Characterization of the Cycle of Iron-mediated Electron Transfer from Adriamycin to Molecular Oxygen*

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The antitumor antitumor drug adriamycin binds iron and these complexes cycle to reduce molecular oxygen (Zweier, J. L. (1984) J. Biol. Chem. 259, 6056–6058). Optical absorption, EPR, and Mössbauer spectroscopic data are correlated with polarographic O₂ consumption and chemical Fe²⁺ extraction measurements in order to characterize each step in this cycle. Fe³⁺ binds to adriamycin at physiologic pH forming an optical absorbance maximum at 600 nm. EPR signals at g = 4.2 and g = 2.01, and a doublet Mössbauer spectrum with isomer shift δ = 0.57 mm/s and quadrupole splitting ΔE₀ = 1.74 mm/s are observed indicating that the Fe³⁺ bound to adriamycin is high spin S = 5/2. Under anaerobic conditions the absorbance maximum at 600 nm decreases with an exponential decay constant = 0.77 h⁻¹, and the EPR and Mössbauer spectra of Fe³⁺-adriamycin similarly decrease as the Fe³⁺ is reduced to EPR silent Fe²⁺. The Fe³⁺-adriamycin complex which is formed exhibits a Mössbauer spectrum with δ = 1.18 mm/s and ΔE₀ = 1.82 mm/s indicative of high spin Fe²⁺. As the EPR spectra of Fe²⁺-adriamycin decrease on reduction of the Fe³⁺ to Fe²⁺ a signal of the oxidized adriamycin free radical appears at g = 2.004 with line width of 8 G. On exposure to O₂ the absorption maximum at 600 nm, the Fe²⁺ EPR, and the Fe²⁺ Mössbauer spectra all return. Polarographic measurements demonstrate that O₂ is consumed and that H₂O₂ is formed. Addition of high affinity Fe²⁺ chelators block O₂ consumption indicating that Fe²⁺ formation is essential for O₂ reduction. This cycle of iron-mediated O₂ reduction can explain the formation of the reactive reduced oxygen and adriamycin radicals which are thought to mediate the biological activity of adriamycin.

Adriamycin is known to chelate iron. Step association constants of 10⁻¹⁸, 10⁻¹⁵, and 10⁻¹² have been estimated for the association of the first, second, and third adriamycin, respectively, with Fe³⁺ yielding an overall association constant of 10⁻³³.(10).

In the past we have shown that adriamycin, through chelation of Fe³⁺, can function as a catalyst for O₂ reduction by the physiologic reducing agents glutathione and cysteine (6, 7). Activation of molecular O₂ to radical species is a feature common to several iron-chelates, but the reaction catalyzed by Fe³⁺-adriamycin appears unusual because the complex binds tightly to erythrocyte-ghost membranes and catalyzes their destruction in the presence of glutathione (6). More recently, we have shown that Fe²⁺-adriamycin is also able to bind to and cleave double-stranded DNA (7). In both sets of experiments, we observed that the chelate formed by adriamycin and Fe³⁺ catalyzes destruction of the erythrocyte ghosts or DNA in the absence of reducing agents. In the case of DNA, this activity is completely blocked by addition of catalase, suggesting that H₂O₂ had been generated and is a critical intermediate in DNA cleavage. The generation of H₂O₂ under these circumstances suggests that iron-adriamycin is able to reduce molecular oxygen in the absence of added electron donors such as the thiols. In support of this hypothesis, it has been reported that a complex of Fe²⁺-ADP and adriamycin can reduce ferricytochrome c in the absence of O₂ (8). Finally, EPR studies of the iron-adriamycin complexes suggest that adriamycin reduces its bound Fe³⁺ to Fe²⁺ with subsequent electron transfer to molecular oxygen (5).

In the present study we definitively demonstrate that the iron-adriamycin complexes cycle to reduce O₂. By correlating optical absorbance, electron paramagnetic resonance, and Mössbauer spectroscopic data with polarographic O₂ consumption and chemical Fe²⁺ extraction measurements each step in the cycle of O₂ reduction is characterized.

EXPERIMENTAL PROCEDURES

Materials

Adriamycin hydrochloride (>98% pure by HPLC²) was supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute or purchased from Aldrich. Bathophenanthroline disulfonic acid disodium salt hydrate, ultrapure NH₄H₂PO₄, acetohydroxamic acid, and ferrous ammonium sulfate β-hydrate (99.999%) were purchased from Aldrich. Ferric-acetohydroxamic acid was prepared daily by dissolving 3 molar eq of the chelating agent in doubly distilled water and adding 1 molar eq of solid FeCl₃·6H₂O (ACS grade, Allied Chemicals). For EPR experiments a ferric chloride standard solution was prepared by the methods of Aisen et al. (11). For the Mössbauer

The anthracycline antibiotic, adriamycin, is one of the most potent antitumor drugs in clinical use. It is effective in the treatment of such common tumors as carcinoma of the lung, breast, and ovary (1). Unfortunately, adriamycin is also toxic to heart muscle, and it is a very active mutagen and carcinogen (2). The drug is thought to mediate its therapeutic and toxic effects via the formation of reactive adriamycin and reduced oxygen radicals; however, the mechanism of radical formation is not known (2–4). Recently a number of laboratories have determined that the binding of iron to adriamycin may be a critical step in the generation of these reactive radicals (5–9).

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1 The abbreviations used are: HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BPS, bathophenanthrolone; AHA, acetohydroxamic acid.

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**Methods**

*Optical Studies*—Optical absorption spectra were obtained with a Hewlett-Packard UV/Vis spectrophotometer model 8450A, which is equipped with microprocessor and storage memory that allows the recording and storage of spectra from 200 to 800 nm each second. The anaerobic time course of the reaction of adriamycin with $Fe^{3+}$ was performed in a quartz anaerobic cuvette equipped with a side arm stoppered with a silicon rubber gas chromatography septum. Four ml of buffered adriamycin were degassed according to previously published techniques (6). Stock ferric acetohydroxamate was added through a gas-tight 10-μl syringe to reach the final concentration desired. The desired amount of the drug in doubly distilled water, mixing the desired amount of $FeCl_3$, and then titrating the pH from the initial value of approximately 2.5 to the desired pH with 0.05 N NaOH. Titration of the pH by addition of 50 mM Hepes, 0.1 M KCl buffer at pH 7.4 yielded spectral results identical to those obtained with NaOH. Anaerobic $Fe^{3+}$- or $Fe^{2+}$-adriamycin complexes were prepared either in vacuum or in 100% nitrogen atmosphere as described previously (5). EPR spectra were recorded with a Varian E-9 spectrometer operating at X band using 100 kHz modulation frequency and a TE 102 cavity. EPR spectra were obtained over the temperature range 10-100 K using an Air Products variable tempeatre apparatus with EPR dewar insert. Spectra were also obtained at 77 K using a liquid nitrogen EPR dewar. The microwave frequency and magnetic field were calibrated using techniques similar to those described previously (12).

*Mossbauer*—Mossbauer complexes were prepared by purging with nitrogen gas in a specially constructed glass apparatus fused to a transfer tube which connects to a lucite Mossbauer cell. After the degassing time the anaerobic sample was poured through the transfer tube into the Mossbauer cell which was then immediately frozen in liquid nitrogen. After freezing the seal between the glass transfer tube and the Mossbauer cell was opened allowing insertion of the cell into the Mossbauer dewar.

Transmission Mossbauer spectra were obtained using a 512-channel spectrometer operated in a constant acceleration mode. A 75-μCi source of $^{57}Co$ diffused into a Rh matrix in conjunction with a Jannis flow cryostat were employed to carry out the measurements at the various temperatures. The theoretical fit of the data was performed assuming a Lorentzian shape for the absorption lines, and the isomer shifts (5) are given relative to metallic iron at room temperature.

*Liquid Chromatographic Experiments*—Solutions of $Fe^{3+}$ and adriamycin were prepared under anaerobic conditions as described for the optical spectroscopy studies. The reaction was monitored spectroscopically for loss of absorbance at 600 nm. After 1.5-h incubation, iron-$Fe^{3+}$ was then extracted according to a modified method (13). This technique depends upon the fact that the $Fe^{2+}$-phenanthroline complex is stable in the presence of oxygen. Four hundred μl of 0.01 M 4,7-diphenyl-1,10-phenanthroline (Sigma) in 95% ethanol was added by a gas-tight syringe to 4 ml of the degassed reaction mixture through the side arm of the anaerobic cuvette. The cuvette was opened, and 5.1 ml of a degassed sointion containing 10% NH$_4$HPO$_4$, glacial acetic acid, and 1 M HCl (20:8:0.3, v/v/v) was added quickly with rapid mixing. The solution had a final pH of 2.5 and was extracted by mixing with 4.4 ml of chloroform/methanol (4:1, v/v). At this pH, only the aglycon forms of adriamycin and the Fe(II)-phenanthroline chromogen can be extracted. The organic phase was extracted as above and serially diluted in methanol in the range of concentrations of 1 to 100 μM. The correlation between the area under the peak eluted at 8 min, and concentration was linear with $r > 0.99$. The same samples described above were analyzed by HPLC according to a previously described method for adriamycin and its metabolites. The analysis showed that less than 5% of the total drug present was extracted as the aglycon plus a poorly resolved peak with the same retention time as adriamycin.

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**Polarographic Experiments**—O_2 consumption studies were performed with a Clark-type electrode (Yellow Spring Instruments) on a Gilson model 5/6 Oxygraph equipped with a water-jacketed cell for temperature control. Superoxide dismutase (Sigma, type I from bovine blood) was used without further purification. Catalase (Sigma, from bovine liver, 2X crystallized) was pretreated by mixing with a slurry of Chelex 100 resin in water at 4 °C for 30 min. This preparative step resulted in higher activity of the enzyme and more reproducible results. Control experiments were performed with enzymes that had been boiled for 10 min. The different buffer solutions were equilibrated with air at 37 °C and assumed to contain the same concentration of dissolved O_2 as water (207 μM at 37 °C). The oxygraph was calibrated daily with a solution of water equilibrated with air for 2 h at 37 °C. All experiments were performed in the dark.

Polarographic studies were performed with ferric-acetohydroxamate as the source of Fe^{3+} for adriamycin. Control experiments were performed with FeCl_3 to rule out possible artifacts due to hydroxamic acid. No significant differences were observed, and ferric-acetohydroxamate was routinely used because it results in a lesser degree of variability at neutral pH.

Calculations and Computer Fitting—Equations were fitted to the data using the MLAB fitting routine program. The rate constant for the 600-nm absorption decay of the anaerobic Fe^{3+}-adriamycin complex was calculated by least squares fit to the equation,

\[ a = a_0 e^{-kt} \]

where \(a\) is the measured absorbance at time \(t\); \(a_0\) the extrapolated absorbance value at time \(0\); and \(K\) the calculated rate constant. The pH titration curves of O_2 consumption were similarly fitted to the Henderson-Hasselbalch equation.

**RESULTS AND DISCUSSION**

The anticancer drug adriamycin forms complexes with Fe^{3+} over the physiologic pH range, pH 6.5–8.5, which give rise to distinctive optical absorption, EPR, and Mössbauer spectra. On addition of Fe^{3+} to adriamycin a new absorption band centered at 600 nm appears along with hypochromicity of the optical absorption band at 600 nm, based on the data of anaerobic preparations of Fe^{3+}–adriamycin (Fig. 1). After 2 h the optical absorption spectra appears identical to the reduction of the bound Fe^{3+} to Fe^{2+} (5).

**Fig. 1**

**Fig. 2** Kinetic graph of the appearance and disappearance of the optical absorption band at 600 nm, based on the data in Fig. 1. In the first min the absorption band rapidly appears on Fe^{3+} coordination to adriamycin. Over the next 100 min a gradual decrease in the magnitude of the absorption band occurs. On addition of O_2 at 100 min the 600-nm absorption band rapidly reappears.

**Fig. 3** EPR spectra of an anaerobic preparation of Fe^{3+}-adriamycin, 0.5 mM in Fe^{3+}, 4 mM in adriamycin, pH 7.4. Microwave frequency, 9.302 GHz; temperature, 77 K. All the spectra are shown at identical gain and instrument settings. (Fig. 4A). Both the temperature behavior of the EPR spectrum and the isomer shift values of the Mössbauer spectra indicate that the Fe^{3+} added to adriamycin is bound as high spin Fe^{3+}, \(S = 5/2\) (14).

It has previously been determined from optical absorbance titrations of Fe^{3+} versus adriamycin that the Fe^{3+}-adriamycin complex has a stoichiometry of 1:3 (6). In the presence of an excess of adriamycin the spectra of aerobic Fe^{3+}-adriamycin complexes remain unchanged. The optical, EPR, and Mössbauer spectra of complexes with an Fe^{3+}-adriamycin ratio of 1:10 are unchanged after 6 h under aerobic conditions at room temperature. Under anaerobic conditions, however, the spectra of the Fe^{3+}-adriamycin complexes dramatically change as a function of time. As shown in Figs. 1A and 2 the addition of Fe^{3+} to an anaerobic solution of adriamycin at pH 7.0 results in the appearance of an absorbance at 600 nm that reaches a maximum in 30 s. After the first 3 min, however, the intensity of the absorbance at 600 nm progressively declines over the next 1.5 h, following a monoeponential decay with an apparent rate constant of 0.77 h^{-1} (Figs. 1B and 2). After 2 h the optical absorption spectra appears identical to that of anaerobic preparations of Fe^{3+}-adriamycin (Fig. 5). Introduction of O_2 rapidly restores the 600-nm absorbance to its initial value (Figs. 1C and 2). The EPR spectra of anaerobic complexes of Fe^{3+}-adriamycin also decrease as a function of time (Fig. 3). As reported previously Fe^{3+}-adriamycin is EPR silent, so the decrease in the Fe^{3+}-adriamycin spectra suggest the reduction of the bound Fe^{3+} to Fe^{2+} (5). A sharp signal appears at \(g = 2.004\) and increases as the Fe^{3+} signals decrease (Fig. 3). The signal at \(g = 2.004\) has a line width of only 8 G, and it is superimposed on the much broader 225-G line width Fe^{3+} signal at \(g = 2.01\). The line width and \(g\) value of this signal suggest that it is due to a free radical. This signal...
appears to correspond to the oxidized adriamycin free radical which is formed as adriamycin reduces its bound Fe$^{3+}$ to Fe$^{2+}$. On re-exposure to O$_2$ the Fe$^{3+}$ EPR spectra reappear, and the free radical signal disappears with both of these spectral changes completed within 5 min. Under anaerobic conditions the Mössbauer spectrum of Fe$^{3+}$-adriamycin also decreases as a function of time. After incubating the sample under anaerobic conditions for 3 h one can notice the emergence of an absorption peak in the high velocity range accompanied by a decrease in the intensity of the higher velocity peak of the original doublet (Fig. 4B). Analysis and simulation of this spectrum indicates that the observed change is due to a decrease in the intensity of the original doublet with the formation of a new component which contributes a doublet with an isomer shift $\delta = 1.18 \pm 0.02$ mm/s and quadrupole splitting $\Delta E_Q = 1.82 \pm 0.02$ mm/s. These parameters are characteristic of Fe$^{2+}$, $S = 2$ (14), which indicates that the Fe$^{3+}$ with $S = 5/2$ is reduced to high spin Fe$^{2+}$, $S = 2$. On exposure to O$_2$ the Fe$^{2+}$ doublet decreases with a corresponding reappearance of the original Fe$^{3+}$ doublet (Fig. 4C). After 5 min the reaction is completed and only the Fe$^{3+}$ doublet remains.

In order to chemically confirm the reduction of Fe$^{3+}$ by the drug, we tested the ability of the specific Fe$^{2+}$ chelator 4,7-diphenyl-1,10-phenanthroline to compete with and displace iron from adriamycin after an anaerobic incubation of the ferric chelate of the drug. Because the drug and Fe$^{2+}$-phenanthroline absorb in the same region, we extracted the Fe$^{2+}$ complex of phenanthroline with CHCl$_3$-CH$_3$OH (4:1, v/v). Fig. 6 shows the results of the HPLC analysis of the organic extract obtained after a 1.5-h anaerobic incubation of adriamycin with Fe$^{3+}$.

Because of the reaction with O$_2$ elucidated by the spectroscopic studies, one would expect the reaction of Fe$^{3+}$ with adriamycin to be associated with O$_2$ consumption. Measurement of O$_2$ concentration by polarography did in fact show that addition of Fe$^{3+}$ to adriamycin triggered the consumption of O$_2$. At pH 7.4 and 0.2 mM adriamycin, the addition of 0.5 eq of Fe$^{3+}$ resulted in a rate of 2.4 $\mu$mol/liter/min. In addition, the rate of O$_2$ consumption was a function of adriamycin concentration yielding a linear double reciprocal plot (Fig. 7). As in the case of ferricytochrome c reduction by adriamycin-Fe$^{3+}$-ADP (8), the rate also progressively increased at more basic pH. The effect of pH on the rate of oxygen consumption...
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FIG. 6. Evidence for Fe\(^{2+} \) presence in anaerobic solutions of Fe\(^{3+} \)-adriamycin. Panel A represents the peak of standard Fe\(^{2+} \)-4,7-diphenyl-1,10-phenanthroline in 10 \( \mu \)l of 25 \( \mu \)M solution. Panel B is the peak present in the sample extracted from an anaerobic solution of adriamycin (100 \( \mu \)M) and ferric acetohydroxamate (25 \( \mu \)M) after 1.5 h of reaction at pH 7.4. The apparent rate constant in this experiment was 0.94 h\(^{-1} \). The peak corresponded to a concentration of 27 \( \mu \)M Fe\(^{2+} \). Panels C and D are the peaks present in samples of ferric acetohydroxamate (25 \( \mu \)M) and buffer alone, with peak height which corresponds to 1 and 1.22 \( \mu \)M, respectively, of Fe\(^{2+} \).

FIG. 7. Oxygen uptake as a function of adriamycin concentration. The triangles represent the titration of 200 \( \mu \)M Fe(AHA)\(_3\) with adriamycin. The points represent the mean of two consecutive experiments. Each experiment was performed by adding stock adriamycin to the desired final concentration in barbital buffer (pH 8.5, 0.1 M) equilibrated with air at 37 °C. The rate after addition of the desired amount of Fe(AHA)\(_3\) was recorded. The final volume in the oxygraph cell was 2 ml. The inset shows the same data values grouped as the double reciprocal.

FIG. 8. Effect of pH on the rate of oxygen consumption by adriamycin and the iron-adriamycin complex. The solid triangles represent the results obtained with addition of 200 \( \mu \)M adriamycin. The results shown in the open circles represent the rate obtained after the addition of 200 \( \mu \)M adriamycin and 100 \( \mu \)M Fe(AHA)\(_3\) minus the rate for adriamycin alone at each pH. From pH 7 to 9, the buffer used was barbital-HCl, 0.1 M. From pH 9-12, the buffer used was glycine-NaOH, 0.1 M. The solid lines represent a least squares fit of the data to the Henderson-Hasselbach equation. The apparent pK was 7.95 for the drug-iron complex and 9.65 for adriamycin.

FIG. 9. Effect of BPS on oxygen uptake by Fe\(^{3+} \)-adriamycin (Dox-Fe(III)). The oxygraph tracings were obtained in Tris-HCl buffer (pH 8.5, 50 \( \mu \)M). The concentrations used in the experiment were 200 \( \mu \)M adriamycin, 100 \( \mu \)M Fe(AHA)\(_3\), and 300 \( \mu \)M BPS.
Adriamycin-Fe(II1) was obtained by adding ferric acetohydroxamate, the concentrations used (see legend to Fig. on adriamycin and BPS for Fe3+ rule out the possibility that, at complex was initiated. The known respective affinities of consumption either before or after the reaction with the peroxo-like form is susceptible to nucleophilic displacement prompted an analysis of the enzyme effects shown in Table I. evolution of two enzymes (superoxide dismutase, 120 units/ml; catalase, still be bound to iron but displaceable as either superoxide or for ferric iron. BPS could be due to successful competition with adriamycin (16). In the case of adriamycin peroxo- or peroxo-like form (15). In some systems, O2 coordinated to iron in a superoxo- or peroxo-like form is susceptible to nucleophilic displacement (16). In the case of adriamycin (200 μM), however, the addition of excess of NaN3 (10 mM), NaCN (10 mM), or NaCl (100 mM) with respect to iron did not inhibit O2 consumption by Fe3+-adriamycin. To rule out the possibility that O2 might still be bound to iron but displaceable as either superoxide or H2O2 rather than O2, nucleophile addition was repeated at the same concentrations used above, but now in the presence of superoxide dismutase and catalase. The introduction of the two enzymes (superoxide dismutase, 120 units/ml; catalase, 400 units/ml) into the oxygraph cell resulted in the immediate evolution of O2 that was, however, unrelated to the presence or absence of the nucleophiles. The latter observation prompted an analysis of the enzyme effects shown in Table 1. Catalase caused a 35% drop in O2 consumption. Since catalase converts each 2 eq of H2O2 to 1 eq of O2, this indicates that H2O2 can account for 70% of the O2 consumed. The initial rates of O2 uptake were affected to the same extent by addition of catalase alone or catalase and superoxide dismutase. Superoxide dismutase alone did not appear to have any measurable specific effect, but boiled superoxide dismutase did decrease the rate of O2 consumption. This surprising decrease could be due to the release of copper and zinc ions which interfere with iron-mediated O2 reduction, or the denatured protein itself may bind the drug-iron complex altering the rate of O2 consumption. From the above experiments, it appears that the observed O2 consumption is not due to the formation of a stable adriamycin-iron-O2 complex between O2 and the drug-iron chelate, but to a reaction that yields H2O2 as the predominant product.

The present investigation demonstrates that iron binds to adriamycin and that these complexes cycle to reduce molecular O2. Optical absorption, EPR, and Mössbauer spectroscopic studies as well as Fe3+ chemical extraction experiments all indicate that the Fe3+ bound to adriamycin is reduced to Fe2+. The isomer shift values observed in the Mössbauer spectrum indicate that the Fe2+ formed is high spin S = 2. The EPR experiments demonstrate that an oxidized adriamycin free radical is formed as Fe3+ is reduced to Fe2+. An unusual aspect of this reaction sequence is the slow rate of Fe2+ reduction, as indicated by the apparent rate constant of the spectral shift seen in Fig. 2. This behavior is confirmed by the EPR and Mössbauer experiments (Figs. 3 and 4) that show a similar slow rate for the disappearance of the Fe2+ signals. This observation is unusual because electron transfer reactions are fast processes, as in the case of Fe2+-thiol complexes (17). The rapidity of the subsequent reaction (Fig. 2) after O2 addition suggests that the Fe3+ reduction is the rate-limiting step in the reaction sequence. This is further corroborated by the ability of BPS to immediately stop the O2 uptake by the drug-metal chelate. Perhaps the closest precedent in the chemical literature for these observations is the reaction of 0-quinones and catechols (o-hydroquinones) with iron. The recent report of phenanthrenequinone-phenanthrene catecholate complexes is of particular interest. These complexes can exhibit either strong apparent charge transfer bands without Fe3+ reduction or, in contrast, full reduction of Fe3+ with formation of a radical semiquinone ligand depending upon the dielectric strength of the solvent (18). Parallel detailed studies do not appear to have been done on hydroquinones analogous to adriamycin. However, this study indicates that the Fe3+-adriamycin complex at neutral pH also exhibits both a strong charge transfer band and slow electron transfer from the ligand to the iron.

The second step in this reaction cycle is the reduction of oxygen accompanied by oxidation of the ferrous iron. Our polarographic experiments, Table I, indicate that 70% of the O2 consumed is transformed to H2O2. Recently we have shown that H2O2 reacts with the complex leading to the oxidative cleavage of DNA. The latter suggests the complication that H2O2 is also a transient product able to react with the iron-chelate of adriamycin. Thus, while it is clear that H2O2 is a product, the formation of other reactive O2 species, such as hydroxyl radical, may also occur. As a result of electron transfer to molecular oxygen Fe3+-adriamycin is regenerated, and in the presence of excess adriamycin this cycle of O2 reduction will continue.

In recent years, oxidative damage to macromolecular targets has been advocated as a mechanism responsible for the cytotoxicity and/or the cardiac toxicity of adriamycin (2-4). The cycle of iron-mediated electron transfer from adriamycin to molecular oxygen results in the formation of reduced oxygen and oxidized adriamycin radicals which may mediate the therapeutic and toxic effects of the drug.

### References

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