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ADRIAMYCIN AND ITS IRON(III) AND COPPER(II) COMPLEXES

GLUTATHIONE-INDUCED DISSOCIATION; CYTOCHROME *c* OXIDASE INACTIVATION AND PROTECTION; BINDING TO CARDIOLIPIN

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Abstract—Some reactions of adriamycin (doxorubicin) and its Fe^{3+} and Cu^{2+} complexes were investigated with a view to understanding the mechanisms by which metal ion–adriamycin complexes damage cellular components. The ability of adriamycin in the presence of Cu^{2+} to inactivate the mitochondrial enzyme cytochrome *c* oxidase was effectively prevented by physiologic levels of glutathione. This result is explained by the observation that glutathione reacts with the Cu^{2+}–adriamycin complex to produce free adriamycin. As sulfhydryl compounds are, in contrast, known to promote Fe^{3+}–adriamycin-induced damage to cellular components, these results suggest that the response of a metal ion–adriamycin system to the presence of sulfhydryl compounds may be indicative of whether or not Cu^{2+}–adriamycin is the damaging species. The partition of adriamycin into the octanol phase of an octanol–water two-phase system was greatly enhanced by the presence of cardiolipin. This result can be explained by the formation of a strong adriamycin–cardiolipin complex in the octanol phase which is one-half formed at an adriamycin concentration of 6 μM.

Adriamycin (doxorubicin) has wide clinical application as an antitumour drug. However, its clinical efficiency is limited by its cumulative dose-dependent cardiotoxicity [1]. Several factors may be involved in this cardiotoxicity. Even though the heart takes up relatively less adriamycin than does the liver or the kidney [2], the fact that the heart contains relatively less of the protective enzymes catalase, glutathione peroxidase and superoxide dismutase [3] suggests that free radical oxidative stress on the heart muscle may be responsible. It has been suggested that iron-dependent hydroxyl radical production during metabolism of adriamycin by NADH dehydrogenase is responsible for injury to cardiac mitochondria [4, 5]. Cardiac mitochondria are a prominent site of injury [1] by adriamycin. Both adriamycin [6, 7] and the Fe^{3+}–adriamycin complex [8] have a high affinity for the phospholipid cardiolipin (diphosphatidylglycerol). Cardiolipin is found in the inner mitochondrial membrane where it constitutes some 25% of the total lipid phosphorus [9] and is the most plentiful in the mitochondria-rich heart. Adriamycin is known to cause an oxygen-dependent lipid peroxidation that is dependent upon trace amounts of iron or copper [10–14]. The degradation of cardiolipin in pig heart submitochondrial particles induced by Fe^{3+}–adriamycin is accompanied by the simultaneous inactivation of several respiratory enzymes [12, 13].

The terminal respiratory enzyme bovine heart cytochrome *c* oxidase, which is located in the inner mitochondrial membrane, requires cardiolipin [15] for full activity. The activity of cytochrome *c* oxidase is decreased in the presence of adriamycin both *in vivo* [16] and *in vitro* [7, 12, 13]. While inactivation of cytochrome *c* oxidase is probably not solely responsible for adriamycin's cardiotoxicity, it may be an important factor and, as a reasonably well characterized mitochondrial cardiolipin-dependent enzyme [17], the mechanism of its inactivation warrants further study.

Adriamycin binds strongly to Fe^{3+} [1, 18, 19]

$$Fe^{3+} + 3 \text{ adriamycin} \rightleftharpoons Fe^{3+}(\text{adriamycin})_3$$

with an overall association constant of $10^{33}\,M^{-3}$ and stepwise association constants of $10^{18}\,M^{-1}$, $10^{11}\,M^{-1}$ and $10^{4.4}\,M^{-1}$ [19]. However, a recent report suggests that the value of $10^{33}\,M^{-3}$ has been overestimated as an overall association constant of $10^{28.4}\,M^{-3}$ has also been measured [20]. Cu^{2+} also binds strongly to adriamycin [19, 21] with $Cu^{2+}(\text{adriamycin})_2$ having an overall association constant of $10^{16.7}\,M^{-2}$. These association constants are so large that the presence of Cu^{2+}- and Fe^{3+}–adriamycin complexes forming *in vivo* must be considered. This may occur in spite of the fact that the concentrations of free or weakly bound Fe^{3+} and Cu^{2+} are very low [3] *in vivo*. A recent report has shown that the transfer of Fe^{3+} from transferrin, the strongly binding Fe^{3+} transfer protein, to adriamycin can occur [22]. Postulating a Cu^{2+}–adriamycin species is less problematic as Cu^{2+} is present *in vivo* as a less strongly bound species [1, 3].

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MATERIALS AND METHODS

Adriamycin, as the hydrochloride, admixed with lactose, was obtained from Adria Laboratories. The Fe^{3+}–adriamycin complex was formed by diluting stock $FeCl_3 \cdot 6H_2O$ (99%) dissolved in 1 mM HCl [to prevent Fe(III) hydroxide polymer formation] into a solution of adriamycin dissolved in water and allowing a few minutes for the color to fully develop. Unless indicated, experiments were conducted in pH 7.0 (NaH_2PO_4-Na_2HPO_4) buffer (ionic strength 0.10 M, 48 mM phosphate). To prevent an EDTA-inhibitable adriamycin-induced inactivation of cytochrome *c* oxidase in the absence of any added metal ion, it was found to be necessary to purify the phosphate buffer of adventitious metal ions by slowly passing it down a 1 × 40 cm Dowex 50W cation exchange column. The adriamycin-induced inactivation of cytochrome *c* oxidase activity in submitochondrial particles has been shown [12, 13] to be counteracted by EDTA and greatly enhanced by exogenous Fe^{3+} and Cu^{2+}.

Cytochrome *c* oxidase (Sigma, bovine heart) was dissolved in pH 7.4 phosphate buffer containing 0.4% Tween 80, and typically 5 μl of this stock would be added to the reaction mixture thus giving a final Tween 80 concentration of 0.002% in a final volume of 1.2 ml. The enzyme concentration (typically 0.4 to 2 nM) was determined spectrophotometrically [23]. The substrate ferrocytochrome *c* (Sigma, horse heart) was dithionite reduced and passed down a Sephadex G-25 column (all under N_2) to remove excess dithionite. The cytochrome *c* oxidase catalyzed aerobic oxidation of ferrocytochrome *c* was followed at 550 nm on a Shimadzu UV-260 spectrophotometer. The initial velocities were estimated using the following millimolar extinction coefficients [24]: E (reduced) 27.7 $mM^{-1}\,cm^{-1}$ and ΔE (reduced-oxidized) 18.5 $mM^{-1}\,cm^{-1}$. The percentage activities, calculated from the initial velocities, are always in reference to controls run under identical conditions. Superoxide dismutase (from bovine erythrocytes), catalase (thymol free, from bovine liver), cardiolipin (from bovine heart, as the sodium salt in methanol), reduced glutathione and 2-mercaptoethanol were all obtained from Sigma.

The *n*-octanol-aqueous buffer partition coefficients, P_{oct}, of adriamycin were measured spectrophotometrically in 48 mM, pH 7.1, phosphate buffer. An equal volume of octanol was added to adriamycin in aqueous buffer, and the two-phase mixture was shaken for about 5 min and then centrifuged to fully separate the two phases. The adriamycin concentration in the aqueous buffer phase was estimated from a Beer's Law plot of the extinction at 480 nm and that in the organic phase was obtained by difference.

RESULTS

Partition of adriamycin into octanol-aqueous buffer in the presence and absence of cardiolipin. The *n*-octanol-aqueous buffer partition coefficient, P_{oct}, was observed to decrease with an increase in the total adriamycin concentration. Thus, with initial adriamycin concentrations of 4.9, 24, 70 and 150 μM in the aqueous buffer phase, the measured P_{oct} values were 2.5, 0.93, 0.35 and 0.21 respectively. A similar decrease in P_{oct} has been observed previously [25] with increasing daunomycin concentration. This effect is due to adriamycin self-association in the aqueous buffer phase which lowers the adriamycin monomer concentration in the aqueous phase and consequently that in the organic phase. The measured values of P_{oct} compare to a value of 0.48 measured at pH 7.8 [26].

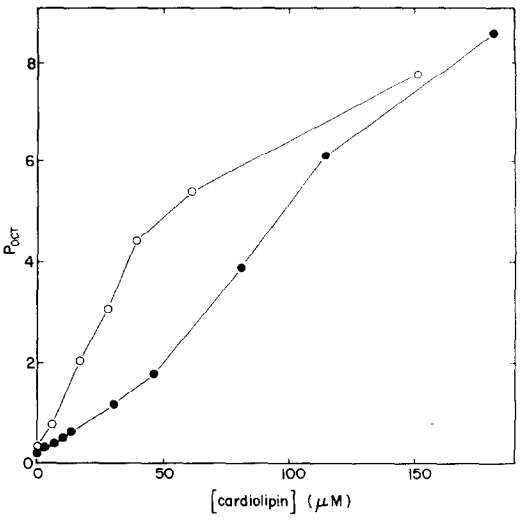

Fig. 1. Partition of adriamycin into an octanol-aqueous buffer (pH 7.1, 48 mM phosphate) two-phase mixture in the presence of added cardiolipin. The total initial adriamycin concentration was held constant at 70 μM (○) and 150 μM (●) respectively. The large increases in the apparent partition coefficient, P_{oct}, are due to formation of a strongly bound complex between adriamycin and cardiolipin in the octanol phase.

Upon the addition of cardiolipin to the octanol phase, the apparent P_{oct} increased up to 40-fold (Fig. 1). The increased uptake of adriamycin into the octanol phase indicates that a strongly bound complex is formed between adriamycin and cardiolipin in the octanol phase. The stoichiometry of the complex was determined by adding small amounts of cardiolipin dissolved in methanol to the octanol phase and measuring the decrease in adriamycin concentration in the aqueous phase. The number of adriamycin molecules that combine with a cardiolipin molecule was thus estimated to be 3.2 ± 0.5 (mean ± SD, N = 5). At the adriamycin concentration of 70 μM (assuming a 3:1 adriamycin:cardiolipin complex), the apparent K_{diss} for the adriamycin–cardiolipin complex was estimated to be about 200 μM^3 in octanol. Thus, the complex would be one-half formed at an adriamycin concentration of 6 μM ($K_{diss}^{1/3}$).

Inactivation of cytochrome c oxidase by Fe^{3+}–adriamycin in the presence of superoxide dismutase, catalase and hydrogen peroxide. The small amount of protection offered by superoxide dismutase (Table 1) may be taken to indicate that superoxide (O_2^-) produced by Fe^{3+}–adriamycin may play some role in the inactivation of cytochrome *c* oxidase. Catalase,

Table 1. Effects of superoxide dismutase and catalase on the Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase*

System	% Activity
Control	100
+Superoxide dismutase (50 units/ml)	100
+Fe^{3+}–adriamycin (10 μM/60 μM)†	76
+Fe^{3+}–adriamycin (10 μM/60 μM) + superoxide dismutase (50 units/ml)	76
+Fe^{3+}–adriamycin (10 μM/60 μM) + superoxide dismutase (120 units/ml)	85
+Fe^{3+}–adriamycin (20 μM/60 μM)	57
+Fe^{3+}–adriamycin (20 μM/60 μM) + superoxide dismutase (120 units/ml)	67
+Fe^{3+}–adriamycin (20 μM/60 μM) + superoxide dismutase (300 units/ml)	67
+Catalase (3000 units/ml)	139
+Fe^{3+}–adriamycin (5 μM/30 μM)	80
+Fe^{3+}–adriamycin (5 μM/30 μM) + catalase (3000 units/ml)	133
+Fe^{3+}–adriamycin (10 μM/30 μM) + catalase (3000 units/ml)	129
+Fe^{3+}–adriamycin (20 μM/60 μM) + glutathione (5 mM)	40

* Experiments were performed in pH 7.0 phosphate buffer (48 mM). Activities were estimated from initial velocities for the aerobic oxidation of ferrocytochrome *c*. The cytochrome *c* oxidase concentration was 2 nM on a heme aa_3 basis. The order of addition was buffer (with or without the protective enzyme); cytochrome *c* oxidase; Fe^{3+}–adriamycin (or not); followed by a 15-min incubation; and then ferrocytochrome *c*. The ferrocytochrome *c* concentration was 20 μM in the superoxide dismutase experiments and 12 μM in the catalase experiments. The addition of Fe^{3+} alone had no effect on the enzyme activity. An activity of 100% corresponds to the oxidation of ferrocytochrome *c* at a rate of 6 μM/min.

† First concentration refers to Fe^{3+}, the second to adriamycin.

however, offered good protection (Table 1) against Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase. Catalase was also observed to reproducibly cause a small increase in the cytochrome *c* oxidase activity (Table 1). The addition of 5 mM glutathione significantly reduced the activity below that due to Fe^{3+}–adriamycin alone.

Since the catalase protection results of Table 1 seemed to suggest that H_2O_2 participated in the Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase, H_2O_2 was added directly to the incubation mixture to test this hypothesis. However, as the results of Table 2 show, up to 250 μM H_2O_2 reproducibly had no effect on the degree of cytochrome *c* oxidase inactivation. Similar results were also obtained at H_2O_2 concentrations of 1 and 10 μM. These results indicate that exogenous H_2O_2 does not promote the Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase. This result may come about because the Fe^{3+}–adriamycin complex itself may produce sufficient H_2O_2 to react in a rate-determining reaction to produce damaging radical species. Hence, the rate of cytochrome *c* oxidase inactivation would be insensitive to exogenous H_2O_2.

Table 2. Effect of hydrogen peroxide on the Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase*

System	% Activity
Control	100
+(catalase)†	110
+Fe^{3+}–adriamycin	38
+Fe^{3+}–adriamycin + H_2O_2 + (catalase)†	53
+Fe^{3+}–adriamycin + (catalase)†	50
−Cytochrome *c* oxidase + Fe^{3+}–adriamycin + H_2O_2 + (catalase)†	0
−Cytochrome *c* oxicase + H_2O_2	(26)‡

* Experiments were performed in pH 7.0 phosphate buffer (48 mM). Activities were estimated from initial velocities for the aerobic oxidation of ferrocytochrome *c* (14 μM). The cytochrome *c* oxidase concentration was 0.4 nM. An activity of 100% corresponds to the oxidation of ferrocytochrome *c* at a rate of 1 μM/min.

† Since H_2O_2 can itself oxidize ferrocytochrome *c* at an appreciable rate under these conditions, a double incubation was carried out. The first incubation of 10 min was carried out in the presence of all the components indicated except catalase. A second subsequent incubation (indicated by the brackets) of 10 min was carried out after the addition of catalase (300 units/ml) to remove any remaining H_2O_2. This was effective in removing any excess H_2O_2 as shown by the data on the last two lines which was obtained in the absence of any enzyme. The Fe^{3+}–adriamycin concentration was 20 μM Fe^{3+} and 60 μM adriamycin, and the H_2O_2 concentration was 250 μM throughout.

‡ Apparent activity due to residual H_2O_2 in the absence of any enzyme. No second catalase incubation was carried out here.

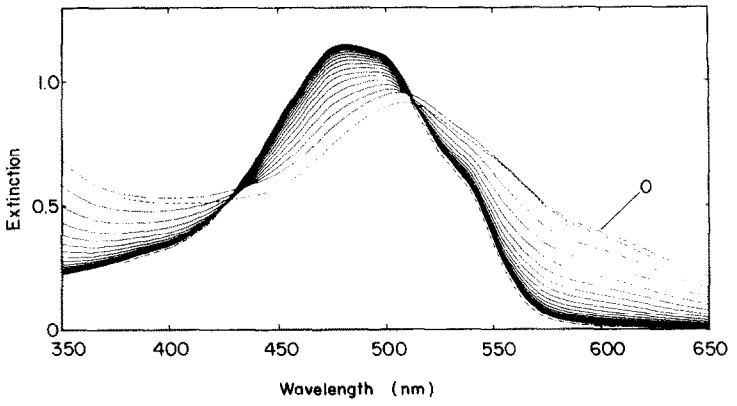

Fig. 2. Cu^{2+}–adriamycin spectrum (----) and the spectral changes that accompany the addition of 1.9 mM glutathione. Reaction conditions: 50 μM Cu^{2+}, 100 μM adriamycin in pH 7.0 (48 mM) phosphate buffer at 25°. The spectrum with the (----) line is that of the Cu^{2+}(adriamycin)$_2$ complex (λ_{max} 512 nm [21]). Upon the addition of 1.9 mM glutathione to the Cu^{2+}(adriamycin)$_2$ solution, slow spectral changes (——) that evolved towards a free adriamycin spectrum were seen. The first spectrum (0) was swept just after the addition of glutathione, and subsequent spectra were recorded at 4.0-min intervals thereafter. The spectrum with the (·—·) line is a reference spectrum at the same concentration of adriamycin and glutathione as above but in the absence of any added Cu^{2+}.

Fe^{3+}- and Cu^{2+}-adriamycin complexes. Copper is present intracellularly and in plasma as more weakly bound species than is iron [1, 3]. Cu^{2+} also forms very stable complexes with adriamycin [19, 21] that could be important in both the antitumor and cardiotoxic effects of adriamycin [1]. Since free Cu^{2+} is known to react with glutathione [27], it was decided to look at the effect of physiologic levels of glutathione on the Cu^{2+}–adriamycin complex and also the ability of glutathione to protect against the inactivation of cytochrome *c* oxidase.

The addition of $CuSO_4$ to buffered adriamycin solutions (Fig. 2) resulted in rapid (less than 5 sec) spectral changes corresponding to formation of a Cu^{2+}–adriamycin complex [21], and this was followed by slower small spectral changes that were complete after 5 min. Based on the spectra (λ_{max} 512 nm) and the reaction conditions, it was concluded that the Cu^{2+}(adriamycin)$_2$ species was formed [21]. Over the time scales of Figs. 2 and 3, there was no observable dissociation of Cu^{2+}–adriamycin. However, after several hours a brick red precipitate formed, indicating possible aggregate formation. Upon the addition of glutathione to fresh Cu^{2+}–adriamycin solutions, there was a small fast initial spectral change followed by a second slower process exhibiting two isosbestic points at 430 and 512 nm (Fig. 2). The spectrum that evolved was close to that of free adriamycin (Fig. 2). Thus, in the presence of glutathione, the Cu^{2+}–adriamycin complex dissociates to give free adriamycin. Shown in Fig. 3 is the time course of the dissociation of the Cu^{2+}–adriamycin complex in the presence of each of glutathione, 2-mercaptoethanol and EDTA. The initial rate for the formation of free adriamycin was almost the same in the presence of 0.02, 0.05, 0.5, 1.0, 1.9 and 5.5 mM glutathione (4.7, 4.2, 4.8, 4.6 and 5.1 μM min^{-1} respectively). Mercaptoethanol and EDTA, likewise, were observed to cause a similar slow dissociation of the Cu^{2+}–adriamycin complex. The initial rates in the presence of 1.9 mM mercaptoethanol (3.8 μM min^{-1}) and 2 mM EDTA (2.4 μM min^{-1}) were smaller than those observed for glutathione. Glutathione, mercaptoethanol, and EDTA probably react with free Cu^{2+} produced by

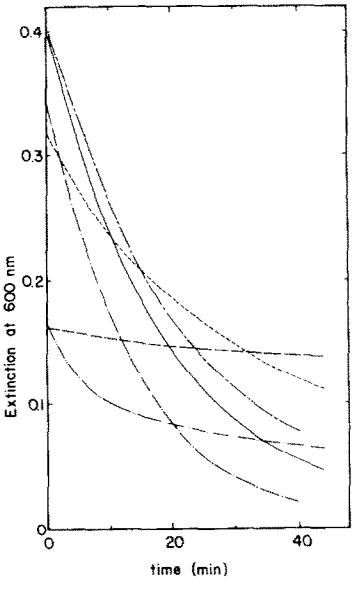

Fig. 3. Change in extinction at 600 nm with time upon the addition of 1.9 mM glutathione (——); 5.5 mM glutathione (—·—); 1.9 mM mercaptoethanol (—··—); and 2 mM EDTA (----) to 100 μM (adriamycin basis) Cu^{2+}–adriamycin complex (Cu^{2+} : adriamycin ratio, 1 : 2). Also shown are the changes in extinction at 600 nm upon the addition of 2 mM EDTA (—·—) and 1.9 mM glutathione (— —) to 100 μM (adriamycin basis) Fe^{3+}–adriamycin complex (Fe^{3+} : adriamycin ratio 1 : 3). The small spectral changes observed upon the addition of glutathione to the Fe^{3+}–adriamycin complex showed no isosbestic points and were generally not consistent with any significant dissociation of Fe^{3+}–adriamycin. Reaction conditions: pH 7.0 (48 mM) phosphate buffer, 25.0°.

Table 3. Effect of glutathione on the Cu^{2+}–adriamycin-induced inactivation of cytochrome *c* oxidase*

[Adriamycin] (μM)	[Cu^{2+}] (μM)	[Glutathione] (μM)	% Activity
0	0	0	100
0	0	2000	100
0	2.8	0	52
0	5.6	0	48
0	2.8	2000	100
50	5.6	0	13
50	2.8	0	38
50	2.8	2000	93
50	2.8	1000	100
50	2.8	500	100
50	2.8	50	94
50	2.8	1	55
50	0	0	100

* Experiments were performed in pH 7.0 phosphate buffer (48 mM). The order of addition was: buffer, Cu^{2+}, glutathione, enzyme and adriamycin which was then followed by a 10-min incubation. The cytochrome *c* oxidase concentration was 0.4 nM. Ferrocytochrome *c* (10 μM) was then added, and its oxidation was followed at 550 nm. An activity of 100% corresponds to the oxidation of ferrocytochrome *c* at a rate of 1 μM/min.

a rate-limiting dissociation of adriamycin from the Cu^{2+}–adriamycin complex or its aggregate. Glutathione is known to reduce Cu^{2+} to Cu^{+} [27] and insoluble colorless polymeric Cu^{+}–sulfhydryl complexes are known to be readily formed by the reduction of Cu^{2+} by sulfhydryl compounds [28]. Thus, these results indicate that Cu^{2+}–adriamycin would not be stable in the presence of glutathione under cellular conditions.

The results of Table 3 indicate that Cu^{2+}–adriamycin greatly enhances the amount of inactivation of cytochrome *c* oxidase over that shown by Cu^{2+} alone. Physiologic levels of glutathione were very effective in protecting cytochrome *c* oxidase from inactivation by Cu^{2+}–adriamycin. The glutathione thus prevents inactivation of cytochrome *c* oxidase by removing free Cu^{2+} from solution before it can complex with adriamycin. Glutathione may also be acting in its capacity as a radical scavenger [3] as well.

DISCUSSION

The increased partition of adriamycin into the lipophilic phase is explained by the formation of a strong complex between cardiolipin and adriamycin in the octanol phase. The high affinity of adriamycin [6, 7, 29] and Fe^{3+}–adriamycin [8] for cardiolipin may result in adriamycin or Fe^{3+}–adriamycin concentrating in or binding to membranes that contain cardiolipin, thus making these membranes and the components they enclose particularly susceptible to adriamycin-induced damage. The inner mitochondrial membrane is particularly rich in cardiolipin [9], and thus the mitochondria may be a target for adriamycin-induced damage. The membrane-bound mitochondrial enzymes that require the presence of cardiolipin for full activity [7, 15, 17, 30] may be damaged by Fe^{3+}–adriamycin or adriamycin that has associated to the cardiolipin bound to the enzyme. A bound Fe^{3+}–adriamycin complex would be located where it could damage the enzyme *in situ* through formation of reactive oxy radicals. Evidence for the role of cardiolipin in the Fe^{3+}–adriamycin-induced damage arises from the observation that the loss of cytochrome *c* oxidase activity in heart submitochondrial particles is accompanied by a simultaneous loss of cardiolipin from the particle and that this inactivation was demonstrated to be reversible upon cholate solubilization of the particles [12, 13].

There are mixed reports on the ability of both catalase and superoxide dismutase to offer protection against various types of adriamycin and Fe^{3+}–adriamycin-induced damage [4, 5, 10, 11, 13, 18, 31, 32]. Both catalase and superoxide dismutase (but to a lesser extent) offer protection against the Fe^{3+}–adriamycin-induced destruction of erythrocyte ghost membranes [18]. Catalase (but not superoxide dismutase) has also been observed to inhibit completely Fe^{3+}–adriamycin-dependent damage to deoxyribose [11] as well as reduce oxygen uptake by the Fe^{3+}–adriamycin complex [31]. Catalase was also observed to inhibit the iron-dependent adriamycin-induced production of methane from dimethyl sulfoxide by heart submitochondrial particles; but superoxide dismutase was only partly effective in inhibiting methane production [4, 5].

There are also mixed reports on the ability of exogenous H_2O_2 to promote adriamycin-induced damage to cellular components [33–35]. Hydrogen peroxide has been observed to increase effectively the adriamycin-stimulated methane production from dimethyl sulfoxide by heart submitochondrial particles [4, 5]. The Fe^{3+}–adriamycin-induced oxidative cleavage of DNA has been shown to be greatly stimulated by micromolar concentrations of exogenous H_2O_2 [33, 34]. On the other hand, deoxyribose degradation by adriamycin was only very slightly enhanced in air by the presence of 180 μM H_2O_2 [35].

Some of the reactions that may lead to adriamycin (ADM) induced damage are:

$$S_{red} + ADM \rightarrow ADM^{\cdot} + S_{ox}$$

semiquinone radical formation (1)

$$ADM^{\cdot} + O_2 \rightleftharpoons ADM + O_2^{\cdot}$$

superoxide formation (2)

$$\left.\begin{aligned} &ADM^{\cdot} + Fe^{3+}\text{–}ADM \rightarrow Fe^{2+}\text{–}ADM + ADM \\ &O_2^{\cdot} + Fe^{3+}\text{–}ADM \rightarrow Fe^{2+}\text{–}ADM + O_2 \end{aligned}\right\} Fe^{3+} \text{ reduction} \quad (3)$$

$$2O_2^{\cdot} + 2H^{+} + O_2 \rightarrow H_2O_2 + O_2$$

hydrogen peroxide formation (5)

$$Fe^{2+}\text{–}ADM + H_2O_2 \rightarrow Fe^{3+}\text{–}ADM + OH^{\cdot} + OH^{-}$$

hydroxyl radical formation (6)

Semiquinone free radical formation (reaction 1) may occur enzymatically by reaction with some reducing

species, S_{red} [1, 4, 5], or it may occur by adriamycin self-reduction [11]. Reaction 2 is fast with $k_{forward} = 3 \times 10^8\ M^{-1}\ sec^{-1}$ and $k_{reverse} = 0.4 \times 10^8\ M^{-1}\ sec^{-1}$ [36]. Under aerobic conditions the equilibrium of reaction 2 would lie far to the right. Though an upper limit of $10^5\ M^{-1}\ sec^{-1}$ has been placed on reaction 3 by analogy with other Fe^{3+} complexes [37], Fe^{3+} reduction may occur through reaction 3 or through 4 [37] as O_2^- is an effective reducing agent [3]. It has also been shown [31] that Fe^{3+} reduction occurs with the formation of an oxidized adriamycin radical and it was also suggested that the Fe^{2+} chelate may react with O_2 to yield O_2^- or H_2O_2. Fe^{2+}–ADM may be involved in production of reactive hydroxyl radicals through a Fenton-type reaction 6 [3–5]. The H_2O_2 required in reaction 6 could be provided by reaction 5 which is fast ($5 \times 10^5\ M^{-1}\ sec^{-1}$) at pH 7.0 [3].

Glutathione has been variously observed to both increase and decrease damage to cellular components induced by adriamycin. Our results suggest a rationale for these observations [1, 4, 5, 14, 18, 33, 38–42]. The metal-ion dependent adriamycin-induced lipid peroxidation of the liver mitochondrial membrane [14] and of the nuclear membrane [39] has been observed to be effectively inhibited by the presence of glutathione. On the other hand, glutathione in the presence of Fe^{3+}–adriamycin caused increased inactivation of cytochrome *c* oxidase (Table 1) and is also known to cause efficient destruction of erthrocyte ghost membranes [18]; to catalyze the reduction of oxygen [18]; to cause the cleavage of DNA [33]; and to cause lipid peroxidation in liver microsomes [40]. It has also been shown that when adriamycin is added to isolated hepatocytes [41] inactivation of the glutathione peroxidase protection system results in a decrease in cell viability and an increase in lipid peroxidation. However, high concentrations of glutathione (15 mM) have no effect on the Fe^{3+}–adriamycin-induced complement susceptibility of human melanoma cells [43]. The sulfhydryl compounds *N*-acetylcysteine and cysteamine have been shown in mice to increase significantly the survival time after acute adriamycin administration without interfering with adriamycin's antitumor activity [42] towards Ehrlich ascites tumor cells either *in vivo* or *in vitro*. These results and the glutathione results taken together suggest that where exogenous sulfhydryl compounds protect against the damaging effects of adriamycin it is the Cu^{2+}–adriamycin species that is responsible, but where sulfhydryl compounds increase the damaging effects of adriamycin it is the Fe^{3+}–adriamycin that is responsible. If this is so, then these results may thus provide the basis for an indicator of which metal ion–adriamycin species is the one responsible for adriamycin's metal-ion induced cytotoxicity. This conclusion is also consistent with the *in vitro* cytotoxic effects of adriamycin on human breast cancer cells [38] in which it was found that *N*-acetylcysteine increases the cytotoxicity of adriamycin. In this case the presence of reactive iron-dependent adriamycin species was inferred by the ability of membrane-permeable iron chelating agents to reduce the cytotoxic effects of adriamycin. Knowing whether a particular cell type, whether normal or cancerous, is further damaged as the result of formation of either endogenous copper– or iron–adriamycin complexes may possibly provide a basis for protecting that cell type or speeding its destruction by the selective removal or addition of these metal ions.

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