Cancer Chemother Pharmacol (2000) 46: 449–458

ORIGINAL ARTICLE

Avner Ramu · Michelle M. Mehta · Jasper Liu
Iva Turyan · Aleksandar Aleksic

The riboflavin-mediated photooxidation of doxorubicin

Received: 3 March 2000 / Accepted: 20 June 2000

Abstract *Purpose*: Previously, it was shown that exposing doxorubicin (ADR) to 365 nm light resulted in the loss of its cytotoxic activity as well as its absorbance at 480 nm. These processes were much enhanced when mediated by riboflavin. In the present study we investigated the quantitative and qualitative aspects of riboflavin-mediated photodegradation of ADR. *Methods*: ADR solutions containing variable concentrations of riboflavin and other agents were exposed to 365 nm light for variable time periods and then the absorbance spectrum of ADR was measured by a double beam spectrophotometer. These measurements were used to calculate the half-time of the ADR degradation process. The degraded ADR solutions were analyzed by chromatography and mass spectrometry. *Results*: Analysis of the riboflavin effect indicated that a maximal rate of photolytic degradation of ADR was obtained only after most of the ADR molecules had formed bimolecular complexes with riboflavin. The retardation of lumichrome formation by ADR and the inhibition of ADR bleaching by excess of ascorbic acid suggested that ADR was degraded by a photooxidation process. Similar spectral changes occurred when ADR was exposed to strong oxidizers such as sodium hypochlorite and dipotassium hexachloroiridate. Cyclic voltammetry revealed that the oxidation-reduction process of ADR was not electrochemically reversible and therefore the oxidation potential could not be determined accurately; however its value should be between 0.23 and 0.78 V. Analysis of the photooxidative process revealed that it was not mediated by the formation of singlet oxygen, superoxide anion radicals, hydrogen peroxide or hydroxyl radicals, and it is suggested that ADR was oxidized directly by the excited triplet riboflavin. The mass spectrograms and the HPLC chromatograms of photooxidized ADR indicate that the central ring of ADR was opened and that 3-methoxysalicylic acid was produced by this cleavage. *Conclusions*: The riboflavin-mediated photodegradation of ADR is an oxidative process resulting in the cleavage of the anthraquinone moiety. 3-Methoxysalicylic acid was identified as one of the resulting fragments. It is possible that some of the large fractions of the ADR metabolites that are non-fluorescent are the result of an in vivo oxidation of ADR and that 3-methoxysalicylic acid may play a role in the different biological activities of ADR.

Key words Doxorubicin · Riboflavin · Photooxidation · 3-Methoxysalicylic acid

A. Ramu (✉) · M. M. Mehta · J. Liu · A. Aleksic
Texas Children's Cancer Center, Texas Children's Hospital, Baylor College of Medicine, 6621 Fannin Street, MC 3-3320, Houston, Texas 77030-2399, USA
e-mail: aramu@bcm.tmc.edu
Tel.: +1-713-7704262; Fax: +1-713-7704107

I. Turyan
Department of Inorganic and Analytical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Introduction

It has previously been reported that irradiating doxorubicin (ADR) with fluorescent or 365 nm (UVA) light results in the reduction of its cytostatic activity against sarcoma 180 cells, the loss of the drug's fluorescence, and of the drug's absorbance in the 400 to 500 nm range [8, 26, 28]. However, neither the changes in chemical structure of ADR nor the mechanism of its photoinactivation were reported in these studies. We have shown recently that when ADR is dissolved in RPMI medium 1640, rather than in phosphate-buffered saline (PBS), the effects of UVA light on the rates of the decrease of the ADR cytostatic activity against P388 murine leukemia cells and on the decrease of the drug's absorbance peak at 480 nm are greatly increased [4]. In that study it was also shown that the major photosensitizing compound in the RPMI 1640 cell-growth medium was riboflavin. The purpose of the present study is to investigate the mechanisms of the riboflavin-mediated photolytic degradation of ADR.

Materials and methods

Chemicals and reagents

Riboflavin, ascorbic acid, sodium benzoate, *tert*-butanol, chloroform, ethyl acetate, sodium hypochlorite, dipotassium hexachloroiridate, deuterium oxide, L-histidine, 1,4-diazabicyclo[2.2.2] octane (DABCO), 1,4-phenylenediamine, anisole, 2-methoxybenzoic acid, 3-methoxybenzoic acid, 3-methoxysalicylic acid, 6-methoxysalicylic acid, cytochrome C, superoxide dismutase (5800 units/mg protein, SOD) and catalase (2200 units/mg protein) were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Dulbecco's phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride was purchased from GibcoBRL (Gaithersburg, Md., USA). Doxorubicin HCl (adriamycin PFS, ADR) was purchased from Pharmacia (Kalamazoo, Mich., USA).

Long ultraviolet light exposure and spectral measurements

ADR and other reagents at concentrations specified in the results section were dissolved in 3 ml PBS solution, placed in 35-mm open cell culture dishes and irradiated in a laminar flow hood with three Blacklight Blue 40-W lamps (Vilber Lourmat, Marne la Vallee, France). The energy flow rate delivered to the solutions, measured with a Cole-Parmer 97503-00 radiometer (Niles, Ill., USA) with a 365 nm sensor, was 4–6 mW/cm^2. The hood air flow was found to be sufficient to prevent warming of the irradiated solutions throughout the duration of the experiments. After the irradiation the 250–800 nm absorbance spectra were measured in a double beam UV-VIS scanning spectrometer (Shimadzu Scientific Instruments, Columbia, Md., USA) The time-dependent decrease in ADR absorbance at 480 nm was used to calculate the half-time of this decrease. In repeated experiments, the standard deviation of this parameter was consistently $<17\%$ of the mean value.

Cyclic voltammetry

Glassy carbon (GC) electrodes were prepared from a GC rod (3 mm diameter, VC-386, Atomergic Chemetals, Farmingdale, N.Y., USA) that was embedded in a Teflon tube and polished first with emery paper (down to 600 grit) followed by alumina slurry (down to 0.05 micrometer). The electrodes were washed with clean water prior to use. A Pt wire served as an auxiliary electrode while an Ag/AgCl electrode was used as a reference electrode. A typical three-electrode cell was employed that was purged with nitrogen for at least 5 min after introducing the solution and before carrying out the measurement.

A BAS 100B (Bioanalytical System, West Lafayette, Ind., USA) electrochemical analyzer was used for all electrochemical experiments.

Chromatography

PBS solutions containing ADR and/or other reagents before or after irradiation with UVA light were acidified with HCl to pH 1 and then extracted twice with two volumes of ethyl acetate. The ethyl acetate phase was evaporated to dryness under a nitrogen stream at 40 °C. The extracts were then redissolved in the mobile phase (methanol/5% acetic acid in water 2.7:7.3 v/v) and injected in a volume of 20 µl into the HPLC system consisting of: Waters 600E multisolvent delivery system, Waters 717 plus autosampler, and Waters 996 photodiode array detector. The guard column was Nova-Pak C18, 4 µM, 3.9 × 20 mm and the separation column was Nova-Pak C18, 4 µM, 3.9 × 150 mm (Waters, Milford, Mass., USA). Isocratic elution was performed at a flow of 1 ml/min. Chromatographic software, Millenium Chromatography Manager, Vers. 3.15.01 (Waters; Milford, Mass., USA), was used for acquisition and processing data. Similarly, anisole, 2-methoxy- and 3-methoxybenzoic acid, 3-methoxy- and 6-methoxysalicylic acid dissolved in mobile phase were also injected into the system as standards.

Fluorescence measurements

Salicylic acid has been reported to exhibit fluorescence with excitation and emission maxima at 310 and 420 nm, respectively [23]. We found that 3-methoxysalicylic acid dissolved in methanol had similar characteristics but the maximal excitation was obtained at 303 nm (data not shown). PBS solutions containing 20 µM ADR, 20 µM riboflavin, or both before or after irradiation with UVA light, were acidified with HCl to pH 1 and then extracted twice with two volumes of ethyl acetate. The ethyl acetate phase was evaporated to dryness under a nitrogen stream at 40 °C. The extracts were then redissolved in methanol. Fluorescence measurements were carried in a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Instruments, Tokyo, Japan).

Results

We have previously shown that in the presence of 20 µM riboflavin, UVA light illumination of ADR dissolved in PBS results in a much faster decrease in the ADR absorbance peak at 480 nm than that observed in the absence of riboflavin (see Fig. 5 in [4]). In an effort to define the quantitative aspects of the photosensitizing activity of riboflavin, the experiment was repeated with different concentrations of ADR and riboflavin. When the concentration of ADR was maintained at 20 µM, the exponential decrease in the drug's absorbance at 480 nm (expressed as $T_{1/2}$) was a function of the riboflavin concentration. However, as shown in Fig. 1, the enhancement is approaching its maximum as the riboflavin/ADR concentration ratio gets closer to a 1:1 molar ratio. Similar results were obtained with ADR

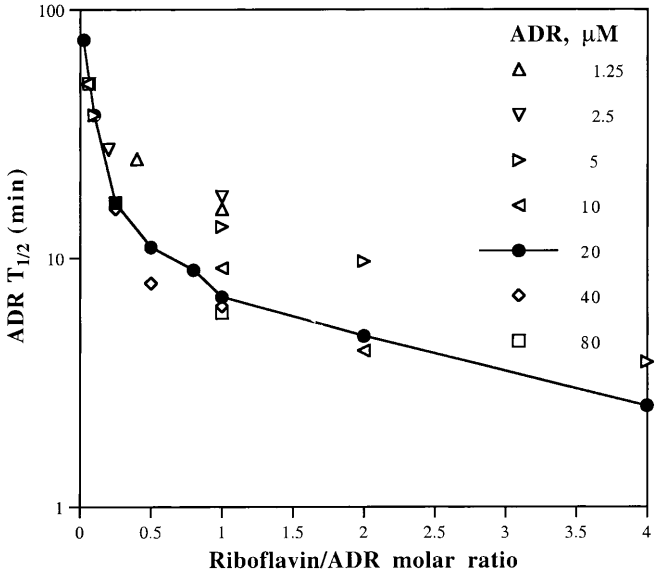


Fig. 1 The effect of UVA irradiation (5 mW/cm^2) of doxorubicin with riboflavin at variable concentrations in PBS (pH 7.2) on the rate of decrease in the doxorubicin absorbance at 480 nm (expressed as $T_{1/2}$)

concentrations between 1.25 and 80 μM, but the results obtained revealed an additional effect. Even at 1:1 molar concentration ratio, the rate of decrease in the ADR absorbance at 480 nm was increased by higher concentrations of ADR (and riboflavin). This concentration effect approached it maximum at 20 μM of ADR and riboflavin.

In the absence of another oxidizable substrate, light-excited riboflavin oxidizes another riboflavin molecule which becomes lumichrome [21]. We have studied whether this process might be affected by the presence of ADR. After UVA illumination of 20 μM riboflavin with or without 20 μM ADR in PBS solutions, chloroform extractions were carried out as lumichrome, unlike riboflavin, is soluble in this solvent. As shown in Fig. 2, the initial rate of lumichrome formation (measured by the increase in 350 nm absorbance) in riboflavin solution that also contained ADR was smaller than that occurring in a solution without ADR. However, after the disappearance of ADR (measured by the decrease in 480 nm absorbance), lumichrome production became much faster. We have previously reported that the decrease in absorbance at 480 nm of UVA-illuminated ADR, dissolved in RPMI medium 1640, was blocked by the presence of ascorbic acid (see Fig. 6 in [4]). A similar result was obtained when ascorbic acid was added prior to illumination to the solution of ADR and riboflavin in PBS. Ascorbic acid also inhibited the formation of lumichrome from light-excited riboflavin (data not shown).

The studies described above suggest that the decrease in the absorbance peak at 480 nm resulted from oxidation of ADR by light-excited riboflavin. Therefore, it was assumed that similar changes in the absorbance of ADR might occur if ADR was exposed to other oxidizers. As shown in Fig. 3, such changes in the absorbance of ADR indeed occurred when it was exposed to sodium hypochlorite. Similar spectral changes also occurred after exposing 20 μM ADR in a PBS solution to dipotassium hexachloroiridate. The absorbance of ADR at 480 nm decreased gradually as the concentration of hexachloroiridate was increased up to 80 μM, at which point it was completely eliminated (data not shown).

To measure the oxidation potential of ADR, cyclic voltammetry (CV) and square wave voltammetry (SWV) examinations of ADR were undertaken. The CV of 0.45 mM ADR on a GC electrode in 0.1 M NaCl and 0.1 M phosphate buffer pH 7 (scan rate 1 $V \cdot s^{-1}$) shows a distinct oxidation wave at 0.78 V vs. Ag/AgCl (Fig. 4). A reduction wave at 0.23 V can be seen on the reverse scan. The oxidation-reduction process was not electrochemically reversible as was evident from the large peak

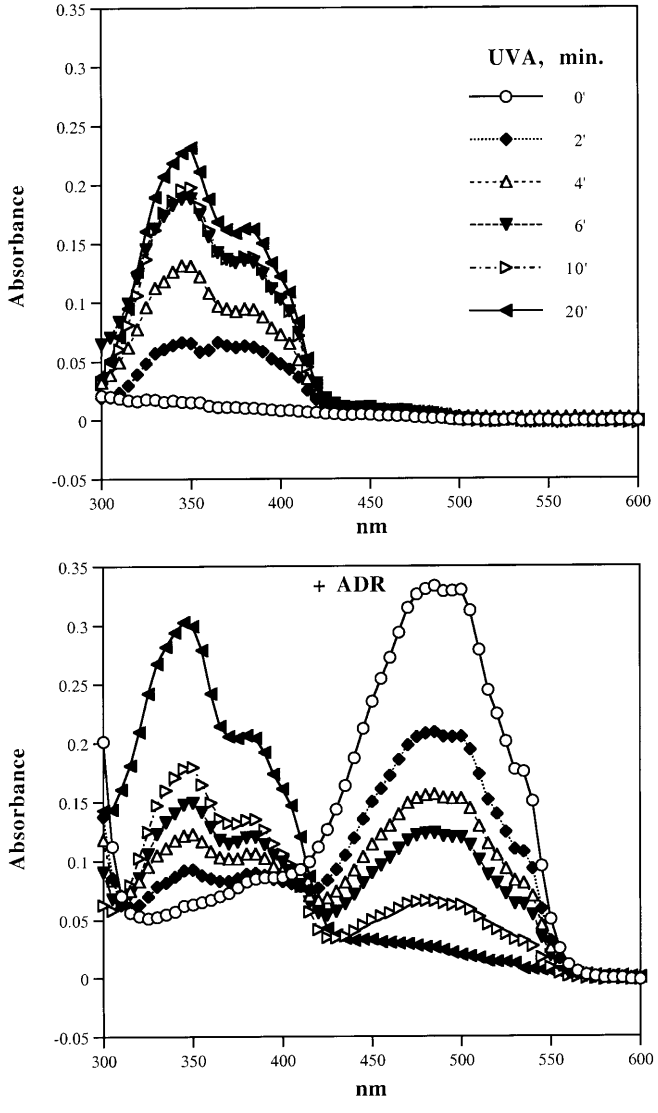


Fig. 2 The effect of UVA irradiation (5 mW/cm^2) of 20 μM riboflavin with or without 20 μM doxorubicin in PBS (pH 7.2) on the absorbance spectrum of the chloroform extract carried out after the irradiation (chloroform/PBS 2:5 v/v)

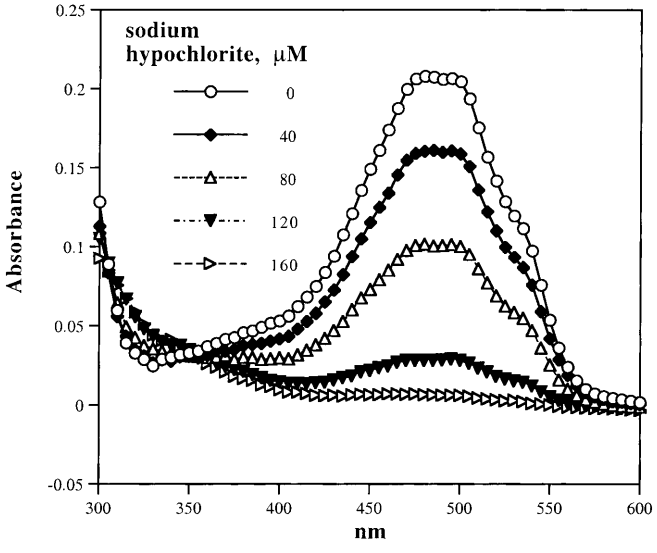


Fig. 3 The effect of sodium hypochlorite on the absorbance spectrum of 20 μM doxorubicin in PBS (pH 8)

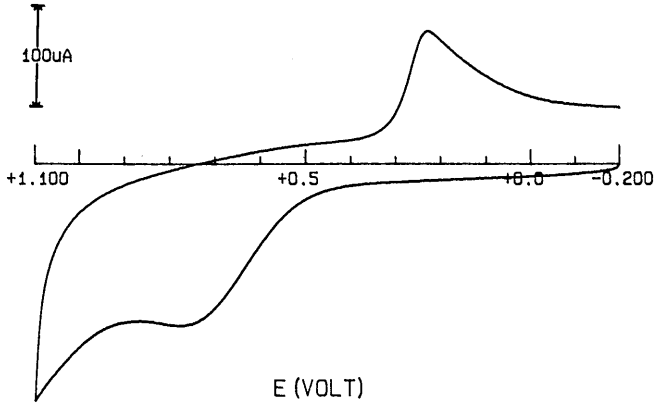


Fig. 4 Cyclic voltammogram of 0.45 mM doxorubicin in phosphate buffer with 0.1 M NaCl (pH 7)

potential separation. The fact that the current of both waves continuously decreased in each cycle when multiple scans were carried out suggested that the electrode had been contaminated by the oxidation product of ADR (data not shown). To better understand the mechanism involved in the electrochemical oxidation of ADR we studied the effect of the scan rate on the anodic and cathodic waves. We found that the peak current of the oxidation wave varied linearly as a function of the square root of the scan rate, as is expected for a diffusion-controlled process. On the other hand, the reduction peak current was linearly dependent on the scan rate, indicating a surface-confined process. This suggested that the oxidation product of ADR was strongly adsorbed onto the GC electrode. For these reasons the ADR oxidation potential could not be determined accurately; however, its value should be between 0.23 and 0.78 V.

Photosensitized oxidation proceeds via type I or type II reactions. We first studied whether the oxidation of ADR by light-excited riboflavin is carried out via a type II reaction. It has been reported that the spontaneous decay of $^1O_2$ in D_2O is at least tenfold slower than in H_2O [14]. It was therefore expected that if the oxidation of ADR occurred via a type II reaction, the UVA irradiation of ADR with riboflavin in PBS solution made from D_2O would result in a greater rate of decrease in ADR absorbance at 480 nm than that observed in PBS solution made from H_2O. However, as shown in Fig. 5, replacing H_2O with D_2O did not result in faster bleaching of ADR. It has also been reported that $^1O_2$ can be efficiently quenched by histidine or by diazabicyclo[2.2.2]octane (DABCO) [7]. It was therefore expected that if $^1O_2$ mediated the light-excited riboflavin bleaching of ADR, the presence of these compounds would block the decrease in ADR absorbance at 480 nm. However, as shown in Fig. 5, these agents did not block the bleaching of ADR by the light-excited riboflavin. In fact they enhanced the rate of ADR bleaching. In the absence of riboflavin or with riboflavin but no UVA light irradiation, these agents did not affect the absorbance spectrum of ADR (data not shown).

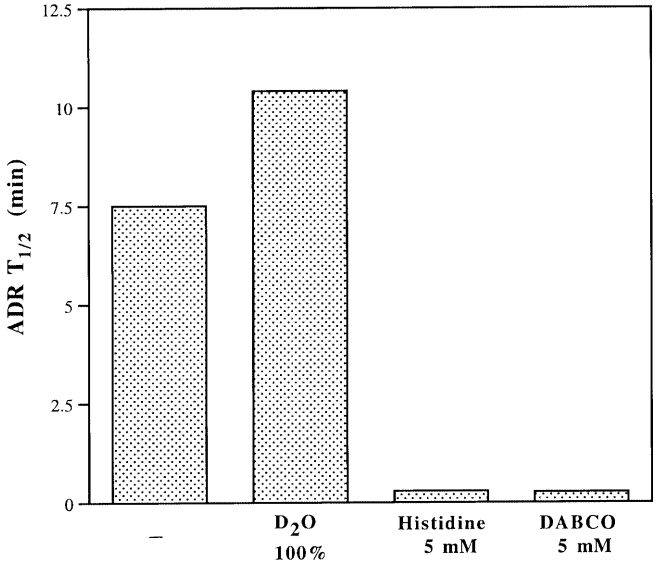


Fig. 5 The effects of deuterated water, histidine or DABCO on the spectral changes of doxorubicin (20 μM) dissolved in PBS (pH 7) with riboflavin (20 μM) under UVA irradiation (4–5 mW/cm^2). The rate of decrease in doxorubicin absorbance at 480 nm is expressed as $T_{1/2}$

Taken together the data did exclude the possibility that the bleaching of ADR by light-excited riboflavin is mediated via a type II reaction.

We then studied whether the oxidation of ADR by light-excited riboflavin is carried out via a type I reaction. As this reaction also leads to the formation of superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, ADR could have been oxidized directly by the excited triplet riboflavin or by one of the reactive oxygen species formed in this system. However, as shown in Fig. 6, since sodium benzoate and *tert*-butanol failed to block the bleaching of ADR by light-excited riboflavin, it was concluded that the hydroxyl radicals did not mediate the oxidation of ADR. The bleaching of ADR in this system was also not mediated by hydrogen peroxide, as it could not be blocked by the addition of catalase. The addition of 300 units/ml of superoxide dismutase accelerated the bleaching of ADR ($T_{1/2}$ 2.05 min). It therefore seemed that the formation of superoxide anion radicals not only did not mediate the oxidation of ADR but also was blocking it. Reducing the ability of the system to form superoxide anion radicals, by purging the oxygen from the ADR-riboflavin solution prior to illumination, also enhanced the rate of ADR bleaching ($T_{1/2}$ 2.87 min).

On the other hand, enhancing the formation of superoxide anion radicals by light-excited 1,4-phenylenediamine [22] considerably reduced the rate of ADR bleaching ($T_{1/2}$ 33.5 min) and this inhibition was partially reversed by superoxide dismutase. These results further supported the conclusion that the formation of superoxide anion radicals (formed by the transfer of an electron from the riboflavin anion radical to dissolved dioxygen) blocked the oxidation of ADR. As shown in

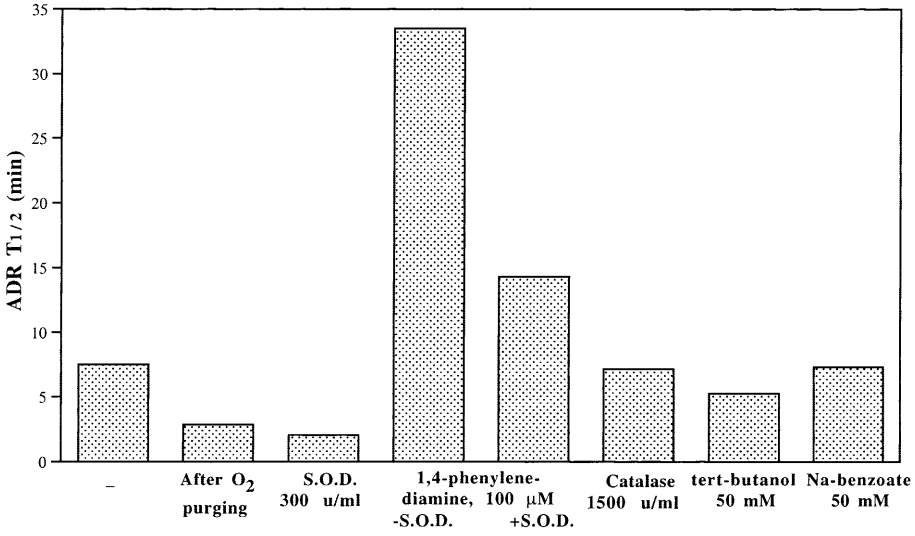


Fig. 6 The effects of various agents on the rate of decrease in doxorubicin absorbance at 480 nm (expressed as $T_{1/2}$) resulting from UVA irradiation (4–5 mW/cm^2) of a PBS solution containing 20 μM doxorubicin and 20 μM riboflavin

Fig. 5, the presence of DABCO (5 mM) also enhanced the bleaching of ADR by light-excited riboflavin ($T_{1/2}$ 0.26 min). One possible explanation is that DABCO might have prevented the effects of superoxide anion radicals in this system. When the production of superoxide anion radicals by light-excited riboflavin was measured by the increase in the absorbance of cytochrome C at 550 nm [5], it was found to be blocked by DABCO in a concentration-dependent manner (Fig. 7).

In an effort to isolate the ADR degradation product, samples of ADR solution before and after oxidation were analyzed by gas chromatography/mass spectrometry at the Baylor College of Medicine Mass Spectrometry Core Facility. As shown in Fig. 8, after oxidation the signal of ADR at 544 m/z disappeared and multiple peaks of smaller mass molecules appeared. As shown in Fig. 9, the HPLC chromatogram of an ethyl acetate extract of the 10-min UVA-irradiated solution containing both 20 μM ADR and 20 μM riboflavin had a peak that had a retention time and absorbance spectrum identical to those of 3-methoxysalicylic acid. Such a peak was not observed in extracts of a solution of ADR with riboflavin that had not been irradiated. This peak was also absent in extracts of UVA-irradiated riboflavin solution and UVA-irradiated ADR solution. It was therefore concluded that 3-methoxysalicylic acid was formed from ADR only when irradiated in the presence of riboflavin. The extracts of these solutions, with or without UVA irradiation, did not display peaks that had retention times and absorbance spectra that matched those of 6-methoxysalicylic acid or of anisole, 2-methoxy- or 3-methoxybenzoic acid (data not shown).

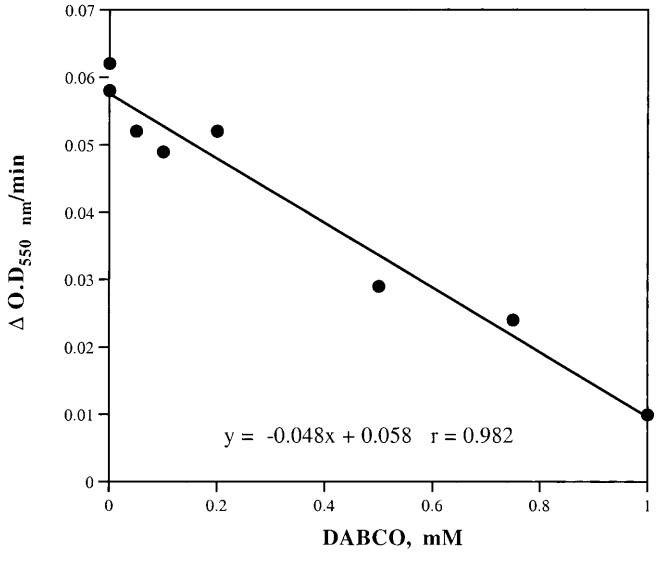
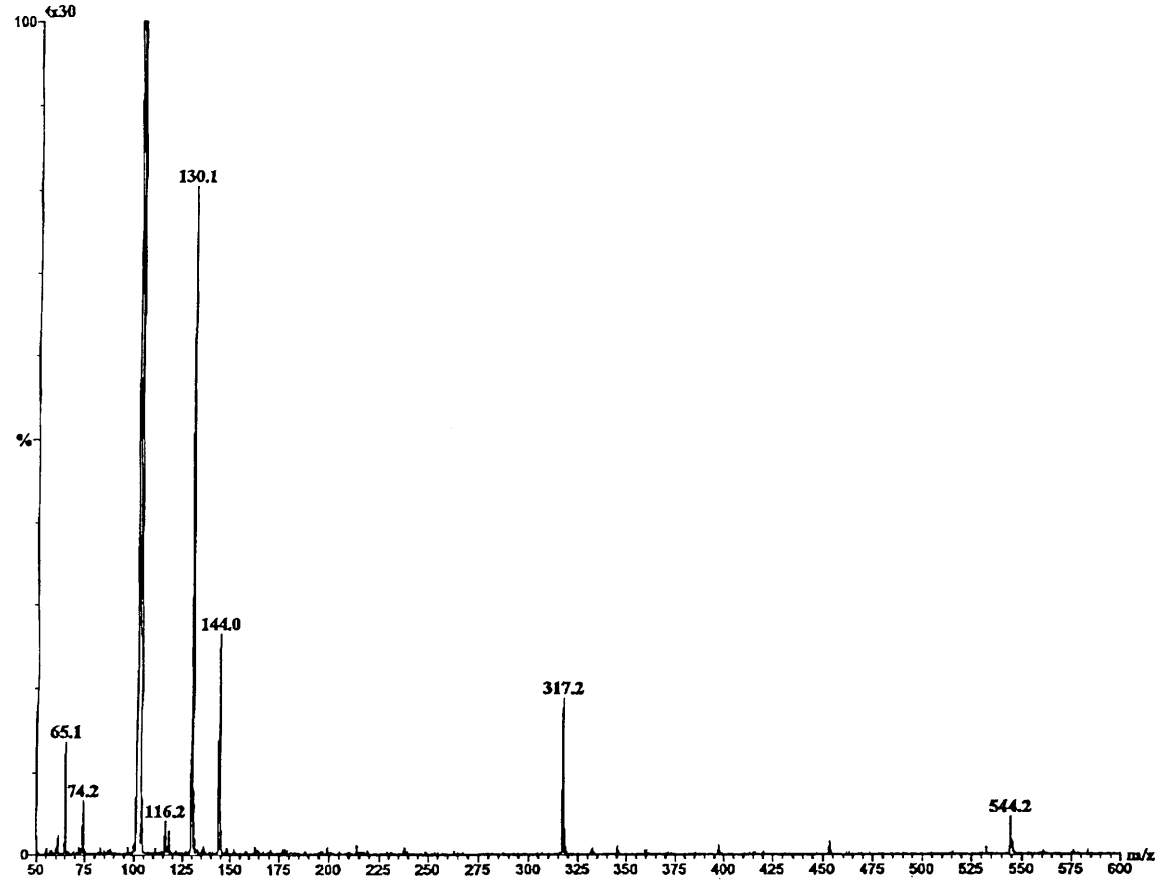


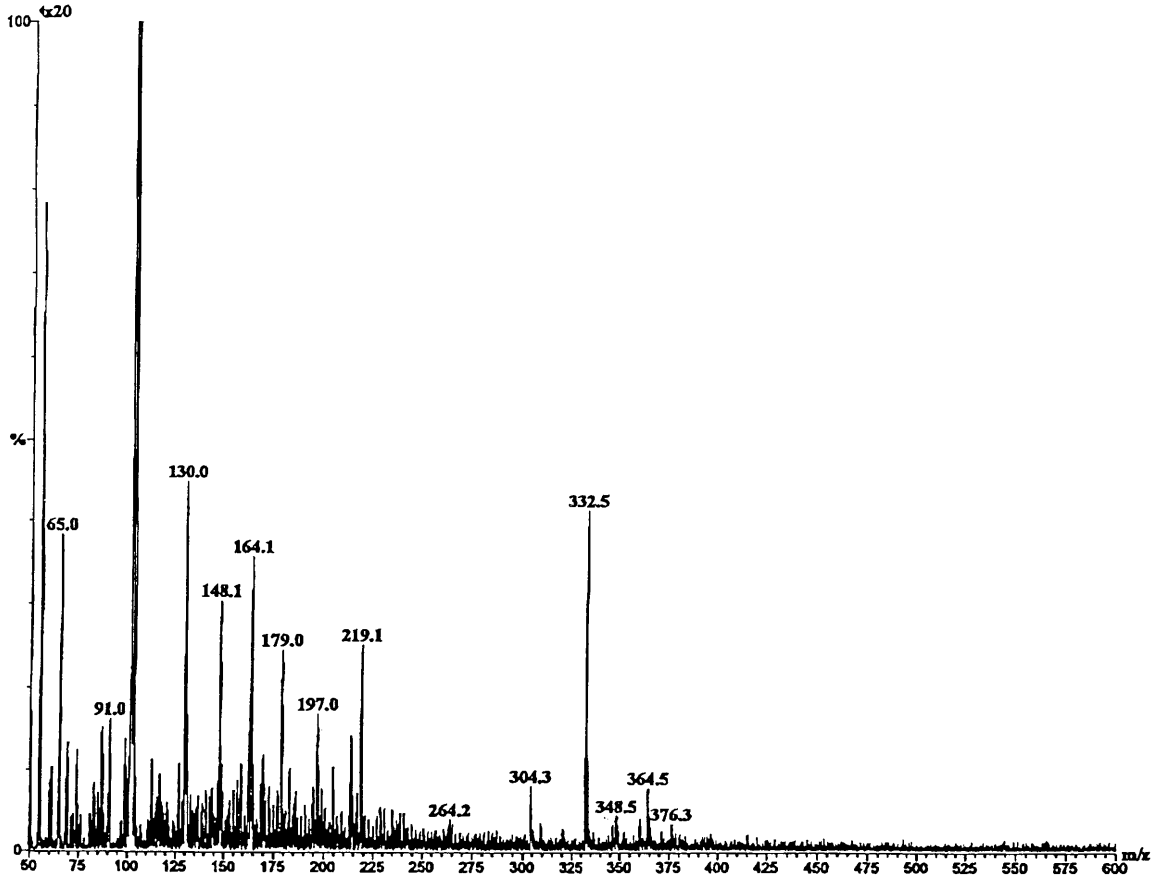
Fig. 7 The effect of UVA irradiation (4.3–4.4 mW/cm^2) of 20 μM riboflavin with 20 μM cytochrome C and increasing concentration of DABCO in PBS (pH 7.2), on the initial rate of increase in the 550 nm absorbance

When excited with 303 nm light, 3-methoxysalicylic acid emits fluorescence with an emission maximum at 420 nm. Such a fluorescence was not found in the ethyl acetate extracts of UVA-irradiated ADR solutions. Ethyl acetate extracts of UVA-irradiated riboflavin solutions contained lumichrome, which emitted fluorescence with a maxium intensity at 448 nm. Ethyl acetate extracts of UVA-irradiated solutions of ADR with riboflavin emitted fluorescence with maximal intensity at 420 nm and this intensity was proportional to the length of irradiation (data not shown). Figure 10 shows that the 420 nm fluorescence intensity of the solution of ADR with riboflavin (after subtraction of the emission resulting from lumichrome) was directly proportional to the decrease in ADR absorbance at 480 nm. These results further indicate that the oxidation of ADR results in the production of 3-methoxysalicylic acid in a quantitative manner.

Discussion

As shown above (Fig. 1), under UVA illumination the rate of the decrease in ADR absorbance at 480 nm


tx30
100
%
0
130.1
144.0
317.2
65.1
74.2
116.2
544.2
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600
m/z


tx20
100
%
0
130.0
332.5
65.0
164.1
148.1
179.0
219.1
91.0
197.0
304.3
364.5
264.2
348.5
376.3
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600
m/z

◀

Fig. 8 Mass spectrograms of a doxorubicin solution before (*upper part*) and after oxidation (*lower part*)

approached a maximal value when the riboflavin/ADR molar concentration ratio approached 1 and their concentrations approached 20 μM. Kharasch and Novak have demonstrated the formation of a flavin–ADR bimolecular complex as a result of a ring current effect, similar to that causing the self-association between ADR molecules or between flavin molecules [18]. Calculation of the dimerization constants for riboflavin and for ADR indicated that a sharp increase in their dimerization occurred as the concentration of these compounds rose from 1 to 50 μM [7, 11, 30]. We therefore suggest that the maximal rate of photodegradation of ADR is reached only after most of the ADR molecules have formed bimolecular complexes with riboflavin. The strong dependence of the ADR bleaching rate on the degree of dimerization between ADR and riboflavin may reflect the short life-time of light-excited riboflavin, and therefore the close proximity between the ring systems of these compounds determines the efficiency of this process.

It is known that light-excited riboflavin which has a reduction potential of 1.89 V can oxidize a large variety of compounds [24, 27]. In fact, in the absence of another oxidizable substrate, light-excited riboflavin could even oxidize another riboflavin molecule (in the ground state) and convert it to lumichrome [21]. The finding that the conversion of riboflavin to lumichrome was blocked by ADR (Fig. 2) implies that the riboflavin-mediated photodegradation of ADR could also reflect an oxidation process. Furthermore, it suggests that ADR is a better substrate for oxidation by light-excited riboflavin than the ground state riboflavin. Another indication that the degradation of ADR by light-excited riboflavin reflects the oxidation of ADR is the fact that it can be blocked by an excess of another oxidizable substrate (e.g. ascorbic acid). If the decrease in the absorbance peak at 480 nm resulted from oxidation of ADR by light-excited riboflavin, then similar spectral changes are also expected to occur when ADR is exposed to other oxidizers. Exposing ADR to sodium hypochlorite or dipotassium hexachloroiridate indeed resulted in the anticipated spectral changes. These results indicated the need to directly measure the oxidation potential of ADR. Previous electrochemical studies of ADR have focused mostly on the cathodic processes occurring at negative potentials as the anodic oxidative waves were ill-defined and a reductive wave was not observed on the reverse scan (e.g. Fig. 7c in [15]). However, in a study where carbon paste, rather than a GC electrode, was employed, a set of waves at approximately +0.5 V vs. saturated calomel electrode was found for ADR at pH 4.5 [3]. In the present study, using a GC electrode to measure the anodic waves of ADR at pH 7, we have shown that the ADR oxidation potential should be between 0.23 and 0.78 V. However, it could not be determined accurately as the ADR oxidation product was adsorbed on the GC electrode. These results suggest that ADR could also be oxidized by hydrogen peroxide. However, when such an oxidation was attempted with the ADR analog, daunorubicin, it failed to occur [19].

Photosensitized oxidation proceeds via type I or type II reactions. The sensitizer, in an excited triplet state, could abstract an electron (or hydrogen atom) from an oxidizable substrate (reaction type I) or could transfer its energy to dissolved molecular oxygen, which will obtain a singlet configuration ($^1O_2$) (reaction type II). Unlike ground state dioxygen that suffers from a kinetic restriction due to the spin-conservation rule, $^1O_2$ could then abstract two paired electrons from organic substrates [13]. It has been shown that 79% of the excited triplet of riboflavin is used to produce $^1O_2$ [6]. We have studied whether the oxidation of ADR by light-excited riboflavin is carried out via a type II reaction. As the riboflavin-mediated photobleaching of ADR was not accelerated in D_2O as compared with water, nor was it blocked by histidine or DABCO, it seemed that $^1O_2$ was not the mediator of photooxidation of ADR.

We then studied whether the oxidation of ADR by light-excited riboflavin is carried out via a type I reaction. Abstraction of an electron from a substrate AH, by excited triplet riboflavin, was shown to result in the formation of a cation radical $AH^+_\bullet$, and riboflavin semiquinone anion radical [16]. The riboflavin semiquinone anion radical could give the electron to $AH^+_\bullet$, undergo dismutation to oxidized and to fully reduced riboflavin, or give the electron to dissolved dioxygen that will then become a superoxide anion radical [13, 17, 25]. The reactions leading from the superoxide anion radical to hydrogen peroxide and to hydroxyl radicals are well established. ADR could have been oxidized directly by the excited triplet riboflavin or by one of the reactive oxygen species formed in this system. However, as indicated by the results shown in Fig. 6, the oxidation of ADR was not mediated by the formation of superoxide anion radical, hydrogen peroxide or hydroxyl radicals. In fact, the results indicated that the formation of superoxide anion radicals blocked the oxidation of ADR, probably by giving an electron to the cation radical form of ADR. The enhancement of the riboflavin-mediated photobleaching of ADR by DABCO (Fig. 5) could also be related to its effect on superoxide anion radicals (Fig. 7). It is suggested that DABCO, reported to be a one-electron donor, could reduce the riboflavin semiquinone anion radical or the superoxide anion radical and therefore allow the degradation of ADR to proceed [12]. These results suggest the ADR is oxidized directly by the excited triplet riboflavin.

It has been reported that 3-methoxybenzoic acid could result from the cleavage of the central ring of 1,4,5-trimethoxyanthraquinones [9]. Therefore it was assumed that the cleavage of the quinone ring of the ADR molecule might result in anisole, 2-methoxy- or 3-methoxybenzoic acid. However, the mass spectrogram

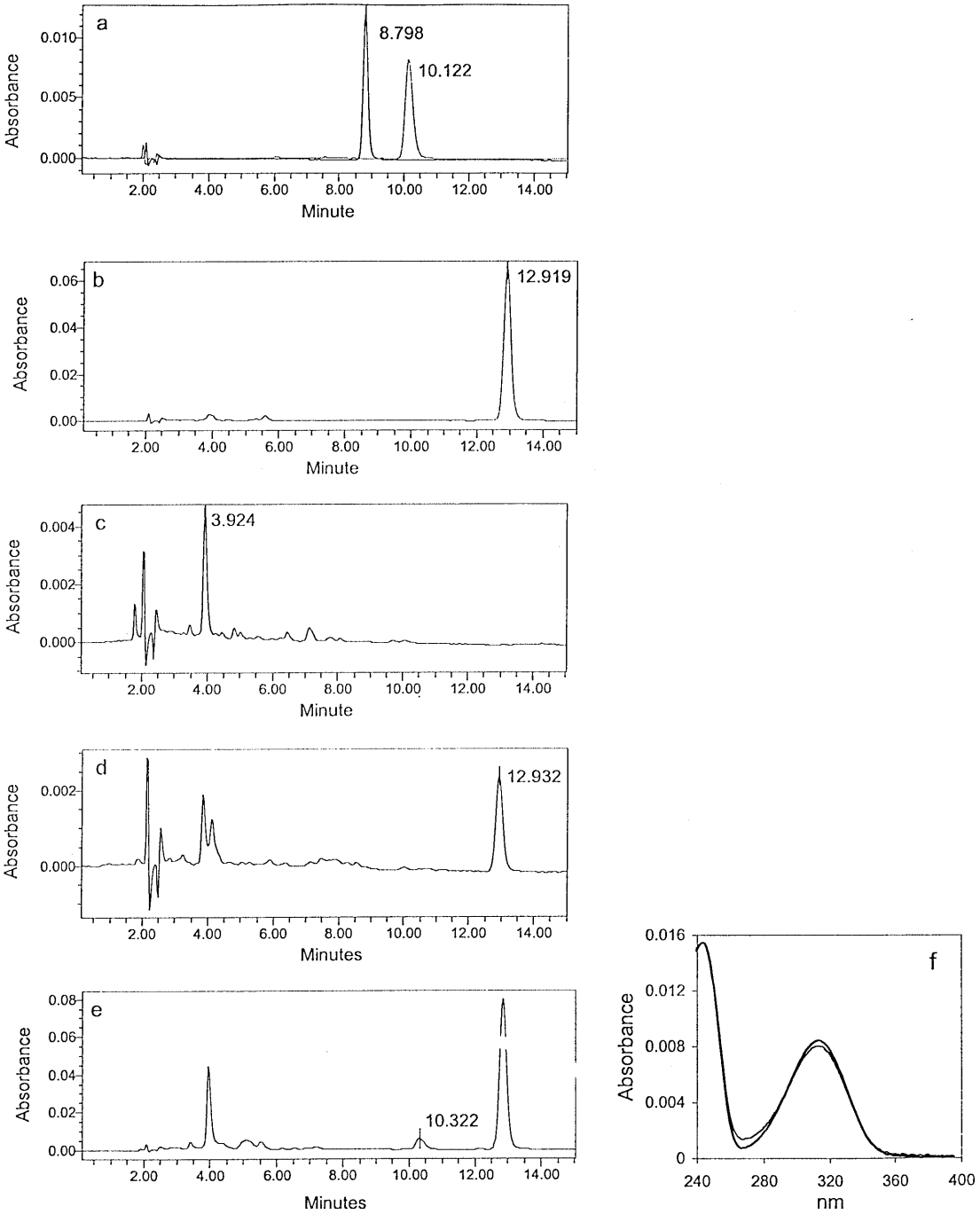


Fig. 9 HPLC chromatograms (detection at 310 nm) of: (**a**) 3-methoxysalicylic acid (peak at 10.122 min) and 6-methoxysalicylic acid (peak at 8.798 min); (**b**) 10 min UVA-irradiated solution of 20 μM riboflavin; (**c**) 10 min UVA-irradiated solution of 20 μM doxorubicin; (**d**) non-irradiated solution of 20 μM doxorubicin with 20 μM riboflavin; (**e**) 10 min UVA-irradiated solution of 20 μM doxorubicin with 20 μM riboflavin. The absorbance spectra of 3-methoxysalicylic acid 10.122 min peak from **a** and of the 10.322 min peak of the UVA-irradiated solution of 20 μM doxorubicin with 20 μM riboflavin are shown in **f**

and the HPLC chromatograms of the oxidized ADR solution did not show peaks corresponding to these compounds. Another possibility was that the oxidative cleavage of the ADR quinone ring might result in 2-hydroxy-3-methoxybenzoic acid (3-methoxysalicylic acid) or 2-hydroxy-6-methoxybenzoic acid (6-methoxysalicylic acid). The mass spectrogram of the oxidized ADR solution showed a peak that could correspond to these molecules (Fig. 8). However, as shown by the HPLC chromatogram (Fig. 9) of a UVA-irradiated solution of ADR with riboflavin, a new peak, with a retention time of 10.322 min, appeared. This peak was not present prior to the irradiation nor was it present in UVA-irradiated solutions of riboflavin or of ADR. The retention time and absorbance spectrum of this new peak were identical to those of 3-methoxysalicylic acid

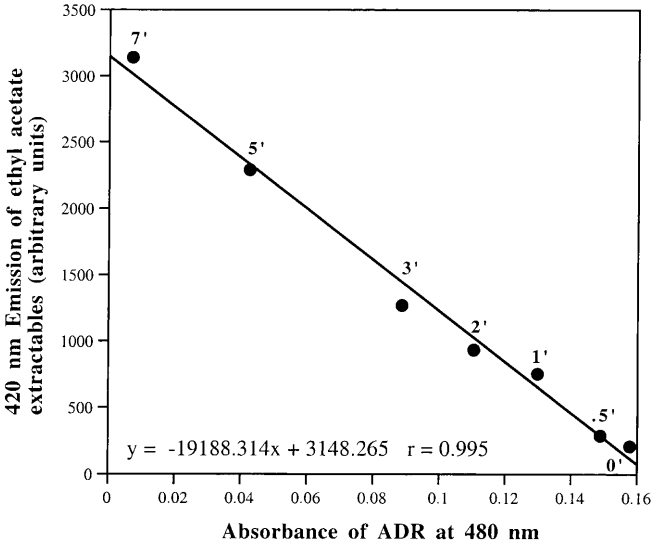


Fig. 10 The effect of UVA irradiation (4.85 mW/cm^2) of 20 μM doxorubicin with 20 μM riboflavin in PBS (pH 7.2) on the absorbance of doxorubicin at 480 nm in the aqueous phase remaining after ethyl acetate extraction and on the fluorescence at 420 nm (ex. 303 nm) of the ethyl acetate extractables dissolved in methanol. The length of UVA irradiation is shown above the corresponding *data points*

but not to those of 6-methoxysalicylic acid. Furthermore, by measuring the increase in fluorescence at 420 nm, it was shown that the photooxidation of ADR results in the production of 3-methoxysalicylic acid in a quantitative manner (Fig. 10).

It is generally accepted that the major metabolite of ADR is doxorubicinol. This metabolite is a result of the drug's C-13 keto group reduction by aldo-keto reductases. Reductive deglycosylation of ADR and doxorubicinol produced the corresponding 7-deoxyaglycones. All these metabolites have excitation and emission spectra that are similar to those of the parent drug [10]. However, as early as 1971, Alberts et al. reported that in patients receiving the tritium-labeled analog of ADR, daunorubicin, the total plasma levels of the drug and its metabolites as determined by the radioassay technique were four- to tenfold higher than those determined fluorometrically (em. 585 nm). As tritium exchange with water accounted for only a fraction of the discrepancy between the two assay methods, it was concluded that the rest was due to the formation of nonfluorescent metabolites of daunorubicin [1]. Others have reported that in rats injected with ^{14}C-labeled ADR the radioactivity associated with very polar unidentified nonfluorescent metabolites extracted from liver tissue and urine was higher than that associated with doxorubicinol and the aglycones [2]. It is therefore suggested that ADR metabolites that have spectral characteristics dissimilar to the parent drug may play an important role in the pharmacology of ADR. The magnitude of the oxidation potential of ADR, as determined in the present study, does not exclude the possibility that ADR could also be oxidized in vivo. If that proves to be the case then it is suggested that one of the missing nonfluorescent metabolites of the drug might be 3-methoxysalicylic acid. This possibility also raises questions regarding the possible role of such a metabolite in the different biological activities of ADR.

Acknowledgement We wish to thank Dr. Daniel Mandler, from the Department of Inorganic and Analytical Chemistry of The Hebrew University of Jerusalem, for his help in the interpretation of the cyclic voltammetry and square wave voltammetry data.

References

1. Alberts DS, Bachur NR, Holtzman JL (1971) The pharmacokinetics of daunomycin in man. Clin Pharmacol Ther 12: 96
2. Arcamone F, Lazzati M, Vicario GP, Zini G (1984) Disposition of ^{14}C-labeled 4′-epidoxorubicin and doxorubicin in the rat. A comparative study. Cancer Chemother Pharmacol 12: 157
3. Baldwin RP, Packett D, Woodcock TM (1981) Electrochemical behavior of adriamycin at carbon paste electrodes. Anal Chem 53: 540
4. Bomgaars L, Gunawardena S, Kelley SE, Ramu A (1997) The inactivation of doxorubicin by long ultraviolet light. Cancer Chemother Pharmacol 40: 506
5. Butler J, Koppenol WH, Margoliash E (1982) Kinetics and mechanism of the reduction of ferricytochrome c by the superoxide anion. J Biol Chem 257: 10747
6. Chacon JN, McLearie J, Sinclair RS (1988) Singlet oxygen yields and radical contributions in the dye-sensitized photooxidation in methanol of esters of polyunsaturated fatty acids (oleic, linoleic, linolenic and arachidonic). Photochem Photobiol 47: 647
7. Dalmark M, Johansen P (1982) Molecular association between doxorubicin (adriamycin) and DNA-derived bases, nucleosides, nucleotides, other aromatic compounds and proteins in aqueous solution. Mol Pharmacol 22: 158
8. Daugherty JP, Hixon SC, Yielding KL (1979) Direct in vitro photoaffinity labeling of DNA with daunorubicin, adriamycin, and rubidazone. Biochim Biophys Acta 565: 13
9. Davies DG, Hodge P, Yates P (1973) Chemistry of quinones. 3. Cleavage of the methyl ethers of some naturally occurring hydroxyanthraquinones. J Chem Soc Perkin I 2299
10. Doroshow JH (1996) Anthracyclines and anthracenediones. In: Chabner BA, Longo DL (eds) Cancer chemotherapy and biotherapy: principles and practice, 2nd edn. Lippincott–Raven, Philadelphia, p 409
11. Drabnet R, Grajek H (1983) The flavin dimers. I. The application of absorption in anti-stokes excitation region to investigate the flavin dimer formation. Biochim Biophys Acta 758: 98
12. Eastland GW, Symons MCR (1977) The triethylenediamine cation radical: an ESR study. Chem Phys Lett 45: 422
13. Foote CS (1968) Mechanisms of photosensitized oxidation. Science 162: 963
14. Foote CS, Clennan EL (1995) Properties and reactions of singlet oxygen. In: Foote CS, Valentine JS, Greenberg A, Liebman JF (eds) Active oxygen in chemistry. Blackie Academic and Professional, London, p 105
15. Gutierrez PL (1988) The influence of ascorbic acid on the free-radical metabolism of xenobiotics: the example of diazoquinone. Drug Metabolism Rev 19: 319
16. Heelis PF (1982) The photophysical and photochemical properties of flavins (isoalloxazines). Chem Soc Rev 11: 15
17. Joshi P, Carraro C, Pathak MA (1987) Involvement of reactive oxygen species in the oxidation of tyrosine and DOPA to melanin and in skin tanning. Biochem Biophys Res Comm 142: 265

18. Kharasch ED, Novak RF (1981) The molecular basis for complexation of adriamycin with flavin mononucleotide and flavin adenine dinucleotide. Arch Biochem Biophys 212: 20
19. Kolodziejczyk P, Reszka K, Lown JW (1989) Enzymatic oxidative activation of 5-iminodaunorubicin. Spectrophotometric and electron paramagnetic resonance studies. Biochem Pharmacol 38: 803
20. Menozzi M, Valentini L, Vannini E, Arcamone F (1984) Self-association of doxorubicin and related compounds in aqueous solution. J Pharm Sci 73: 766
21. Moore WM, Spence JT, Raymond FA, Colson SD (1963) The photochemistry of riboflavin. 1. The hydrogen transfer process in the anaerobic photobleaching of flavins. J Am Chem Soc 85: 3367
22. Munday R (1988) Generation of superoxide radical, hydrogen peroxide and hydroxyl radicals during the autoxidation of N,N,N′,N′-tetramethyl-p-phenylenediamine. Chem Biol Interact 65: 133
23. Schachter D, Manis JG (1958) Salicylate and salicyl conjugates: fluorometric estimation, biosynthesis and renal excretion in man. J Clin Invest 37: 800
24. Seely GR (1978) The energetics of electron-transfer reactions of chlorophyll and other compounds. Photochem Photobiol 27: 639
25. Silva E, Godoy J (1994) Riboflavin sensitized photooxidation of tyrosine. Int J Vit Res 64: 253
26. Tavolini N, Guarino AM, Berk PD (1980) Photolytic degradation of adriamycin. Comm J Pharm Pharmacol 32: 860
27. Taylor MB, Radda GK (1971) Flavins as photosensitizers. Methods Enzymol 18: 496
28. Williams BA, Tritton TR (1981) Photoinactivation of anthracyclines. Photochem Photobiol 34: 131