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Molecular Pharmacology of the Interaction of Anthracyclines with Iron.

Xu, X., Persson, H.L., and Richardson, D.R.

[1]*Children's Cancer Institute Australia for Medical Research, Iron Metabolism and Chelation Program, PO Box 81, High St, Randwick, Sydney, New South Wales, 2031, AUSTRALIA (X.X and D.R.R.), and* [2]*Divisions of Pulmonary Medicine and Pathology II, Faculty of Health Sciences, University of Linköping, SE-581 85 Linköping, SWEDEN (H.L.P.)*

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Running Title: Interaction of Anthracyclines with Iron

Author for Correspondence: Dr. D.R. Richardson, Children's Cancer Institute Australia for Medical Research, Iron Metabolism and Chelation Program, PO Box 81, High St, Randwick, Sydney, New South Wales, 2031, Australia. Email:d.richardson@ccia.org.au; FAX: +61-2-9382-0060; Ph: +61-2-9382-0046.

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Abbreviations: DMT1, divalent metal transporter 1; HFE, human gene for hemochromatosis; IRP, iron-regulatory protein; IRE, iron responsive element; PIH, pyridoxal isonicotinoyl hydrazone; SIH, salicylaldehyde isonicotinoyl hydrazone; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

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Abstract

Although anthracyclines such as doxorubicin are widely used anti-tumor agents, a major limitation for their use is the development of cardiomyopathy at high cumulative doses. This severe adverse side-effect may be due to interactions with cellular iron metabolism, since iron-loading promotes anthracycline-induced cell damage. Conversely, anthracycline-induced cardiotoxicity is significantly alleviated by iron chelators, e.g. desferrioxamine and dexrazoxane. The molecular mechanisms by which anthracyclines interfere with cellular iron trafficking are complex and still unclear. Doxorubicin can directly bind iron and can perturb iron metabolism by interacting with multiple molecular targets including the iron regulatory proteins 1 and 2 (IRP1 and IRP2). The RNA-binding activity of these molecules regulates synthesis of the transferrin receptor 1 and ferritin, which are crucial proteins involved in iron uptake and storage, respectively. At present, it is not clear whether doxorubicin affects IRP1-RNA-binding activity by intracellular formation of doxorubicinol and/or by generation of the doxorubicin-iron(III) complex. Furthermore, doxorubicin prevents the mobilization of iron from ferritin by a mechanism that may involve lysosomal degradation of this protein. Prevention of iron mobilization from ferritin would probably disturb vital cellular functions, due to inhibition of essential iron-dependent proteins, such as ribonucleotide reductase. This review discusses the molecular interactions of anthracyclines with iron metabolism and the development of cardioprotective strategies such as iron chelators.

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1. General Introduction

Anthracyclines are potent anti-neoplastic agents used extensively to treat a range of cancers, including leukemias, lymphomas, sarcomas, and carcinomas (for review see Gewirtz, 1999). Doxorubicin, daunorubicin and epirubicin are clinically used anthracyclines (Figure 1). The intricate and complex cellular responses to anthracyclines hinder efforts to unveil the mechanisms involved in their cytostatic and cytotoxic actions. However, anthracyclines are proposed to disrupt macromolecular biosynthesis by various mechanisms, including DNA intercalation and the inhibition of DNA polymerase and topoisomerase II (Gewirtz, 1999). Anthracyclines can also induce DNA damage by the generation of free radicals that react with a variety of macromolecules, thus inhibiting cellular proliferation or causing apoptosis (Gewirtz, 1999). Generally, the anti-tumor effect of anthracyclines are mainly attributed to their DNA-binding and damaging abilities. Indeed, the pharmacological aspects of these drugs have been extensively reviewed (for reviews see Myers, 1998; Minotti et al., 2004), and will not be discussed in depth by this article.

A major problem with the clinical use of anthracyclines is their cardiotoxicity, which limits administration exceeding an accumulated dose of approximately 550 mg/m^2 (for review see Singal et al., 1997). The toxic effects of anthracyclines to cardiomyocytes are not due to inhibition of DNA synthesis, since these cells do not replicate (Myers, 1998). While the reasons for the cardiotoxicity of these drugs are not fully understood, a number of observations suggest that the interactions of anthracyclines with iron are of great importance (Minotti et al., 1998; Kotamraju et al., 2002; Kwok and Richardson, 2002, 2003). The redox state of iron can be converted between the iron(II) and iron(III) states by interaction with anthracyclines, generating toxic reactive oxygen species (ROS) (Myers, 1998; Minotti et al., 2004). Before beginning a description of the reactions of anthracyclines with iron, we will first briefly detail the molecular mechanisms involved in the processing and trafficking of intracellular iron.

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2. Cellular and Molecular Physiology of Iron Metabolism

2.1 The Transferrin – Transferrin Receptor Mechanism of Iron Uptake

Iron is a crucial element for living cells and is found in two functional forms of macromolecules, i.e. heme and non-heme iron-containing proteins (for review see Richardson and Ponka, 1997). Briefly, ferric iron [iron(III)], is transported through the body in a soluble form bound to transferrin (M_r = 80 kDa), a protein mainly synthesized by the liver and also sanctuary sites such as the brain and testis. Transferrin donates iron to cells through binding to the dimeric transferrin receptor 1 (TfR1) on the cell membrane, which is subsequently endocytosed (Figure 2). Within late endosomes at acidic pH, iron is liberated from transferrin, and in the ferrous form [iron(II)] is then transported into the cytosol by the divalent metal transporter 1 (DMT1; for review see Napier et al., 2005). The transferrin-TfR1 complex, on the other hand, is returned to the cell surface, where apotransferrin is released from its receptor into the extracellular space (Figure 2). The binding of transferrin to the TfR1 and the subsequent uptake of iron is regulated by a number of factors, including: (1) TfR1 expression, which is modulated by intracellular iron levels (see Section 2.2.2); (2) the competitive binding of transferrin to the TfR1 by the product of the hemochromatosis (*HFE*) gene (Lebron et al., 1999); and (3) the saturation of transferrin with iron, since apotransferrin has a very low affinity for the TfR1 in contrast to diferric transferrin.

The recent description of the detailed structure of the iron-transferrin-TfR1 complex has unveiled important insights into the iron uptake process (Cheng et al., 2004). This latter investigation revealed that HFE and transferrin compete for the same binding site on each of the TfR1 monomers which is in agreement with the findings of a previous study (Lebron et al., 1999). Interestingly, the apical part of the receptor within the transferrin-TfR1 complex remains free and potentially accessible to interaction with other molecules. It is possible that DMT1 and/or the postulated

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ferrireductase, which reduces iron(III) to iron(II) within transferrin, could associate with the TfR1 at this site (Cheng et al., 2004).

A recently identified second transferrin receptor (TfR2; Kawabata et al., 1999) probably plays an important role in iron homeostasis, since mutations of this molecule can lead to hemochromatosis (Camaschella et al., 2000). Even though TfR2, like TfR1, is a type II membrane protein with a large C-terminal ectodomain and a small N-terminal cytoplasmic domain, the affinity of TfR2 for transferrin is approximately 25-times less than TfR1 (Kawabata et al., 1999). On the other hand, TfR1 binds to HFE with nanomolar affinity (Lebron et al., 1999), while HFE binding to the TfR2 is not detectable (West et al., 2000). In contrast to the iron-dependent, post-transcriptional regulation of TfR1, the expression of TfR2 is regulated, at least in part, by the erythroid transcription factor, GATA-1 (Kawabata et al., 2001). Presently, it is unclear whether there is direct interaction between TfR1 and TfR2. However, Vogt et al. (2003) have suggested that these molecules form heterodimers due to their similar internalization and co-localization patterns.

2.2 Intracellular Iron Metabolism

2.2.1 Hephaestin, Ferroportin and Hepcidin

Apart from transferrin, TfR1 and TfR2, more recently several other proteins have been implicated in the trafficking and release of intracellular iron, including: hephaestin (Vulpe et al., 1999), ferroportin1 (Donovan et al., 2000) and hepcidin (for review see Ganz, 2003). The hephaestin molecule is a trans-membrane ceruloplasmin homologue that is markedly expressed in the intestine and was first identified in the *sla* mouse (Vulpe et al., 1999). The mutation in this animal leads to reduced iron release into circulation, resulting in iron accumulation within enterocytes (Vulpe et al., 1999). Therefore, hephaestin may play a role in facilitating iron release in cooperation with the iron transporter, ferroportin1, which is believed to be responsible for iron release from enterocytes into

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the bloodstream (Donovan et al., 2000).

Studies over the last 3 years have shown that hepcidin, a peptide hormone secreted by the liver, is critical in iron homeostasis by acting as a iron regulatory hormone (Ganz, 2003). Under conditions of iron overload, hepcidin is highly expressed in the liver (Pigeon et al., 2001). It is thought that hepcidin negatively regulates intestinal iron absorption, maternal-fetal iron transport across the placenta and iron release from hepatic stores and macrophages (Ganz, 2003). Once in the circulation, hepcidin may bind to ferroportin1 on the cell membrane leading to its internalization and degradation (Nemeth et al., 2004). This results in reduced iron efflux from enterocytes and completes a homeostatic loop, whereby iron regulates hepcidin secretion that then affects ferroportin-1 expression (Nemeth et al., 2004). In addition to these molecules, the serum protein, ceruloplasmin, is also involved *in vivo* in mediating iron efflux from cells (Richardson and Ponka, 1997).

2.2.2 The Regulation of Iron Homeostasis by Iron-Regulatory Proteins

The iron-regulatory proteins 1 and 2 (IRP1 and IRP2; M_r 90-95 kDa) are mRNA-binding molecules involved in the control of normal iron homeostasis (Figure 3; for review see Hentze and Kuhn, 1996). The IRP1 contains an [4Fe-4S] cluster and is identical to cytoplasmic aconitase. Iron-responsive elements (IREs) are present in the 5' or 3'-untranslated regions of mRNAs of a variety of molecules that play a role in iron metabolism, including the TfR1 and ferritin. Within ferritin mRNA, the IRE is found in the 5'-untranslated region and its binding with either IRP inhibits translation, thereby decreasing iron storage (Hentze and Kuhn, 1996). However, in the case of *TfR1* mRNA, the IRE is in the 3'-untranslated region and IRP-IRE binding leads to increased translation by stabilization of the mRNA against degradation, in turn causing enhanced iron uptake via the TfR1. The mRNA-binding activity of IRP1 is determined by the presence of the [4Fe-4S] cluster

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within the protein (Hentze and Kuhn, 1996). In cells that are iron-deplete, the [4Fe-4S] cluster is absent (apo-IRP1) and allows IRP1-IRE binding (Figure 3). Conversely, when intracellular iron levels are high, the [4Fe-4S] cluster forms within the protein (holo-IRP1) and prevents IRP1-IRE binding (Hentze and Kuhn, 1996). To date, two forms of IRP1 have been well characterised; a high-affinity binding type which spontaneously binds mRNA and a low affinity form unable to bind IREs (Figure 3). In cellular assays, the low affinity form can be converted to the high affinity IRP-RNA-binding molecule by the addition of β-mercaptoethanol. This allows an estimate of the total IRP-RNA binding activity, thus representing the total IRP present in the cell (Hentze and Kuhn, 1996).

Notably, IRP1 and IRP2 share extensive sequence homology apart from a 73 amino acid sequence unique to IRP2 that mediates its degradation (Richardson and Ponka, 1997). The IRP2 molecule contains no [4Fe-4S] cluster and in iron-replete cells, IRP2 is degraded by a proteasome-dependent mechanism (Guo et al., 1995). Although both IRPs are ubiquitously expressed, IRP1 is more abundant in most tissues (Kim et al., 1995). However, it should be noted that IRP2 is not a null protein. In fact, IRP2(-/-) mice develop neurodegeneration and movement-disorder symptoms due to significant iron accumulation in white matter tracts and nuclei of the brain (LaVaute et al., 2001). In contrast, IRP1(-/-) mice demonstrate normal serum chemistry and all major tissues are without histological abnormalities (Meyron-Holtz et al., 2004). Consequently, it has been argued that IRP2 is highly expressed in many tissues, and in comparison to IRP1, appears to dominate the regulation of iron metabolism (Meyron-Holtz et al., 2004).

2.2.3 The Intracellular Labile Iron Pool

After iron(II) is transported out of the endosome, it enters the intracellular iron pool or labile iron pool (Figure 2). This entity is not well understood, although it is classically thought to be composed of chelatable iron [iron(II) and iron(III)], associated with low M_r ligands, such as citrate or ATP

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(Richardson and Ponka, 1997). More recent work has failed to demonstrate the presence of low M_r intermediates in the iron uptake process and the possible involvement of high M_r iron-binding chaperone molecules have been suggested (Petrak and Vyoral, 2001). Alternatively, or in combination with iron-binding chaperone proteins, interactions between organelles such as the endosome and mitochondrion might be involved in intracellular iron trafficking (Richardson and Ponka, 1997; Zhang et al., 2005). Irrespective of its character, the labile iron pool donates iron to a variety of iron-containing molecules required for cellular metabolism and also for iron storage in ferritin.

The labile iron pool is generally referred to as being cytosolic and represents < 5% of total cellular iron (for review see Esposito et al., 2002). A transit pool of chelatable iron is also required in the mitochondrion during heme synthesis, and chelatable redox-active iron may exist within other organelles (for review see Napier et al., 2005). However, the size and molecular nature of these different subcellular iron pools remains to be investigated. A growing body of evidence suggests that a significant amount of iron, mainly in a redox-active form, is located within the lysosome (Persson et al., 2001). The concentration and distribution of chelatable iron in different intracellular compartments in rat hepatocytes has been determined via quantitative laser scanning microscopy using the fluorescent chelator, Phen Green (Petrat et al., 2001). The highest concentrations of iron (15.8 ± 4.1 μM) was in a subgroup of endosomes and/or lysosomes that may be responsible for degrading iron-containing proteins and mitochondria (Petrat et al., 2001). In comparison, all other cellular compartments demonstrated significantly lower concentrations of chelatable iron, for instance the mitochondria and nucleus had 3-fold lower iron levels.

2.2.4 Iron Storage in Ferritin and Lysosomal Iron Recycling

Iron which is not immediately required for cell function or synthesis of hemoproteins is deposited in

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the iron-storage protein, ferritin (Figure 2). Ferritin is composed of 24 subunits categorized into two subtypes: a heavy subunit (M_r = 21 kDa) and a light subunit (L-Ft; M_r = 19 kDa) that polymerize into a high M_r polymer (M_r = 430-450 kDa) (for review see Harrison and Arosio, 1996).The H- and L-ferritin subunits display about 55% amino acid sequence identity, and have a similar three-dimensional structure. The ferritin molecule is able to accommodate approximately 4,500 iron atoms in its protein shell. Ferritin stores iron(II) by forming a solid oxo-mineral in its core (Harrison and Arosio, 1996). It is suggested that the ferritin H-subunit subunits induce a rapid oxidation of iron(II) to iron(III) through a ferroxidase site composed of seven conserved residues. The L-ferritin subunit on the other hand, has a nucleation site, which is involved in the formation of the iron core (Harrison and Arosio, 1996).

Present knowledge favours the lysosomal pathway as being a significant route for ferritin degradation and re-utilization of iron (Radisky and Kaplan, 1998; Persson et al., 2001). Lysosomes degrade various macromolecules, including metalloproteins such as ferritin, and damaged cell organelles. Due to lysosomal autophagy, lysosomes become particularly rich in iron (Persson et al., 2001; Petrat et al., 2001). Solubilized lysosomal iron is either transported to the cytosol by an unknown mechanism or is stored within lysosomes as iron(III) in hemosiderin, i.e., partially degraded ferritin (Persson et al., 2001).

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3. Iron-Related Mechanisms for Anthracycline-Induced Cardiotoxicity

The cardiotoxic effects of anthracyclines have been suggested to be a result of a number of different mechanisms. These are discussed below with emphasis on the role of iron.

3.1 Cardiotoxicity Mediated by Iron-Anthracycline Complexes and Free Radical Generation

Anthracyclines bind avidly to iron, forming a 1:1, 2:1 or 3:1 drug-to-metal complex (Figure 1) with

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an overall association constant of 10^{18} (Gianni and Myers, 1992). doxorubicin can directly bind iron and in the presence of oxygen it can cycle between the iron(II) and iron(III) states (Figure 4). The doxorubicin-iron(III) complex can be reduced to the doxorubicin-iron(II) complex in the presence of reducing agents such as NADPH cytochrome P450 reductase, glutathione and cysteine. These reactions are accompanied by the formation of $O_2^{\bullet -}$, and the conversion of anthracycline quinone moieties to semiquinone free radicals (Figure 4). The quinone structure of anthracyclines has the potential to act as an electron acceptor from enzymes such as flavin reductases, NADH dehydrogenase and cytochrome P450 reductase (Gianni and Myers, 1992). Through the iron-catalyzed Haber-Weiss reaction, H_2O_2 and extremely reactive hydroxyl radicals are generated. The semiquinone radical may transform to an aglycane C7-centered radical, which is a potent alkylating agent (Figure 4) (Jung and Reszka, 2001). It is well-known that such ROS generation by anthracyclines causes DNA damage and apoptosis (Minotti et al., 2004).

Although neither H_2O_2 nor $O_2^{\bullet -}$ are particularly reactive, in the presence of redox-active iron even low quantities of ROS are cytotoxic. Since all cells contain small amounts of redox-active iron, formation of hydroxyl radicals, or similarly reactive iron-centered (ferryl and perferryl) radicals, can be promoted under appropriate conditions. These highly reactive species can attack almost all cellular constituents and a number of organelles, and create chain reactions that lead to cell death (Myers, 1998). The increased activity of the anti-oxidant pathways, such as catalase, glutathione peroxidase, and glutathione transferase, in anthracycline-exposed cardiomyocytes further support the great importance of ROS formation in cardiac injury secondary to anthracycline treatment (Jung and Reszka, 2001). However, cardiac tissue is generally recognized to be quite vulnerable to free radical damage due to the low activity of anti-oxidant enzyme systems (Gianni and Myers, 1992). Finally, it should also be noted that there is evidence that the cytotoxic mechanisms of anthracyclines can be independent of ROS generation (Keizer et al., 1990; Wu and Hasinoff, 2005).

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3.2 Anthracyclines Mediate Dysregulation of Iron Homeostasis

A number of studies have provided evidence for mechanisms that could be involved in anthracycline mediated cardiotoxicity that are both independent and dependent on iron. Below, we focus upon the iron-dependent mechanisms of anthracycline-mediated cardiotoxicity.

3.2.1 Effect of Doxorubicin on Major Regulators of Iron Homeostasis: IRP1 and IRP2

3.2.1.1 The Role of Doxorubicinol in Decreasing IRP-RNA-Binding Activity

The effect of doxorubicin on cellular iron homeostasis, including IRP levels, has been suggested to be a factor contributing to its cardiotoxicity (Minotti et al., 1995, 1998). The mechanisms involved in the effect of doxorubicin on IRP1 remain controversial, as a number of research groups have shown different results. Initially, doxorubicinol, a secondary alcohol metabolite of doxorubicin, was described to interact with the [4Fe-4S] cluster of IRP1, resulting in the release of iron(II), and a decrease in cytoplasmic aconitase activity (Minotti et al., 1995; Minotti et al., 1998; Brazzolotto et al., 2003). Considering classical IRP theory, decreased cytoplasmic aconitase levels may result in increased IRP-RNA-binding (Hentze and Kuhn, 1996). In contrast, doxorubicinol led to a decrease in IRP-RNA-binding and this could not be reversed by the reducing agent, β-mercaptoethanol (Minotti et al., 1998). It was suggested that doxorubicinol in the presence of the aconitase substrate, *cis*-aconitate, directly removed iron(II) from the [Fe-S] cluster from IRP1 by a mechanism independent of free radical generation (Minotti et al., 1998) (Figure 5; Scheme 1). Moreover, a generalized model was proposed indicating that the interaction of doxorubicinol with IRP1 resulted in iron(II) release from the [4Fe-4S] cluster and the reoxidation of doxorubicinol to doxorubicin. The iron(II) released then formed a complex with doxorubicin that irreversibly inactivated IRP1 (Minotti et al., 1998).

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In the investigation of Minotti et al. (1998) described above, lysates from homogenized hearts incubated with iron salts and cysteine were implemented to reconstitute the [4Fe-4S] cluster of IRP-1. Clearly, this system is undefined and the exact molecular site of the iron mobilization observed in the lysates was not identified. Indeed, it was not clear if the iron release in the lysates after incubation with anthracyclines was due to mobilization of iron from IRP1 or other molecules (Minotti et al., 1998). Later studies by the same authors showed that incubation of a cardiomyocyte cell line with doxorubicin increased active IRP-RNA-binding but decreased aconitase activity (Figure 5; Scheme 2; Minotti et al., 2001). These authors suggested that the sequential action of doxorubicinol and ROS on IRP1 leads to the generation of the null protein. However, it was not clear from this latter article why an increase in IRP1-RNA-binding was observed, which was in contrast to the decrease previously observed in heart lysates (Minotti et al., 1998).

Further studies by other investigators assessed the effect of doxorubicin in GLC_4 small cell lung carcinoma cells resistant (GLC_4^R) and sensitive (GLC_4^S) to this agent (Brazzoloto et al., 2003). These authors showed that incubating doxorubicin with the sensitive cell type resulted in a decrease in IRP-IRE-binding activity, while it had no effect on the resistant clone (Brazzoloto et al., 2003). When recombinant human IRP1 was incubated with very high concentrations of doxorubicin or doxorubicinol (120 µM), aconitase activity was significantly reduced only after doxorubicinol treatment in the presence of oxygen. These concentrations of anthracyclines are well above those encountered within human patients administered doxorubicin, where serum concentrations reach micromolar levels. In contrast, there was no effect of doxorubicin on purified recombinant IRP2, suggesting that doxorubicin targeted IRP1 (Brazzolotto et al. 2003). This latter result was not consistent with previous studies which showed that incubation of cardiomyocyte cell lines or primary cultures with doxorubicin reduced IRP2-RNA-binding activity (Minotti et al., 2001; Kwok and Richardson, 2002).

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3.2.1.2 The Role of the Doxorubicin-iron Complex in Decreasing IRP-RNA-Binding Activity

In subsequent studies by others, three anthracyclines, namely doxorubicin, daunorubicin and epirubicin, had a complex effect on IRP-RNA-binding activity when incubated with cells in culture (Kwok and Richardson 2002). In these experiments, active IRP-RNA-binding activity decreased over a 6 h incubation with anthracyclines and then subsequently increased, while total IRP-RNA-binding decreased as a function of time. In contrast to a previous investigation by Minotti and co-workers (1998), experiments using cell lysates demonstrated that doxorubicinol in the presence or absence of *cis*-aconitate had no effect on IRP-RNA-binding (Kwok and Richardson, 2002). In contrast, anthracycline-iron and -Cu complexes reduced active IRP-RNA-binding and this was reversible upon the addition of β-mercaptoethanol (Kwok and Richardson, 2002). These latter results differed to those of Minotti and associates (1998) using tissue homogenates, which suggested that the doxorubicin-iron complex irreversibly inactivated IRP-RNA-binding. This inhibitory effect could be due to the ability of the doxorubicin-iron complex to oxidize critical sulfhydryl groups involved in IRP-mRNA-binding activity (Philpott et al., 1993) (Figure 5B; Scheme 3). In this way, the doxorubicin-iron complex would act similarly to other agents that react with sulfhydryl groups, such as diamide (Philpott et al., 1993). Considering this, it is well known that the doxorubicin-iron complex catalyzes a range of redox reactions. For instance, it reacts with reductants including glutathione to yield oxidized thiols and oxygen radicals (Gianni and Myers, 1992).

Using primary cultures of cardiomyocytes, Kwok and Richardson (2002) also reported that doxorubicin reduced IRP2-RNA-binding binding activity. These studies are similar to those reported using the H9c2 cardiomyocyte cell line, where doxorubicin irreversibly decreased IRP2-RNA-binding activity (Minotti et al., 2001). Since IRP2 does not possess an [Fe-S] cluster, it can be

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speculated that the effects of doxorubicin may also be mediated by its ability to oxidize sulphydryl groups involved in mRNA-binding activity. Indeed, anthracycline-mediated free radical production may be involved in this process since 5-iminodaunorubicin that generates far lower levels of free radicals does not affect IRP2-RNA-binding activity (Minotti et al., 2001).

3.2.1.3 Doxorubicin Increases IRP-RNA-Binding Activity in Endothelial Cells

Studies using endothelial cells have shown different effects of doxorubicin on IRP-RNA-binding activity than those described above. In fact, in an investigation by Kotamraju and colleagues (2002), doxorubicin increased IRP-RNA-binding activity within 8 h and there was also increased iron uptake and TfR1 expression. These experiments suggested that doxorubicin-induced iron uptake occurred via increased IRP-RNA-binding activity and the subsequent elevation of TfR1 levels (Kotamraju et al., 2002). The authors proposed that oxidative stress generated by doxorubicin activated IRP-RNA-binding, as anti-oxidants such as ebselen and Mn(III) tetrakis (4-benzoic acid) porphyrin complex (MnTBAP) inhibited the effect of doxorubicin on TfR1 levels and iron uptake. These results were clearly different to those using lysates from the homogenized heart (Minotti et al., 1998) or neoplastic cell lines where doxorubicin decreased IRP-RNA-binding and may indicate a cell type-specific response.

In conclusion, the results above demonstrate that the effects of doxorubicin on IRP-RNA-binding activity are complex. In general, in most cell types, doxorubicin decreased active IRP-RNA-binding activity and this will probably result in important downstream effects on cellular iron metabolism. Further studies to clarify the precise molecular mechanisms involved need to be performed.

3.2.2 Effect of Doxorubicin on Iron Trafficking Pathways: Doxorubicin Induces Iron Accumulation in Ferritin

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Initial studies assessing the effects of doxorubicin on cellular iron metabolism reported that doxorubicin released iron from ferritin (Thomas and Aust, 1986). However, these experiments were performed *in vitro* using the purified ferritin protein and their physiological significance remained unclear. More recent investigations showed that a 24 h incubation of a range of neoplastic and normal cells with diferric transferrin and doxorubicin (1-10 μM) lead to 3- to 8-fold higher ferritin-iron levels compared to control cells incubated with diferric transferrin alone (Kwok and Richardson, 2003; Kwok and Richardson, 2004). This accumulation of ferritin-iron was due to the fact that incubation of cells with anthracyclines prevented iron release from this molecule (Figure 6). Moreover, the slight increase in ferritin protein levels observed after incubation with doxorubicin (to 130% of the control at 5 μM doxorubicin) could not account for the 3 to 8-fold increase in ferritin-iron accumulation (Kwok and Richardson, 2003).

Considering the mechanism of ferritin-iron accumulation after incubation with doxorubicin, the general process of iron mobilization from this protein is poorly understood. However, catabolism of ferritin by lysosomes has been suggested to be a likely mechanism (Radisky and Kaplan, 1998; Persson et al., 2001). In addition, anthracyclines are known to accumulate in lysosomes (Hurwitz et al., 1997), and this organelle may be a target for these drugs (Figure 6). Recent studies have shown that ferritin iron mobilization is an energy-dependent process that also requires protein synthesis (Kwok and Richardson, 2004). This latter observation was based on studies inhibiting protein synthesis using cycloheximide, which prevented ferritin iron release (Kwok and Richardson, 2004). It can be speculated that this effect could be due to the requirement for translation of a protein that is involved in ferritin iron mobilization.

Additional evidence for the involvement of the lysosome in the doxorubicin-mediated inhibition of ferritin iron mobilization was provided by implementing a number of lysosomal protease inhibitors

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(Kwok and Richardson, 2004). Depression of lysosomal protease activity using pepstatin A, E64d, or leupeptin, demonstrated that pepstatin A had no effect, while E64d and leupeptin increased ferritin iron-loading to a level similar to doxorubicin. Considering this, since pepstatin A is an aspartic protease inhibitor, while E64d and leupeptin are cysteine or cysteine and serine protease inhibitors, respectively, it can be suggested that aspartic proteases are not involved in ferritin-iron mobilization (Kwok and Richardson, 2004). Further support for the role of the lysosome and/or proteasome in ferritin-iron mobilization was achieved through the use of the lysosomotropic agents, NH_4Cl, chloroquine and methylamine, and the proteasomal inhibitors, MG-132 and lactacystin, that also prevented ferritin-iron mobilization (Kwok and Richardson, 2004). Thus, the lysosome/proteasome pathway may be an anthracycline target, inhibiting ferritin iron release that is vital for iron-requiring processes, e.g., DNA synthesis (Kwok and Richardson, 2004).

In view of the effect of anthracyclines at inhibiting iron mobilization from ferritin, it must be noted that this is only one of the many effects of these drugs that contributes to their cytotoxicity. At present, compared to the other cytotoxic effector mechanism of anthracyclines, the extent to which the inhibition of ferritin iron mobilization contributes to cardiotoxicity is not clear. Furthermore, it must be noted that the potential cytotoxicity induced by inhibiting ferritin iron mobilization is at odds with the ability of iron chelators to prevent anthracycline-mediated cardiotoxicity (see Section 4 below). In terms of trying to understand this apparent dichotomy, the complexity of the mechanisms of action of anthracyclines must be considered (Minotti et al., 2004). In fact, the iron pool or other molecular sites that are targeted by chelators to prevent anthracycline-mediated cardiotoxicity are not known and it is of interest that the chelator, dexrazoxane, does not completely prevent this problem (Swain et al., 1997b). Additional studies investigating the mechanisms of how chelators inhibit anthracycline-mediated cardiotoxicity are required.

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Interestingly, similarly to anthracyclines, a number of free-radical generating agents (i.e., menadione and paraquat) have also been shown to be effective at increasing ferritin iron accumulation, an effect that can be at least partially reversed by free radical scavengers (Kwok and Richardson, 2003). It has been proposed that the ability of free radical generators to induce ferritin iron accumulation may be mediated via the effects of these agents on lysosomal function (Kwok and Richardson, 2004). Further evidence for a role of free radical generation in inducing the alterations in ferritin-iron metabolism was obtained by Corna and associates (2004) comparing doxorubicin and the redox active anthracycline analogues, mitoxantrone and 5-iminodaunorubicin. These ROS-generating compounds were found to significantly induce ferritin protein expression, especially the H-ferritin subunit, suggesting that doxorubicin regulates ferritin levels via ROS formation (Corna et al., 2004). Further experiments revealed that enhanced ROS production and ferritin accumulation after doxorubicin treatment can be prevented by ROS scavenger, *N*-acetylcysteine. Therefore, it was suggested that doxorubicin-mediated ROS production was involved in ferritin induction in the H9c2 cardiomyocyte cell line (Corna et al., 2004). Concomitant experiments showed that preincubation of doxorubicin or mitoxantrone could paradoxically protect H9c2 cells from cytotoxicity induced by iron loading with ferric ammonium citrate, while incubation with 5-iminodaunorubicin did not protect the cell. Considering that 5-iminodaunorubicin reportedly produces less ROS than either doxorubicin or Mitox, these studies suggested that the protective effect of these compounds to iron-loading appeared to correlate with their ability to act as ROS generators (Corna et al., 2004).

Apart from the effect of doxorubicin on ferritin iron-loading, further evidence that interactions between doxorubicin and iron metabolism are involved in the cytotoxic effects of these drugs has been provided by studies examining HFE knockout mice (Miranda et al., 2003). After doxorubicin treatment, HFE knockout mice accumulate more iron in the serum and several organs compared to

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their wild-type counterparts, suggesting that HFE deficiency may increase susceptibility to doxorubicin-induced toxicity (Miranda et al., 2003). Doxorubicin treated HFE-deficient mice also have higher mortality rates and a greater degree of mitochondrial damage compared with the control (Miranda et al., 2003).

4. Agents that Prevent Doxorubicin-Mediated Cardiotoxicity by Interacting with Iron

The evidence presented above indicates that anthracyclines markedly disturb intracellular iron metabolism and a variety of studies have clearly shown that iron plays an important role in the cardiotoxicity mediated by this drug. Apart from this, it is well known that the iron chelator, dexrazoxane (also known as ICRF-187), is an effective cardioprotective agent against the effects of doxorubicin. Below, we discuss the potential of iron chelators as agents to prevent anthracycline-mediated cardiotoxicity.

4.1 Dexrazoxane

The only clinically approved chelator that is currently used to alleviate doxorubicin-induced cardiotoxicity is dexrazoxane (Figure 7; Swain et al., 1997a,b; Minotti et al., 2004). It is suggested that *in vivo*, dexrazoxane permeates the cell membrane and can be rapidly hydrolysed to its metal ion-binding metabolite, ADR925, thus decreasing anthracycline-iron binding and ROS formation (Hasinoff, 1998). ADR-925 quickly displaces iron(III) and copper(II) from their complexes with anthracyclines, indicating that ADR925 chelates iron(III) more strongly than doxorubicin (Hasinoff, 1998). Interestingly, metal ions, including the anthracycline-iron(III) complex, promote formation of ADR-925 and potentiate its metal chelating effect (Hasinoff, 1998).

Dexrazoxane has shown significant protection against cardiotoxicity caused by doxorubicin in numerous animal models, for example, mouse, rat, hamster, rabbit and dog (Minotti et al., 2004). In

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addition, clinical trials showed that dexrazoxane protects patients with advanced breast cancer from doxorubicin-induced cardiotoxicity (Swain et al., 1997b). With dexrazoxane therapy, patients treated with dexrazoxane-doxorubicin (ratio 10:1) were only 38% as likely to develop cardiac complications compared to treatment with doxorubicin alone (Swain et al., 1997b). Dexrazoxane also shows short-term cardioprotection against doxorubicin in childhood cancers (Minotti, 2004). Because the outcomes of long-term cardioprotection are not clear, dexrazoxane is only recommended for adult patients who have received an accumulated dosage of doxorubicin ≥ 300 mg/m^2 (Gewirtz, 1999). However, this agent does not confer absolute cardioprotection (Swain et al., 1997b) and causes myelosuppression (Curran et al., 1991). Considering this, other regimens of chelation therapy using a variety of ligands have been investigated and these are discussed below.

4.2 Desferrioxamine

Desferrioxamine (Figure 7) is a hexadentate iron chelator widely used for iron overload disease such as β-thalassemia major (for review see Richardson and Ponka, 1998). Desferrioxamine significantly reduces iron storage and ferritin levels and has been used for many years to control the iron-loading observed in transfusion-dependent anemias (Richardson and Ponka, 1998). However, in comparison, there has been relatively few investigations assessing its protective effects against anthracycline-mediated cardiotoxicity. An early study found that after incubating isolated mice atria with doxorubicin (30 μM), desferrioxamine (200 μM) was more effective than dexrazoxane (200 μM) at preventing the doxorubicin-induced decrease in contractile force (Voest et al., 1994). In addition, Saad et al. (2001) found that desferrioxamine was highly effective at protecting against doxorubicin-induced acute cardiotoxicity when used at a dose that was 10-fold greater than doxorubicin. Treatment with desferrioxamine either prior to or after doxorubicin administration reduced the doxorubicin-mediated elevation of cardiac isoenzymes such as creatine kinase

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isoenzyme and lactate dehydrogenase, which are indicators of myocardial damage and compromised cellular integrity, respectively (Saad et al., 2001). However, desferrioxamine is limited to subcutaneous or intravenous infusion because of its poor absorption from the gastrointestinal tract and its short plasma half-life (Aouad et al., 2002). Moreover, considering that chelator permeability is critical to the ability of these compounds to inhibit anthracycline-mediated cardiotoxicity (Voest et al., 1994), the limited membrane permeability of desferrioxamine probably explains the need for high levels of this chelator to inhibit the effects of doxorubicin (Saad et al., 2001). These disadvantages have encouraged the design of orally active chelators with high lipophilicity and membrane permeability that can access intracellular iron pools to inhibit anthracycline-mediated cardiotoxicity.

4.3 Other Iron Chelators with Potential Cardioprotective Activity

Pyridoxal isonicotinoyl hydrazone (PIH; Figure 7) is a relatively lipophilic orally effective tridentate iron chelator that has high membrane permeability and possesses marked iron chelation efficacy (for review see Richardson and Ponka, 1998). In fact, PIH is able to remove iron from a variety of rodent models via the biliary route (Richardson and Ponka, 1998; Link et al., 2003). Moreover, low doses of PIH (30 mg/kg/day) given to human patients increased iron excretion from iron-loaded patients (Richardson and Ponka, 1998). In a recent *in vivo* study, PIH pre-treatment protected rabbits from daunorubicin-mediated toxicity, although its efficacy was not as great as dexrazoxane (Simunek et al., 2005). This study showed that repeated administration of daunorubicin to rabbits (3 mg/kg, i.v. once a week for 10 weeks) led to 4 deaths, while all animals survived when PIH was administered 60 min prior to daunorubicin. Salicylaldehyde isonicotinoyl hydrazone (SIH; Figure 7), a PIH analogue, also showed cardioprotective potential by restoring a loss in cytochrome P450 activity after daunorubicin treatment (Schroterova et al., 2004). Hence, this class of chelators show promise as potential cardioprotective agents and require further

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investigation.

Other chelators with possible potential in the treatment of anthracycline-mediated cardiotoxicity include the new orally active chelator, ICL670A (4-[3,5-bis-(hydroxyphenyl)-1,2,4-triazol-1-yl]-benzoic acid) (Figure 7). This compound belongs to the synthetic tridentate and iron-selective ligands of the bis-hydroxyphenyl-triazole class (Nisbet-Brown et al., 2003). Using the hypertransfused rat model, ICL670A was able to remove iron from parenchymal iron stores 4 to 5 times greater than desferrioxamine, and combination of desferrioxamine and ICL670A demonstrated an additive effect at the lower dose range of 25 to 50 mg/kg (Hershko et al., 2001). Phase I and II clinical trials of ICL670A were successful in patients with transfusional iron overload (Nisbet-Brown et al., 2003). However, a recent study on neonatal rat cardiac myocytes showed that ICL670A was unable to protect these cells from doxorubicin-mediated cardiotoxicity as measured by lactate dehydrogenase release, while dexrazoxane significantly prevented this (Hasinoff et al., 2003). The reason for inability of ICL-670A to prevent anthracycline mediated cardiotoxicity is puzzling, but it could be related to its inability to access appropriate pools of iron that could be the potential targets of anthracyclines. Understanding why some chelators are protective against anthracycline-mediated cardiotoxicity while others are not is important for the rationale design of new cardioprotective agents and understanding the mechanism of action of these drugs.

5. Summary

Anthracyclines avidly bind iron and form anthracycline-iron(III) complexes that may serve as a regulator of cellular iron homeostasis, by generally decreasing IRP-RNA-binding activity. However, the precise molecular mechanism of this effect remains controversial with the doxorubicin-iron complex and/or doxorubicinol, being involved. Another molecular target of doxorubicin includes the iron-storage protein ferritin. Recent experiments suggest that anthracyclines prevent ferritin iron

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mobilization through a mechanism which may involve inhibition of lysosomal function that is involved in ferritin degradation. The prevention of iron release from ferritin may be detrimental to the cell due to the importance of iron for DNA synthesis and energy metabolism. On the other hand, it has been suggested that doxorubicin-mediated ferritin iron accumulation is a protective effect against anthracycline-induced free radical generation.

Due to the role of iron in anthracycline-mediated cardiotoxicity, both dexrazoxane and other chelators such as desferrioxamine have proven to be successful cardioprotective agents. Because these two chelators have limitations, including myelotoxicity and a cumbersome administration regimen, respectively, other chelators that are orally effective are being developed. Indeed, the ligands PIH, SIH and ICL670A are under investigation and their ability to alleviate anthracycline-mediated cardiotoxicity is important to evaluate in further studies.

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Figure Legends

Figure 1. Illustration of the structures of Doxorubicin (Dox), Daunorubicin (Daun), Epirubicin (Epi) and the iron-complex of Doxorubicin.

Figure 2. Schematic illustration of iron metabolism in human cells. Diferric transferrin in the serum avidly binds to the transferrin receptor 1 (transferrinR1) on the cell membrane. The transferrin-TfR1 complex is then internalized into an endosome by receptor-mediated endocytosis. The iron is released from transferrin by a decrease in endosomal pH that is mediated by a proton pump in the endosome membrane. Once iron is released from transferrin it is thought to be reduced by a ferrireductase and is then transported through the endosomal membrane by the divalent metal transporter 1 (DMT1). Upon leaving the endosome the iron becomes part of a poorly characterized compartment known as the intracellular labile iron pool. Iron can be redistributed from the labile iron pool for cellular use, stored in ferritin, or potentially pumped out of the cell by ferroportin1. Doxorubicin and other anthracyclines bind iron to form drug-iron(III) complex that is known to generate reactive oxygen species (ROS) and lead to cellular damage and apoptosis.

Figure 3. The mRNA binding activity of IRP1 is regulated by the presence of an [4Fe-4S] cluster within the protein. When cellular iron levels are high, the [4Fe-4S] cluster is present in the IRP1, which abrogates mRNA-binding and is known as holo-IRP1 or cytoplasmic aconitase. Conversely, when cells are iron-deplete, the [4Fe-4S] cluster is absent, and the protein has mRNA-binding activity and is known as apo-IRP1.

Figure 4. Illustration of doxorubicin-mediated redox cycling. Doxorubicin can bind to iron by

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forming doxorubicin-iron(III) complexes, which may lead to ROS formation and cellular damage. One electron reduction of doxorubicin generates the doxorubicin-semiquinone that induces DNA damage and lipid peroxidation by ROS formation. The semiquinone radical can be transformed to a C7 radical that can also mediate cellular damage. The reduction of doxorubicin by 2 electrons generates a secondary alcohol metabolite, doxorubicinol. Although it is clear that doxorubicin affects cellular iron regulatory protein 1 (IRP1) RNA-binding activity and iron homeostasis, it is debatable whether it is doxorubicinol, the doxorubicin-iron(III) complex, or both that are the active molecular effectors.

Figure 5. Proposed mechanisms of action of doxorubicin on IRP1-RNA-binding activity. There are three main hypotheses of how doxorubicin regulates cellular IRP1 levels: Scheme 1, doxorubicinol, a secondary alcohol metabolite of doxorubicin, together with *cis*-aconitate may act to remove iron from the [4Fe-4S] cluster of holo-IRP1. The removal of iron from the cluster results in the oxidation of doxorubicinol to doxorubicin and generation of the doxorubicin-iron complex which irreversibly converts IRP1 to a null protein (Minotti et al., 1998); Scheme 2, doxorubicin removes the [4Fe-4S] cluster of holo-IRP1 and increases IRP-IRE binding (Minotti et al., 2001; Kotamraju et al., 2002); Scheme 3, The doxorubicin-iron complex catalyzes disulfide bridge formation between crucial IRP1 thiol groups inhibiting IRP-IRE binding (Kwok and Richardson, 2002).

Figure 6. Schematic illustration of the effect of doxorubicin on cellular iron metabolism. Doxorubicin leads to iron accumulation in ferritin due to inhibition of iron mobilization from this protein. There is no change in the total amount of iron in the cell, rather only the intracellular distribution is markedly affected. The mechanism of how doxorubicin inhibits iron release from ferritin is not known, but may, at least in part, involve disturbance to lysosomal function. Indeed,

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the lysosome has been reported to be involved in ferritin degradation and the release of iron from this protein. The inability of ferritin to release iron after exposure to doxorubicin may lead to cytotoxic effects due to the requirement of essential metabolic processes for iron e.g., DNA synthesis. Clearly, this potential growth inhibitory mechanism is not the only one mediated by anthracyclines.

Figure 7. Illustration of the chemical structures of the iron chelators: desferrioxamine, dexrazoxane, pyridoxal isonicotinoyl hydrazone (PIH), 4-[3,5-bis-(hydroxyphenyl)-1,2,4-triazol-1-yl]- benzoic acid (ICL670A) and salicylaldehyde isonicotinoyl hydrazone (SIH).

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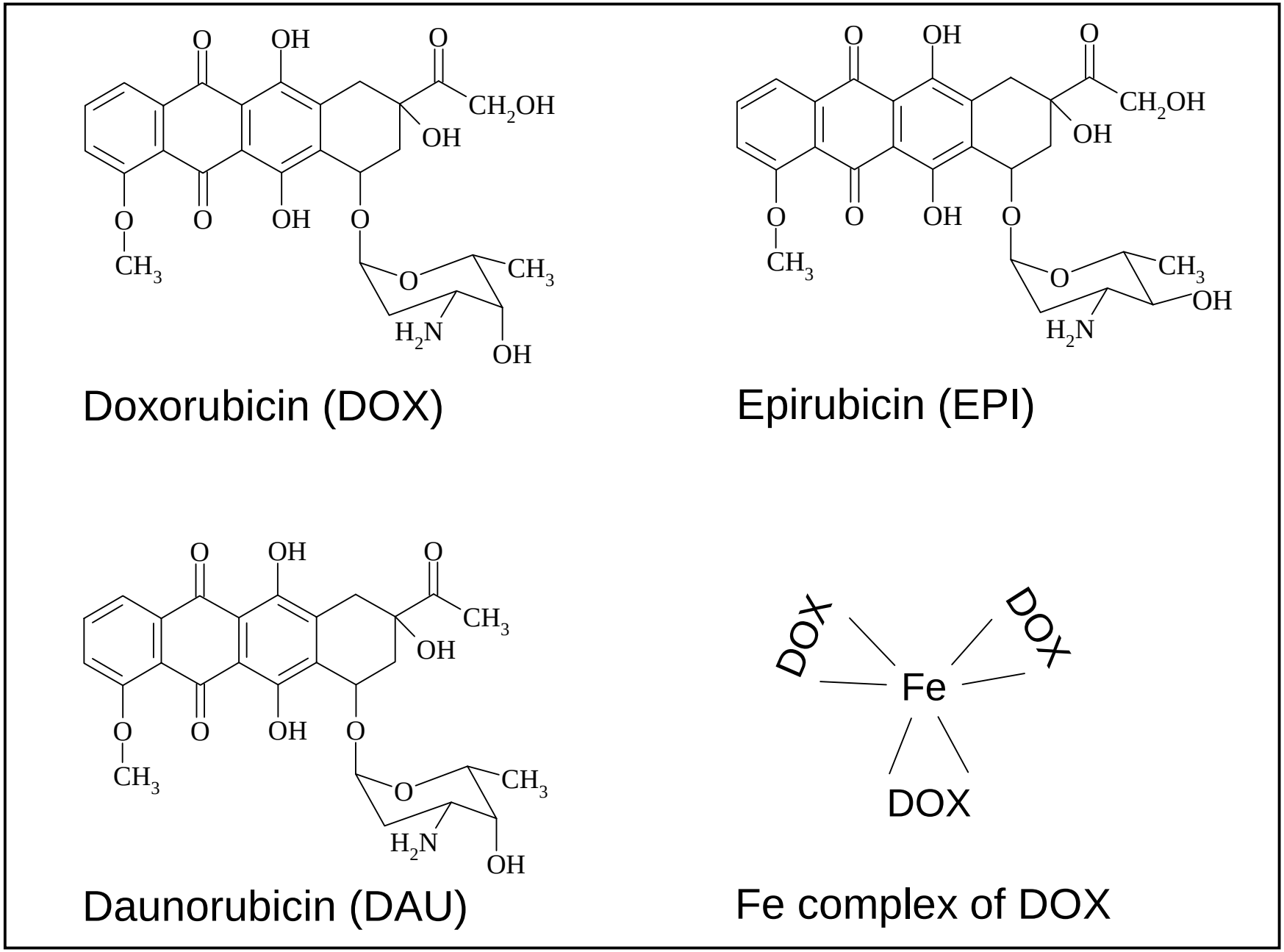

Figure 1

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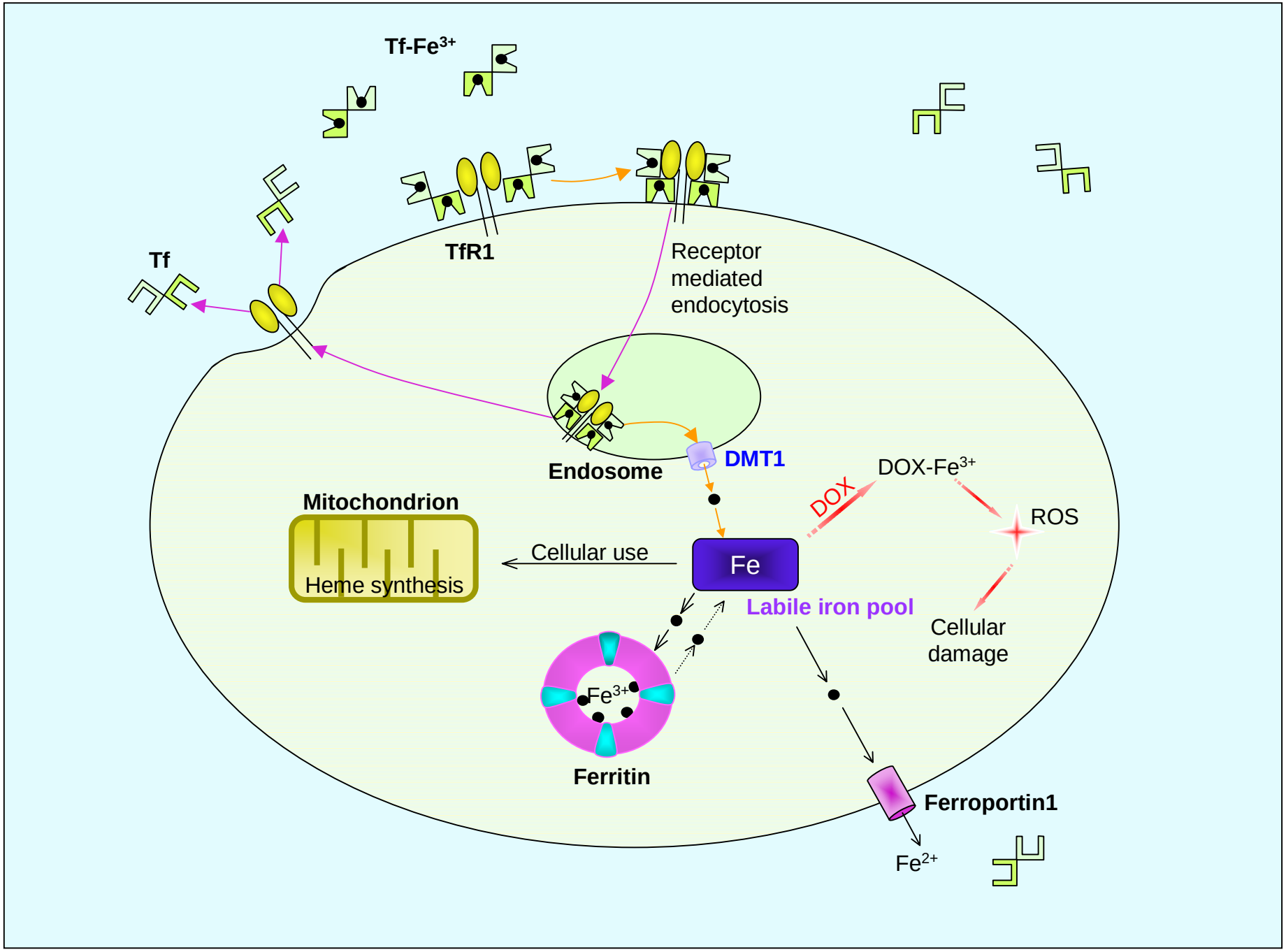

Figure 2

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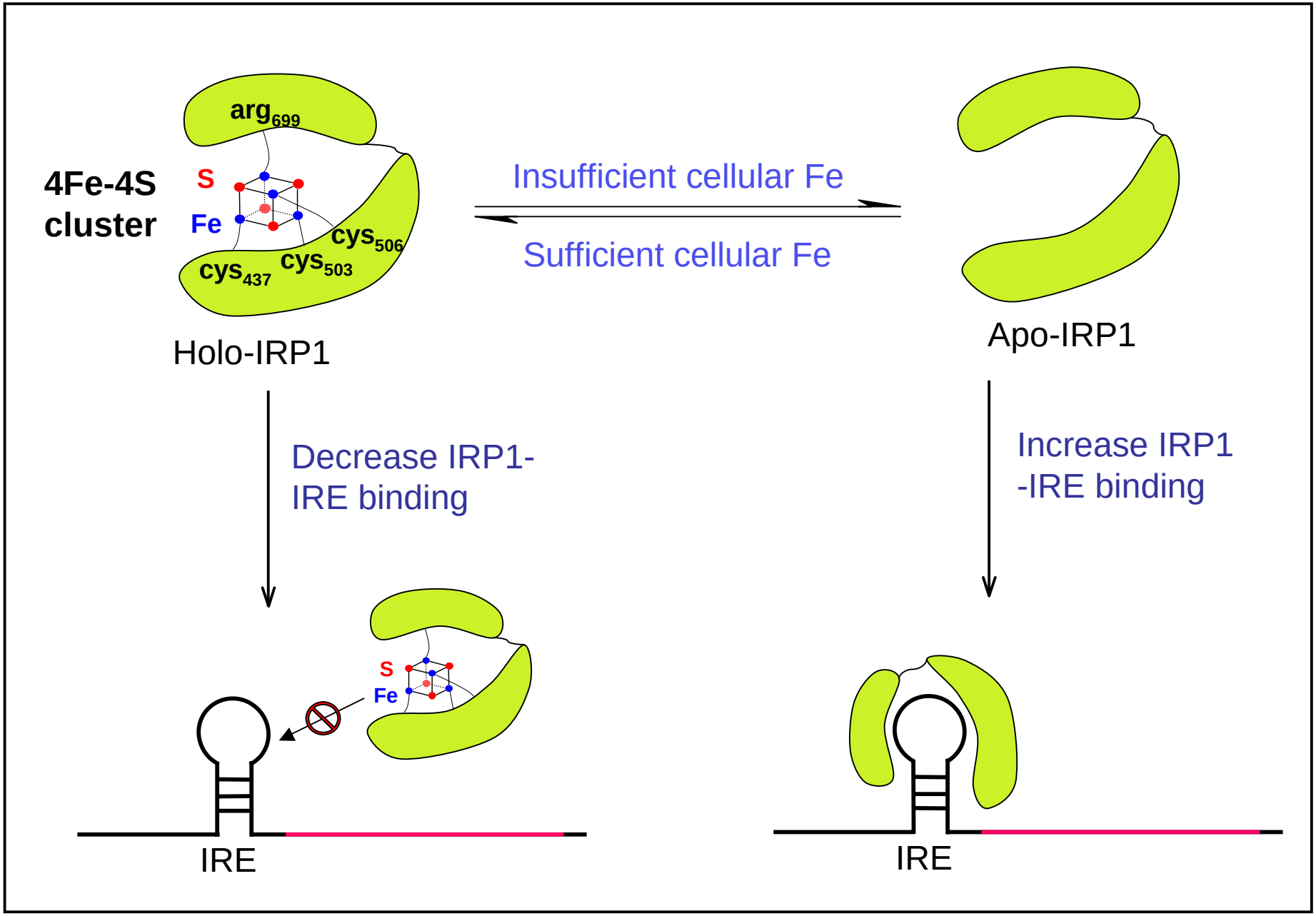

Figure 3

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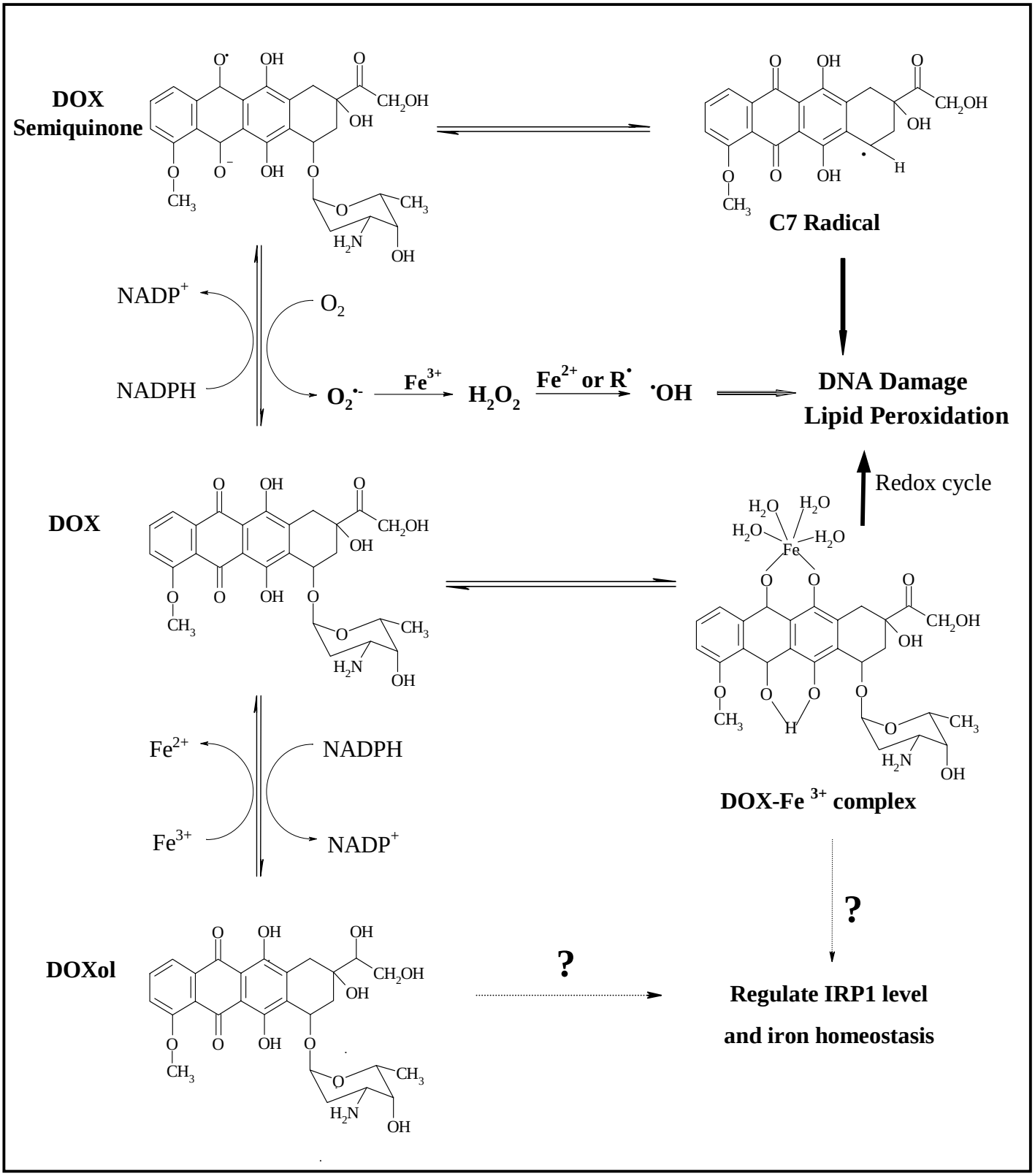


Figure 4

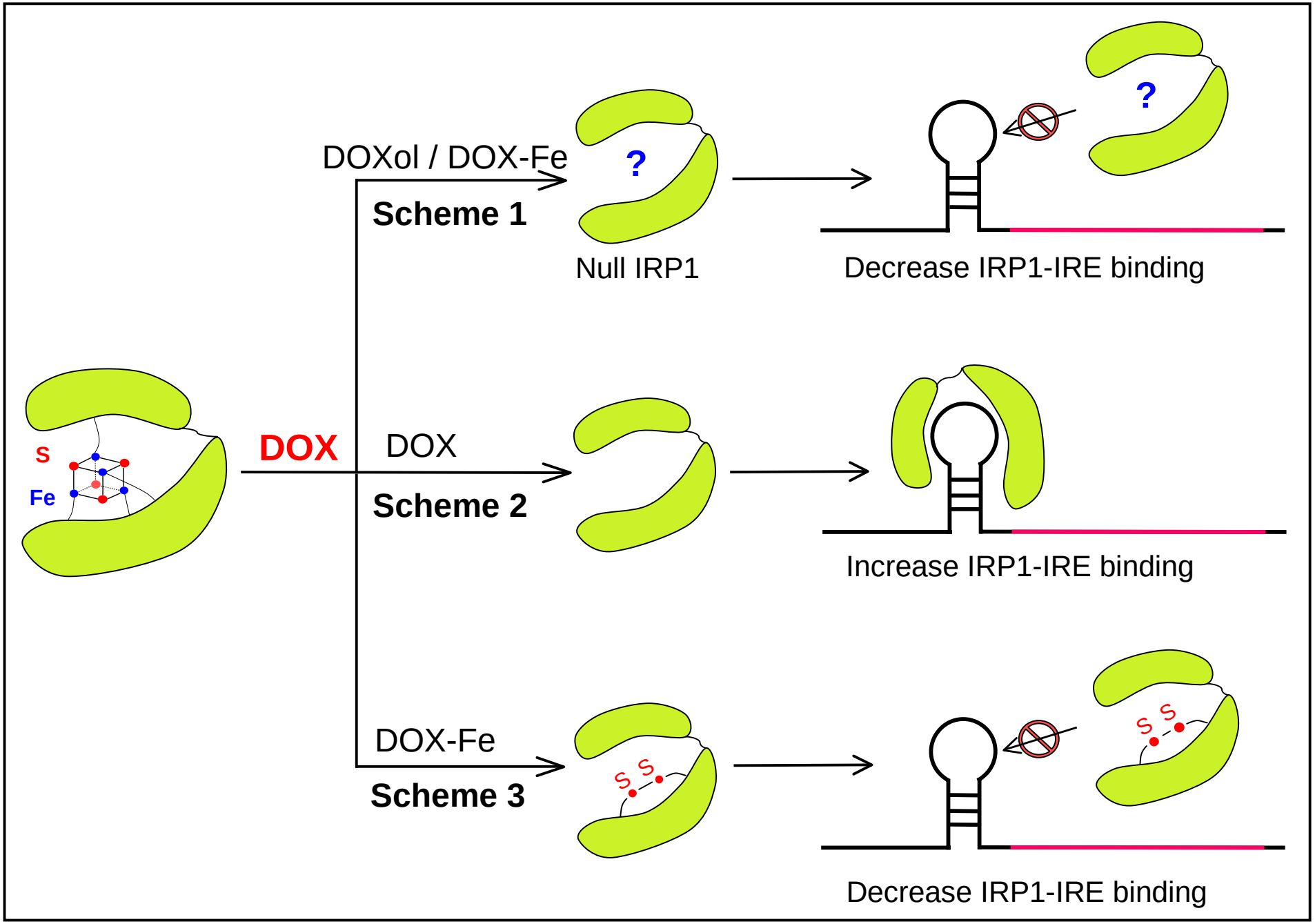


Figure 5

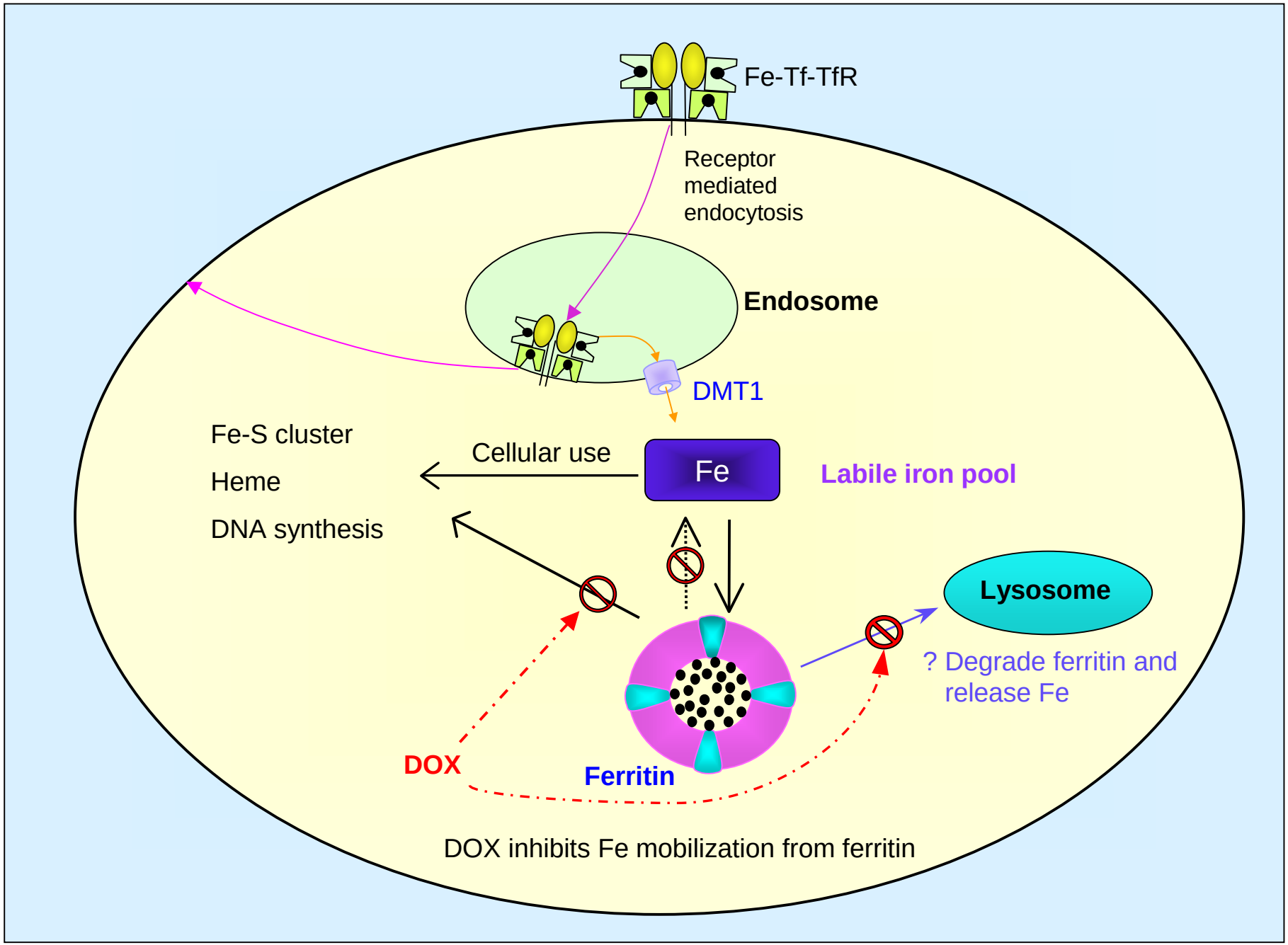
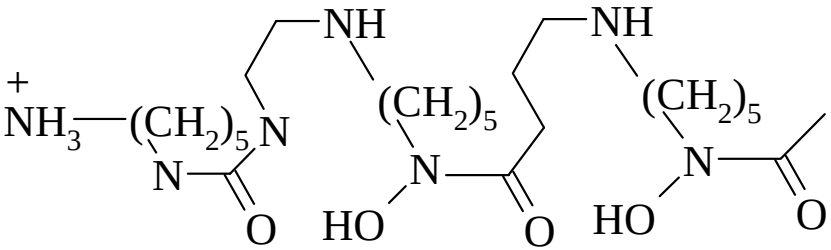
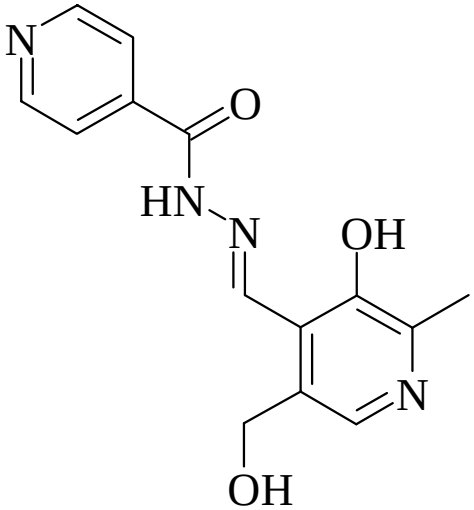


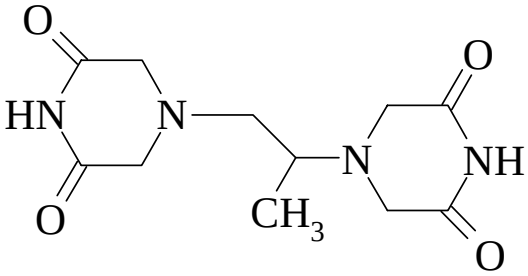
Figure 6



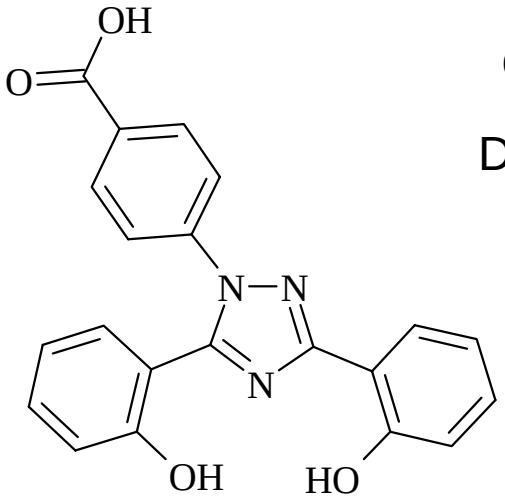
Desferrioxamine (DFO)



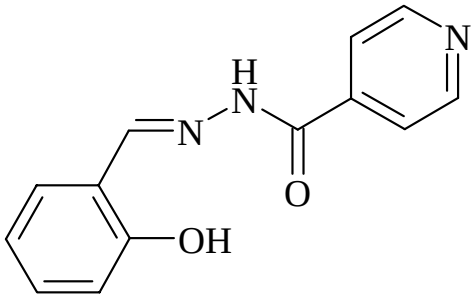
Pyridoxal isonicotinoyl hydrazone (PIH)



Dexrazoxane (DXR)



4-[3,5-bis-(hydroxyphenyl)-1,2,4-triazol-1-yl]- benzoic acid (ICL-670A)



Salicylaldehyde isonicotinoyl hydrazone (SIH)

Figure 7