation by anthracyclines that involve iron. Fe – iron, $O_2^{\bullet-}$ – superoxide radical, SOD – superoxide dismutase, H_2O_2 – hydrogen peroxide, $OH^{\bullet}$ – hydroxyl radical, NAD(P) – nicotinamide adenine dinucleotide (phosphate), Fp – flavoprotein, GSH/GSSG – reduced/oxidized glutathione

Oxidative stress in anthracycline-induced cardiotoxicity

Free radical-mediated myocyte damage was the first and most thoroughly studied mechanism proposed to explain the cardiotoxicity of ANTs. The ability of ANTs to induce ROS formation could be predicted from their chemical structure, which contains a quinone moiety (Fig. 1 and 3). This moiety is notorious for its ability to undergo redox cycling. Indeed, the production of free oxygen radicals by both DOX and DAU was soon shown *in vitro* [36, 41, 118]. Concurrently, Myers et al. showed that acute DOX cardiotoxicity can be reduced using the free radical scavenger alpha-tocopherol [103]. By the time of these studies, it was already widely accepted that the antitumor activity of ANTs was due to their ability to interfere with DNA replication, but it seemed unlikely that this mechanism would take part in cardiotoxicity, given that terminally differentiated cardiac cells do not divide and other DNA-damaging drugs are not cardiotoxic. Thus, the hypothesis that ROS-induced ANT cardiotoxicity could be prevented without compromising antitumor action was presented in 1977 in the journal *Science* [104]. The idea subsequently gained its current popularity, as it seemed to be confirmed by the cardioprotective efficiency of DXZ. Since then, ROS formation in the cardiac cells in response to ANT treatment has been repeatedly and unequivocally demonstrated in numerous studies [8, 18, 117, 161]. Several studies with transgenic animals overexpressing physiological antioxidants such as catalase [65], mitochondrial superoxide dismutase [159], thioredoxin-1 [122] or metallothionein [136] have shown beneficial effects on DOX cardiotoxicity. Consistent with the previous findings, glutathione peroxidase 1-deficient mice have been shown to be more susceptible to acute DOX-induced cardiotoxicity [31].

All these reports have reinforced the view of ROS as important culprits in ANT cardiotoxicity.

One-electron reduction of ring C of the ANT tetracycle leads to the formation of a semiquinone free radical (Fig. 3). This radical is relatively stable in an anoxic environment, but under normoxic conditions its unpaired electron is donated to oxygen, forming superoxide radicals. Suitable flavoproteins such as complex I catalyze the formation of reduced semiquinone radicals by accepting electrons from NADH or NADPH and donating them to ANT. This sequence of reactions, known as "redox cycling", can be highly damaging, because a relatively small amount of ANT is sufficient for the formation of numerous superoxide radicals [67]. The redox cycling of ANTs has been described in cytoplasm, mitochondria and sarcoplasmic reticulum [23]. The first targets of ANT-mediated free radical damage to be recognized, and the targets most often discussed, are various cellular membranes, which are rich in lipids prone to peroxidation. This radical damage results in production of many relatively stable and highly toxic aldehydes, such as malondialdehyde (MDA). These aldehydes can easily diffuse within the cell or even cross the plasma membrane and attack macromolecular targets far from where they were generated and thus act as "second cytotoxic messengers" [90].

A legitimate question is why would the heart be so much more susceptible than other tissues to the oxidative stress produced by ANTs? Several responses have been proposed: ANTs have been shown to be retained within cardiomyocytes more than in other cell types [58]. Doroshow et al. have proposed that cardiac tissue has weak antioxidant activity, since it lacks catalase [21]. Furthermore, this research group has shown that DOX selectively down-regulates glutathione peroxidase [26], suggesting that cardiomyocytes are exposed to high levels of hydrogen peroxide. In addition, cardiomyocytes are rich in mitochondria, which represent up to 50% of cardiomyocyte mass and which serve as both source and target of ROS [8, 144, 162]. Moreover, an important role has been attributed to exogenous NADH dehydrogenase. Unlike cardiac mitochondria, liver mitochondria lack the NADH-related pathway of reducing equivalents from the cytosol to the respiratory chain. As a result liver mitochondria do not generate significant amounts of ANT semiquinones [106]. Finally, ANTs are thought to enter mitochondria and to inhibit the respiratory chain by binding to cardiolipin, which is a relatively cardiospecific phospholipid that is rich in polyunsaturated fatty acids and that is found in the inner mitochondrial membrane. Cardiolipin has a high affinity for ANTs [38].

In addition to ROS, reactive nitrogen species (RNS) are also implicated in ANT cardiotoxicity [30]. This implies cross-talk between ANT and NO production. On both the mRNA and protein levels, repeated ANT administration *in vivo* has been shown to induce NO production within the myocardium by increasing the expression of inducible NO synthase (iNOS, NOS2). In contrast, ANT administration did not alter the expression of two other isoforms of this enzyme [89]. However, studies with a model of acute ANT cardiotoxicity have suggested the involvement of the endothelial NOS isoform (eNOS, NOS3) [105]. In studies with this model, genetic knock-out of iNOS led to a paradoxical worsening in ANT-induced cardiotoxicity [19], whereas knockout of eNOS provided protection against this type of toxicity [105]. The authors of the last study also hypothesized that the eNOS reductase domain may be responsible for generating cardiac superoxide in myocardium exposed to ANTs. Similar findings pointing out redox activation of ANTs by eNOS and consequent triggering of apoptotic cell death have been also reported with endothelial cells cultured *in vitro* [63]. However, the direct translatability of these findings to clinically relevant settings remains uncertain, since no investigation using a clinically relevant model of chronic cardiotoxicity has been performed to date.

The concomitant overproduction of NO and ROS is known to yield highly reactive nitrogen species, which may attack and destroy important cellular biomolecules [151]. Peroxinitrite certainly ranks among the most important and toxic nitrosative stress mediators. Numerous studies, mostly involving acute ANT exposure, have measured significantly elevated levels of nitrotyrosine in the myocardium of ANT-treated animals; this molecule is a nitrosative stress biomarker [4, 108, 151]. It is worth noting that nitrosative stress may occur secondarily to oxidative stress [16], which may be consistent with the above theoretical assumptions.

The above data on the involvement of oxidative (and nitrosative) stress in ANT cardiotoxicity seemed to give a perfect rationale for cardioprotection by antioxidants. Effective protection in a clinical setting would be useful not only for reducing the cardiac risk of cancer patients, but it would also be the best and

most direct confirmation of the hypothesis that free radical injury is the main culprit behind ANT cardiotoxicity. Indeed, since the mid-1970s, numerous different agents have been experimentally studied using various experimental models. Although most of these studies rely on models of acute or subacute exposure, they announce "promising" new antioxidant substances on a monthly basis [55, 56]. Often these substances are of natural origin.

Unfortunately, these agents have failed to measure up to the claims when they are tested in animal experiments and clinical trials involving chronic ANT exposure. The first disappointment came in 1983 with N-acetylcysteine (NAC). Although highly effective in acute preclinical experiments [22], NAC showed no activity against chronic DOX-induced cardiac toxicity in a randomized, controlled clinical trial [101]. Another example of widely studied antioxidant agents are polyphenols, either naturally occurring substances or their semisynthetic analogs. Among these molecules, the flavonoid 7-monohydroxyethylrutoside (monoHER) proved effective in numerous experiments of acute or subchronic ANT administration to experimental animals [145]. MonoHER was even well tolerated by patients during a Phase I clinical trial [153], but it failed to provide any cardioprotection in a chronic animal study [14]. Moreover, during Phase II clinical evaluation, monoHER seemed to enhance DOX-induced cardiotoxicity rather than provide cardioprotection [13]. Other agents showing promise in preclinical experiments but not in clinical studies include coenzyme Q10, L-carnitine, and the combination of vitamins E and C and NAC [147]. Probucol, a lipid-lowering and antioxidant drug, has shown very encouraging results in preclinical studies [84, 130], but to our knowledge, no clinical trial has been reported and no further signs to develop it as a clinically applicable cardioprotectant can be found in recent literature. Similarly, a peroxynitrite decomposition catalyst showed significant cardioprotective potential in an animal model of acute ANT exposure [108], but no data are available from animal experiments of chronic exposure or from clinical trials.

The discovery of DXZ and the assumed elucidation of its mechanism as iron chelation-mediated ROS inhibition has transformed the "ROS hypothesis" of ANT cardiotoxicity into the "ROS and iron hypothesis", which has guided subsequent research in this field [100].

Iron in anthracycline-induced cardiotoxicity

Iron (Fe) is a crucial biogenic element indispensable for all living cells, where it is essential for oxidation-reduction catalysis and bioenergetics. However, unless appropriately sequestered, this metal plays a crucial role in the formation of ROS [40]. There are two main pathways by which Fe may promote ROS formation in ANT-exposed cells: the first mechanism involves the Fenton and Haber-Weiss reactions, while the second involves formation of ANT-Fe complexes.

As shown in previous sections and in Fig. 3, the ability of ANT aglycone to undergo redox cycling results in formation of superoxide free radicals ($O_2^{\bullet-}$). The dismutation of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) is catalyzed by superoxide dismutase (SOD), or it may occur spontaneously. H_2O_2 is a relatively stable molecule of low toxicity and, under physiological conditions, excess amounts are eliminated by catalase and glutathione peroxidase. However, H_2O_2 and $O_2^{\bullet-}$ may generate highly toxic hydroxyl radicals ($OH^{\bullet}$). This takes place during the Haber-Weiss reaction (eq. 1), which is very slow unless catalyzed by transition metals, especially Fe [40]. The Fe-catalyzed Haber-Weiss reaction can be divided into two parts: in the first step, ferric ion (Fe^{3+}) is reduced to ferrous ion (Fe^{2+}) by $O_2^{\bullet-}$ (eq. 2); in the second step, the Fenton reaction (eq. 3) occurs between of Fe^{2+} and H_2O_2.

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet} \text{ (eq. 1)}$$

$$O_2^{\bullet-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \text{ (eq. 2)}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\bullet} + Fe^{3+} \text{ (eq. 3)}$$

Given the key catalytic role of free cellular Fe in the production of dangerous $OH^{\bullet}$, organisms are equipped with specific proteins designed for Fe acquisition, transport, and storage, as well as with sophisticated mechanisms that regulate intracellular Fe homeostasis. The labile pool of cytosolic Fe, which apparently corresponds to Fe in transit between the transporter transferrin and the storage protein ferritin, is sensed by the iron regulatory proteins (IRPs). These proteins control the expression of transferrin receptor and ferritin at the translational level in order to maintain the intracellular free Fe pool at a low level [98]. However, Thomas and Aust have shown that ANTs may increase the amount of free, redox-active Fe by

generating $O_2^{\bullet-}$, which mediates the slow reductive release of Fe from ferritin [142].

The hydroxyl radical ($OH^{\bullet}$) formed in this way has a very short half-life and extremely high reactivity. This makes the compound dangerous to cells. Unlike $O_2^{\bullet-}$ or H_2O_2, both of which can be readily detoxified by enzymatic systems, $OH^{\bullet}$ cannot be eliminated by enzymes. Instead, $OH^{\bullet}$ reacts with any oxidizable compound in its vicinity and thus it can induce damage in all types of macromolecules, including lipids (peroxidation), nucleic acids (mutations), and proteins [40].

The second mechanism by which Fe promotes oxidative stress involves formation of ANT-Fe complexes. Two distinct mechanisms have been described, one dependent on the presence of a reducing system and another in which radicals are formed from the ANT-Fe complex itself, in the absence of a reducing system [67]. In the presence of a reducing system, which can be NADH cytochrome P450 reductase or the thiols of cysteine or glutathione, ANT-Fe^{3+} is reduced to ANT-Fe^{2+}. It can react with O_2 to form $O_2^{\bullet-}$, which in turn dismutates to H_2O_2 and/or enters the Haber-Weiss reaction resulting in $OH^{\bullet}$. Alternatively, ANT-Fe^{2+} can react with H_2O_2 to yield $OH^{\bullet}$ directly (Fig. 3). In the absence of a reducing system, ANT-Fe^{3+} can reduce its chelated Fe through an intramolecular redox reaction, either by oxidation of the side chain on C9 or the hydroquinone moiety at ring C, forming an ANT free radical chelate with Fe^{2+} (ANT$^{\bullet}$-Fe^{2+}). In the presence of O_2, this complex can be oxidized to yield $O_2^{\bullet-}$ and it can also react with H_2O_2 to generate $OH^{\bullet}$ (Fig. 3).

Involvement of Fe in ANT-induced oxidative stress and cardiotoxicity is supported by a large body of experimental evidence. As early as the early 1980s, Myers et al. observed formation of ANT-Fe complexes and the resulting potentiation of oxidative damage of biomembranes *in vitro* [102]. These ANT-Fe complex-mediated lesions were not blocked by classical ROS scavengers. More recently, an Fe-mediated increase in ANT cardiotoxicity has been demonstrated in isolated rat cardiomyocytes where the toxicity of DOX was increased by prior Fe loading, leading to the higher release of lactate dehydrogenase (LDH) and more pronounced reduction of cell contractility. The toxicity of the combination of Fe and DOX was apparently not a simple additive effect, because at the concentrations used, Fe had only a minimal effect on LDH release and no effect on contractility, and DOX alone had only a minor effect on contractility [51]. Fe loading of DOX-treated rats resulted in severe weight loss and a two-fold increase in mortality [86]. In both of the latter studies, the unfavorable effects of Fe on DOX cardiotoxicity was eliminated using the Fe chelator deferoxamine. Panjrath et al. observed substantially increased DOX cardiotoxicity by giving dietary Fe to rats, and the authors concluded that body stores of Fe, as well as its bioavailability in tissue, may be important independent predictors of susceptibility to DOX cardiotoxicity in humans [109]. Hfe-deficient mice serve as a model of human hereditary hemochromatosis, which is a genetic disorder resulting in excessive levels of Fe in the body. Using these mice, Miranda et al. have shown them to have a significantly greater sensitivity to DOX-induced cardiotoxicity. Mortality after chronic DOX treatment was higher in both $Hfe^{-/-}$ and $Hfe^{+/-}$ mice than in wild-type animals. Moreover, DOX-treated $Hfe^{-/-}$ mice had a higher degree of mitochon-

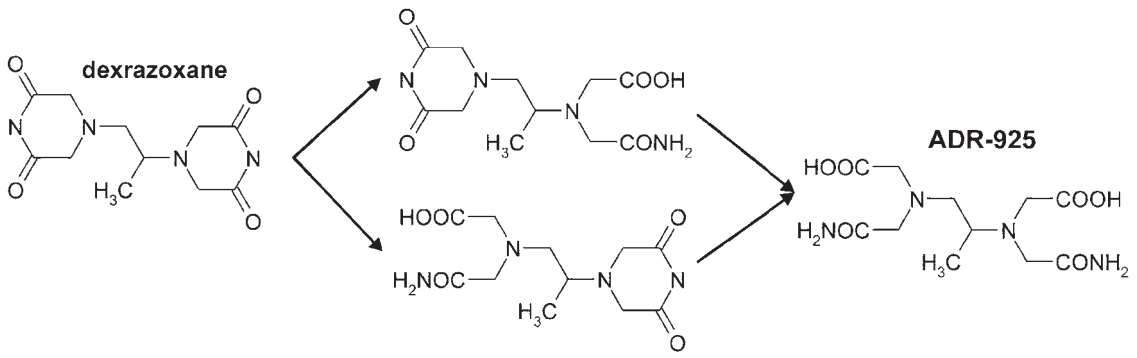

Fig. 4. Chemical structure of dexrazoxazone (ICRF-187) and its hydrolysis into the presumably active iron-chelating metabolite ADR-925

drial damage and Fe deposits in the heart than did wild-type mice [97].

The second main factor that suggests the important role of Fe in ANT-induced cardiotoxicity is the efficient cardioprotection afforded by the clinically approved DXZ. As already mentioned above, DXZ is believed to act by undergoing hydrolysis to yield the Fe-chelating metabolite ADR-925, which structurally resembles ethylenediaminetetraacetic acid (EDTA, Fig. 4). The current prevailing hypothesis is that DXZ exerts its cardioprotective effects by binding free Fe, loosely bound Fe, and Fe complexed to ANTs thus preventing site-specific oxygen radical production that damages cellular components [39].

This suggests the rationale for studying the cardioprotective potential of other Fe chelators than DXZ. Given the disappointing clinical results with classical antioxidants, it is reasonable to hypothesize that it is more effective to prevent ROS formation than to scavenge free radicals already formed, in particular the extremely toxic $OH^{\bullet}$. Studies on cardioprotection provided by Fe chelation began in the early 1990s with deferoxamine (DFO, also called desferrioxamine), a well-established chelating agent that has long been used to remove excess Fe from the body. DFO has been shown to protect cultures of isolated rat neonatal cardiomyocytes against DOX toxicity. However, this has been shown only in Fe-overloaded cells. In contrast, DFO treatment of normal heart cells had no measurable cardioprotective effect against DOX toxicity, regardless of whether DFO was administered before or simultaneously with DOX [51]. Using a model of isolated mice atria acutely exposed to DOX, Voest et al. showed that DFO at a concentration of 200 μM was the most effective of the six chelators tested, including DXZ [148]. However, increasing the DFO concentration slightly to 500 μM caused its protective effects to disappear completely. In other studies of acute cardiotoxicity, DFO protected against lesions in the hearts of rats given a single injection of very high DOX doses (15 and 25 mg/kg) [2, 116]. The only paper assessing the protective potential of DFO in a model of chronic ANT cardiomyopathy is that of Herman et al., who used a well-established experimental setup with spontaneously hypertensive rats. In this study, DFO failed to achieve significant cardioprotection and it did not reduce DOX-induced mortality [48]. High molecular weight and hydrophilicity were suggested as reasonable explanations of the low protective efficacy of DFO. Both of these physicochemical variables of DFO can cause low plasma membrane permeability and insufficient distribution of the drug into cardiac myocytes. While DFO is among the fastest and most efficient Fe chelators in solution, its access to the intracellular labile Fe pool is very limited [160]. Therefore, during the next years, the research interest shifted towards smaller and more lipophilic ligands.

Deferiprone (L1, CP 20) is an orally effective Fe chelator. Two *in vitro* studies demonstrated that treatment with L1 resulted in an inhibition of DOX toxicity in both Fe-loaded and normal cardiac culture cells [6, 86]. Deferiprone also protected spontaneously beating isolated rat atria from the contractility impairment induced by acute DOX exposure [157]. However, in a well-characterized and DXZ-validated model of chronic DAU cardiotoxicity in rabbits, L1 failed to afford any protection against DAU-induced mortality, lipoperoxidation of left ventricular tissue, systolic and diastolic cardiac dysfunction, morphological cardiac deteriorations, or the increase in plasma cardiac troponin T [111].

Deferasirox (ICL670) is another novel synthetic oral Fe chelator. ICL670, tested in the same *in vitro* model as L1, did not protect myocytes against DOX [43]. Depending on the concentration used, ICL670 either failed to affect DOX cytotoxicity in cardiac cells, or it increased it. Interestingly, this occurred in spite of the fact that ICL670 quickly and efficiently removed Fe^{3+} from its complex with DOX, rapidly entered myocytes and displaced Fe from an intracellular Fe-calcein complex. The fact that the ferric complex of ICL670 was less toxic than ICL670 alone suggested that ICL670A was cytotoxic by either removing or withholding Fe from critical Fe-containing proteins [43]. So far, no *in vivo* study with ICL670 has been reported.

Aroylhydrazone Fe chelators are represented by pyridoxal isonicotinoyl hydrazone (PIH) and its analogs. These ligands are small, lipophilic molecules with a neutral charge at physiological pH. These properties allow them to pass easily through cell membranes and gain access to intracellular labile Fe pools [15]. To date, the potential cardioprotective effects against ANT cardiotoxicity have been evaluated in three different aroylhydrazone drugs: PIH, salicylaldehyde isonicotinoyl hydrazone (SIH) and *ortho*-chlorbenzoyl isonicotinoyl hydrazone (*o*-108). These agents have shown high antioxidant potential: PIH and some of its analogues inhibited lipid peroxida-

tion, ascorbate oxidation, 2-deoxyribose degradation, plasmid DNA strand breaks and 5,5-dimethylpyrroline-N-oxide (DMPO) hydroxylation mediated by either the Fenton reagent (Fe^{2+} + H_2O_2) or Fe^{3+}-EDTA + ascorbate. Compared with hydroxyl radical scavengers (dimethyl sulfoxide, salicylate and mannitol), PIH has been shown to be approximately two orders of magnitude more active in protecting 2-deoxyribose from degradation [50]. The chelator SIH very efficiently prevented oxidative stress-induced mitochondrial injury and cell death in H_2O_2-exposed H9c2 cardiomyoblast cells [123]. All these results argued for testing the ability of these agents to prevent cardiotoxicity induced by ANTs. In a rabbit model of chronic DAU cardiomyopathy, PIH was found to reduce DAU-induced mortality [125]. The chelators SIH and *o*-108 were then shown to prevent both premature death as well as significantly improve the DAU-induced impairment of cardiac function (left ventricular ejection fraction, left ventricular dP/dt_{max} index), biochemical markers of cardiotoxicity (plasma levels of cardiac troponin T) as well as histopathological parameters. However, even a slight increase of 2- or 2.5-fold in the dose of all three of these chelators not only failed to provide additional protection, but it hampered virtually all the beneficial effects of the chelators on both overall mortality and cardioprotection [128, 134, 135]. Importantly, each of these chelators was non-toxic and well tolerated when administered on its own to animals at these high doses. Theoretically, the equivocal results of aroylhydrazone chelators *in vivo* could have the pharmacokinetic background. The inability of aroylhydrazones to provide the same degree of protection as DXZ may be due to their relatively rapid hydrolysis and short plasma half-life, while the disappearance of the cardioprotective effect at higher doses may be due to high peak plasma concentrations [70–73].

Therefore, in an attempt to shed some light on the results of *in vivo* experiments, a study examining how SIH protects against DAU cardiotoxicity was conducted *in vitro*, using primary cultures of neonatal rat ventricular cardiomyocytes. SIH has been shown to protect cardiomyocytes against the effects of a 48-h incubation with 10 μM DAU. However, the protection was only partial and showed no apparent dose dependence: the reduction in DAU-induced toxicity caused by 3 μM SIH (22%) was comparable to that observed with 100 μM SIH (25%). This contrasted sharply with the protection against 500 μM H_2O_2 (a model oxidative injury), in which SIH pretreatment resulted in dose-dependent protection with an EC_{50} value of 2.1 μM and 100% protection at SIH concentrations of at least 10 μM [127]. The observed protection with SIH against both DAU and H_2O_2 was dependent on Fe chelation, since it could be blunted by increasing the concentration of Fe salts in the incubation medium. An interesting insight into the mechanisms of cardioprotection was obtained using a lipoperoxidation assay involving HPLC determination of MDA levels. As expected, SIH completely abolished the H_2O_2-induced increase in the MDA level in isolated ventricular myocytes. However, SIH provided protection against DAU cardiotoxicity, which is associated with significantly increased malondialdehyde content, but SIH did not decrease this lipid peroxidation [127]. This strongly suggests that the protection against ANT toxicity provided by the Fe chelator may be ROS-independent. Notably, this last study revealed that Fe chelation modulated ANT toxicity differently in cardiac and cancer cells. At concentrations at which SIH significantly reduced DAU toxicity in rat cardiomyocytes, it increased the antiproliferative action of DAU in the acute promyelocytic leukemia cell line HL-60, confirming that ANT cytotoxicity occurs through different mechanisms in cardiac and cancer cells [127].

The study of Kaiserova et al. [60] called into question whether prevention of $OH^{\bullet}$ formation through Fe chelation can be regarded as a major pathway for preventing or decreasing the toxicity of ANTs. This study compared the effects of various Fe chelators on the prevention of oxidative stress induced by the Fenton reagent (H_2O_2 + Fe^{2+}) and DOX. The ability of chelators to prevent $OH^{\bullet}$ formation was investigated using EPR spectroscopy, and their efficiency was found to decrease in this order: PIH = SIH > DXZ > DFO > monoHER. When these compounds were tested in a model of A549 human lung adenocarcinoma cells, only DFO and SIH were found to prevent H_2O_2 + Fe^{2+}-induced oxidative damage, as monitored by LDH release, MDA formation and cellular glutathione levels. In contrast, when toxicity was induced with DOX, DFO and SIH were not protective, whereas DXZ and monoHER significantly decreased DOX-induced oxidative damage [60].

Flavonoids can bind Fe, and it has been proposed that Fe-chelating activity is an important part of their antioxidant action [146]. However, analysis of a series

of 10 different flavonoids (including quercetin and monoHER) showed no correlation between their Fe-chelating efficiency and the degree of protection provided to cultures of isolated rat neonatal cardiomyocytes against the toxicity induced by exposure to 1 μM DOX for 72 h [62].

To sum up, studies with various different Fe chelators have yielded rather mixed and often unexpected results that contradict the classical "ROS and iron" hypothesis of ANT cardiotoxicity and DXZ cardioprotection. Clearly, none of the Fe chelating agents has reached the high protective efficacy of DXZ, despite the fact that ADR-925 has a lower affinity for Fe than other chelators. It is therefore clear that the ability of a ligand to chelate Fe is not a major determinant of its cardioprotective action [61]. This challenges the notion that DXZ cardioprotection against ANT cardiotoxicity is mediated by Fe chelation. Interestingly, among the group of various bisdiketopiperazines including DXZ (ICRF-187) and its six analogs (ICRF-154, ICRF-192, ICRF-197, ICRF-198, ICRF-239, ADR-559), no correlation has been found among their protective activity against chronic DOX cardiotoxicity in an *in vivo* rat model; their rates of hydrolysis to yield their corresponding Fe-chelating forms; and the ability of these metabolites to bind Fe and subsequently cause a decrease in the formation of ROS [49].

Notably, the complex of ADR-925 with Fe has been shown to undergo redox cycling and to stimulate ROS production [91]. This was not very surprising since ADR-925 is a close analog of EDTA, which because of its redox potential, does not keep Fe from redox cycling and it promotes the Haber-Weiss reaction [40]. EDTA nonspecifically chelates many various di- and trivalent metals, and its resemblance to ADR-925 suggests that the latter is also unlikely to be a selective Fe ligand. Indeed, the hydrolysis product of razoxane (DXZ racemate) gave similar formation constants for iron, copper, calcium, magnesium and manganese [54]. This is in contrast to the other chelators tested (DFO, L1, ICL 670, aroylhydrazones), all of which are highly Fe-specific. Interestingly, a study showed that chronic cardiomyopathy and systolic heart failure induced by DAU were accompanied by chronic calcium overload of the myocardial tissue, and that DXZ restored normal levels of calcium. At the same time, neither DAU nor its combination with DXZ had any significant effect on the myocardial level of Fe [126].

The study by Minotti et al. gave paradoxical and unexpected results when they showed that DOX did not increase, but actually decreased the myocardial release of conjugated dienes and hydroperoxides [94]. The same group also reported that DOX protected cardiomyocytes against Fe-mediated cytotoxicity [20]. Another recent study by Ferreira et al. has shown that the myocardium of chronically DOX-treated rats has greater antioxidant activity than that of control animals [29].

These findings do not, however, necessarily rule out a role for Fe in ANT cardiotoxicity. Indeed, during the last decade, ANTs have been shown to interfere with cellular Fe in a very complex manner and this is certainly not limited to the mere production of toxic free oxygen radicals. ANTs and/or their metabolites perturb cellular Fe metabolism by interacting with multiple molecular targets, including the Fe regulatory proteins 1 and 2 (IRP1 and IRP2). The RNA-binding activity of these molecules regulates expression of transferrin receptor 1 and ferritin, which are key regulatory proteins involved in Fe uptake and storage, respectively. It is possible that this occurs as a response to oxidative stress as well as in an ROS-independent manner [69, 96]. Moreover, it has been shown that DOX can cause the accumulation of Fe in ferritin and prevent its mobilization [76], which may create a relative deficiency of Fe for metabolic use. Several review articles have recently been published covering this novel and perspective research area in detail [64, 95, 158].

Concluding remarks

Due to their excellent anticancer efficiency, ANT chemotherapeutics will certainly remain an important component of numerous chemotherapeutic regimens in the future. Since treating cardiac complications is very troublesome and expensive, clinicians should provide the best available level of cardioprotection to patients undergoing ANT-containing chemotherapy. Well-designed pharmacological cardioprotective intervention can be an effective option; however, the ability to rationally develop such agents largely depends on our understanding of the molecular mechanisms involved in both the cardiotoxic and anticancer effects of ANTs.

Both laboratory and clinical studies have generated unequivocal evidence that ANTs induce increased ROS formation. Iron has clearly been shown to potentiate both ANT-induced oxidative stress as well as cardiac toxicity. However, careful evaluation of all available data raises a question: is the Fe-catalyzed burst of free radicals, detected by the vast majority of studies, a primary cause of ANT cardiotoxicity? Several recent studies, as well as some theoretical arguments have challenged the traditional and still popular notion that Fe-promoted ROS formation is the primary pathway of ANT-induced oxidative stress and cardiotoxicity. In addition, the expectations for effective cardioprotection by both ROS scavengers and Fe chelators have so far remained unfulfilled.

All of these arguments suggest that oxidative stress may be neither the main trigger nor the key executor of ANT cardiotoxicity, and that increased ROS formation may be merely a secondary consequence of previous cellular and mitochondrial damage. Further studies using modern molecular methodologies, clinically relevant doses of ANTs and preferably *in vivo* experiments involving chronic ANT treatment are needed to shed more light on these questions. In addition, important lessons can still be learned from the "gold standard" cardioprotectant, DXZ. Hopefully, the upcoming years will bring novel data that will significantly advance our understanding of the mechanisms of ANT cardiotoxicity and allow us to rationally design safer and more effective cardioprotectants.

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