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ORIGINAL ARTICLE

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The enhancement of riboflavin-mediated photo-oxidation of doxorubicin by histidine and urocanic acid

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Abstract *Purpose*: Previously we have shown that doxorubicin (Adriamycin, ADR) can be inactivated by light-excited riboflavin. The inactivation of the drug results from its direct oxidation by the excited triplet riboflavin in a type I photosensitization reaction, and 3-methoxysalicylic acid is an ADR breakdown product. In the present study, we investigated the enhancement of this process by histidine and some other imidazole analogs. *Methods*: ADR solutions containing various concentrations of riboflavin and other agents were exposed to 365 nm light for various time periods and then the absorbance spectrum of ADR was measured by a double beam spectrophotometer. These measurement were used to calculate the half-time of the ADR degradation process. The degraded ADR solutions were analyzed by HPLC. *Results*: The rate of bleaching of ADR by light-excited riboflavin was enhanced in the presence of histidine in a concentration-dependent manner. This enhancement was more pronounced at higher riboflavin concentrations. Histidine also enhanced the riboflavin-mediated photobleaching of *N*,*N*-dimethyl-4-nitrosoaniline (RNO), a compound known to be resistant to oxidation by singlet oxygen but sensitive to oxidation by the *trans*-annular peroxide of histidine. RNO was found to block the histidine enhancement of the riboflavin-mediated photobleaching of ADR in a competitive manner. Among the imidazole analogs of histidine tested, urocanic acid was found to be the most efficient enhancer of the riboflavin-mediated photobleaching of ADR. Superoxide anion radicals which retard the oxidation of ADR were quenched by urocanic acid but not by histidine. It was shown that the oxidation of ADR by the *trans*-annular peroxide of histidine resulted in the formation of 3-methoxysalicylic acid. *Conclusions*: In contrast to singlet oxygen, the *trans*-annular peroxide, formed by the interaction of histidine and the singlet oxygen produced by photoexcited riboflavin, is an efficient oxidizer of ADR. The enhancement of the riboflavin-mediated photobleaching of ADR by histidine analogs depends on the rate of their conversion to a *trans*-annular peroxide and on the efficiency of these products in oxidizing ADR. However, for some analogs of histidine, as shown for urocanic acid, other mechanisms could also be involved. The presence of urocanic acid in the skin suggests that significant degradation of ADR could occur in the presence of biologically relevant concentrations of riboflavin if patients treated with ADR are exposed to sunlight. The finding that histidine also enhanced the degradation of ADR to 3-methoxysalicylic acid, suggests that the process of ADR oxidation by the *trans*-annular peroxides is similar to the direct oxidation of ADR by excited triplet riboflavin.

Key words Doxorubicin · Riboflavin · Histidine · Urocanic acid · 3-Methoxysalicylic acid

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Introduction

We have reported that the irradiation of a solution of doxorubicin (Adriamycin, ADR) in RPMI-1640 medium with longwave ultraviolet light (UVA) results in a rapid decline in the drug's cytostatic activity against P388 murine leukemia cells and in the loss of the drug's absorbance in the 400 to 550 nm range. Of the 40 components of this growth medium, riboflavin was found to be the major photosensitizer [1]. We have also shown subsequently that the bleaching of ADR results from its oxidation by photoactivated riboflavin in a type I photosensitization reaction and that the oxidation of the drug results in the formation of 3-methoxysalicylic acid [12]. Although four-fifths of the light-excited triplet riboflavin is consumed to convert the solution's

dissolved oxygen to singlet oxygen ($^1O_2$), the photo-oxidation of ADR is $^1O_2$ independent [3]. Moreover, the purging of oxygen from the solution prior to its exposure to light enhances the riboflavin-mediated photo-oxidation of ADR, indicating that the oxygen could retard the oxidation of ADR. Further studies have shown that this inhibition is due to the formation of superoxide anion radicals [12].

We have also reported that the amino acid component group of RPMI-1640 medium accelerates the rate of riboflavin-mediated photobleaching of ADR. In further experiments it has been found that riboflavin-mediated photobleaching of ADR can be enhanced by an amino acid subgroup comprising histidine, methionine, tryptophan and tyrosine or even by histidine alone [1, 12]. These results were somewhat surprising as it is known that these amino acids can also be oxidized by light-excited riboflavin and therefore would be expected to compete with ADR on the light-excited oxidizing agent rather than to augment the drug's oxidation [6]. Previously we have shown that the enhancement by 1,4-diazabicyclo{2.2.2}octane (DABCO) of the riboflavin-mediated photo-oxidation of ADR results from its ability to quench superoxide anion radicals [12]. It is therefore possible that the enhancement of the photobleaching of ADR by these amino acids might also be related to such an effect. Another potential explanation, at least for the histidine enhancement of the photo-oxidation of ADR, could be related to its ability to interact with $^1O_2$ [14]. It can be assumed that the product of such an interaction might mediate the oxidation of ADR.

The purpose of the present study was to investigate the mechanisms by which histidine enhances the riboflavin-mediated photolytic degradation of ADR.

Materials and methods

Chemicals and reagents

Riboflavin, ethyl acetate, L-histidine, imidazole, 4-imidazole carboxylic acid, 4-imidazole acetic acid, urocanic acid, *N*,*N*-dimethyl-4-nitrosoaniline (RNO), 3-methoxysalicylic acid, 6-methoxysalicylic acid, and cytochrome *c* were purchased from Sigma-Aldrich (St. Louis, Mo.). Dulbecco's phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride was purchased from GibcoBRL (Gaithersburg, Md.). Doxorubicin HCl (Adriamycin PFS, ADR) was purchased from Pharmacia (Kalamazoo, Mich.).

Longwave 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 for 0, 2, 4, 6, 8, 10, 15 and 20 min in a laminar flow hood with three Blacklight Blue 40-W lamps (Vilber Lourmat, Marne La Vallée, France). The energy flow rate delivered to the solutions, measured with a Cole-Parmer 97503-00 Radiometer (Niles, Ill.) 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 experiments. After irradiation the 250–800 nm absorbance spectra of the solutions were measured in a double-beam UV-VIS scanning spectrometer (Shimadzu Scientific Instruments, Columbia, Md.). 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. Experiments in which the calculated half-time decrease in the ADR absorbance was shorter than 5 min were repeated with irradiation times of 0, 0.25, 0.5, 0.75, 1, 1.5, 2 and 3 min.

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 ºC. 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 a Waters 600E multisolvent delivery system, a Waters 717 plus autosampler, and a Waters 996 photodiode array detector. The guard column was a Nova-Pak C18, 4 µm, 3.9 × 20 mm and the separation column was a Nova-Pak C18, 4 µm. 3.9 × 150 mm (Waters, Milford, Mass.). Isocratic elution was performed at a flow of 1 ml/min. Chromatographic software (Millennium Chromatography Manager, v 3.15.01; Waters) was used for acquisition and processing of data. Similarly, 3-methoxy- and 6-methoxysalicylic acids dissolved in mobile phase were also injected into the system as standards.

Results

We have previously shown that histidine at a concentration of 5 m*M* enhances 26-fold the rate at which ADR (20 µ*M*) is bleached by UVA-irradiated riboflavin (20 µ*M*) (see Fig. 5 in reference 12). In an effort to further define the quantitative aspects of this enhancement, the experiments were repeated with various concentrations of histidine or riboflavin. As shown in Fig. 1, at a constant concentration of riboflavin (20 µ*M*) the rate of ADR bleaching increased as an exponential

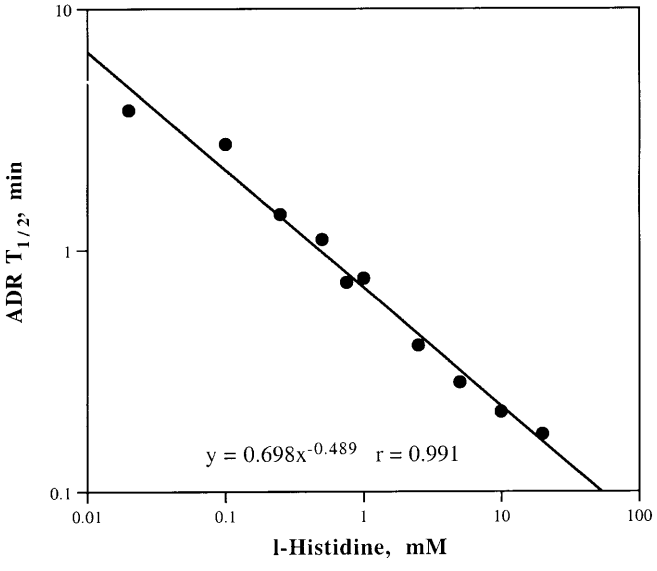

Fig. 1 The effect of UVA irradiation (4–5 mW/cm^2) of 20 µ*M* ADR with 20 µ*M* riboflavin and increasing concentration of L-histidine in PBS (pH 7.2) on the rate of decrease in the ADR absorbance at 480 nm (expressed as $T_{1/2}$)

function of the histidine concentration. At a constant concentration of histidine (5 m*M*) the rate of ADR bleaching increased as an exponential function of the riboflavin concentration (Fig. 2). Over the range of riboflavin concentrations tested (0.5 to 80 μ*M*), the ratio of the ADR bleaching $T_{1/2}$ measured in the absence of histidine to that measured in its presence (at 5 m*M* concentration), became greater with the rise in riboflavin concentration. In other experiments the rate of ADR bleaching by UVA irradiation was tested with solutions containing 1 to 20 μ*M* ADR with 0, 0.5, 1 or 2 μ*M* riboflavin and with or without 5 m*M* histidine. In these experiments, the addition of histidine enhanced by at least tenfold the rate of ADR bleaching (data not shown). The results indicated that at the higher riboflavin concentrations a histidine-dependent mechanism assumed an increasing role among the mechanisms responsible for the riboflavin-mediated photobleaching of ADR.

As quenching of superoxide anion radicals or the prevention of their production was found to enhance the riboflavin-mediated photobleaching of ADR, we tested whether histidine has such effects. However, the increase in absorbance at 550 nm that followed the UVA irradiation of a solution of 20 μ*M* riboflavin with 20 μ*M* cytochrome *c* could not be blocked by histidine. In fact, histidine enhanced the rate of increase in the 550 nm absorbance in a concentration-dependent manner (data not shown). This increase indicates that in this system histidine enhanced the production of superoxide anion radicals. It is suggested that histidine serves as a convenient source of electrons for the conversion of light-excited riboflavin to riboflavin anion radical which then could reduce dissolved oxygen to superoxide anion radicals.

We have also investigated whether a product of the interaction between histidine and the photogenerated $^1O_2$ could mediate the oxidation of ADR. The decrease in absorbance at 440 nm of RNO caused by a variety of light-excited dyes is enhanced in the presence of histidine or of some other imidazoles. An imidazole compound reacts with the photogenerated $^1O_2$, producing a *trans*-annular peroxide which then bleaches RNO in a secondary reaction [10, 13]. As riboflavin was not among the photosensitizers described in these reports, it was important to study whether light-excited riboflavin could also bleach RNO and the extent to which this process was affected by the presence of histidine. As shown in Table 1, even after 20 min of UVA irradiation, in the absence or presence of 1 m*M* histidine, bleaching of RNO was not detected. In the presence of 20 μ*M* riboflavin (but not 0.5 μ*M*), a small percentage of RNO was bleached by the UVA irradiation. Histidine (1 m*M*) greatly enhanced the bleaching rate of RNO by light-excited riboflavin and the effect was even larger at a concentration of 5 m*M*.

If the enhancement of riboflavin-mediated photobleaching of ADR by histidine is dependent on the formation of such a *trans*-annular peroxide of histidine as reported for the bleaching of RNO, then a competition between RNO and ADR on this product is expected and RNO should block the histidine enhancement of the riboflavin-mediated photobleaching of ADR. As shown in Table 2, RNO (10 μ*M*) did not affect the rate of

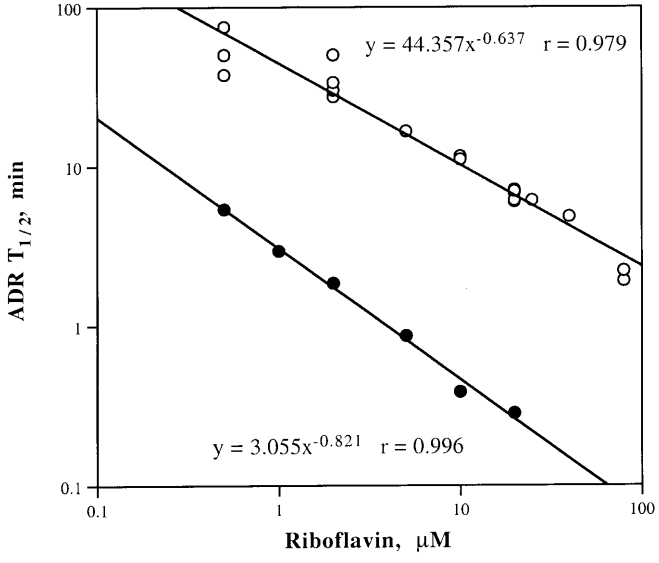

Fig. 2 The effect of UVA irradiation (4–5 mW/cm^2) of 20 μ*M* ADR with increasing concentration of riboflavin and with (●) or without (○) 5 m*M* L-histidine in PBS (pH 7.2) on the rate of decrease in the ADR absorbance at 480 nm (expressed as $T_{1/2}$)

Table 1 The effect of UVA irradiation (4–5 mW/cm^2) of 10 μ*M* RNO with 0, 0.5 or 20 μ*M* riboflavin and with 0, 1 or 5 m*M* L-histidine in PBS (pH 7.2) on the rate of decrease in RNO absorbance at 440 nm (expressed as $T_{1/2}$ in minutes)

Histidine (m*M*)	Riboflavin (μ*M*)		
	0	0.5	20
0	–[a]	–[a]	113 ($r=0.994$)
1	–[a]	21.5 ($r=0.999$)	2.62 ($r=0.995$)
5	–[a]	8.36 ($r=0.996$)	0.766 ($r=0.998$)

[a] No measurable decrease in RNO absorbance was observed over 20 min of UVA irradiation

Table 2 The effect of UVA irradiation (4–5 mW/cm^2) of 20 μ*M* ADR with or without 1 m*M* L-histidine, 0, 0.5 or 20 μ*M* riboflavin and with or without 10 μ*M* RNO in PBS (pH 7.2) on the rate of decrease in the ADR absorbance at 480 nm (expressed as $T_{1/2}$ in minutes)

	Riboflavin, μM		
	0	0.5	20
–	75.3 ($r=0.967$)	60.2 ($r=0.950$)	6.14 ($r=0.984$)
Histidine	60.2 ($r=0.965$)	14.3 ($r=0.998$)	0.766 ($r=0.995$)
RNO	60.2 ($r=0.956$)	43.0 ($r=0.966$)	5.8 ($r=0.992$)
Histidine + RNO	60.2 ($r=0.988$)	50.2 ($r=0.914$)	4.56 ($r=0.989$)

bleaching of ADR in the presence or absence of histidine (1 m*M*), nor did it affect the rate of ADR bleaching measured in the presence of 0.5 or 20 μ*M* riboflavin. However, the enhancement of the riboflavin-mediated ADR photobleaching by histidine was blocked by RNO. In other experiments it was found that the histidine (1 m*M*) enhancement of the riboflavin-mediated (20 μ*M*) photobleaching of ADR (20 μ*M*) was blocked by RNO as a linear function of the RNO concentration between 3 and 7 μ*M* (data not shown). These results suggest that RNO was a better substrate for oxidation by the histidine *trans*-annular peroxide than was ADR. Furthermore, if ADR and RNO were competing on the same *trans*-annular peroxides, then it could be expected that raising the concentration of histidine might increase the formation of the *trans*-annular peroxide and thus alleviate the RNO blockade of the histidine enhancement. As shown in Table 3, the ability of RNO (5 μ*M*) to block the histidine enhancement of the riboflavin-mediated photobleaching of ADR did indeed decrease at higher concentrations of histidine.

It has been reported that the magnitude of the enhancement of dye-mediated photobleaching of RNO by imidazole compounds is determined by the nature of the groups attached to the imidazole ring [8]. We therefore tested the ability of imidazole, as well as its analogs 4-carboxylic acid, 4-acetic acid and urocanic acid to enhance the riboflavin-mediated photobleaching of RNO and ADR. The effects of these compounds on the rate of conversion of light-excited riboflavin to lumichrome was also measured. In the presence of these compounds at a concentration of 1 m*M* the rates (expressed as $T_{1/2}$) of the bleaching of RNO (10 μ*M*) by light-excited riboflavin (20 μ*M*) were 1.7, 1.41, 0.546 and 1.13 min, respectively (compared to 2.62 min for histidine and 113 min in the absence of imidazoles). The rates of conversion of light-excited riboflavin to lumichrome (determined in terms of the decrease in riboflavin absorbance at 450 nm) were 4.07, 7.0, 1.3 and 20.1 min, respectively (compared to 4.63 min for histidine and 6.84 min in the absence of imidazoles). The effects of the imidazole analogs on the rate of the riboflavin-mediated photobleaching of ADR are shown in Table 4. Urocanic acid was by far the strongest enhancer of riboflavin-mediated photobleaching of ADR. In the absence of riboflavin, urocanic acid did not enhance the photobleaching of ADR (data not shown).

Table 3 The effect of UVA irradiation (4.20–4.22 mW/cm^2) of 20 μ*M* ADR with 20 μ*M* riboflavin and increasing concentration of L-histidine and with or without 5 μ*M* RNO in PBS (pH 7.2) on the rate of decrease in the ADR absorbance at 480 nm (expressed as $T_{1/2}$ in minutes)

RNO (μ*M*)	Histidine (m*M*)		
	1	2	4
0	0.766 ($r=0.995$)	0.663 ($r=0.993$)	0.442 ($r=0.991$)
5	3.2 ($r=0.997$)	1.43 ($r=0.991$)	0.689 ($r=0.990$)

Table 4 The effect of UVA irradiation (4–5 mW/cm^2) of 20 μ*M* ADR with 20 μ*M* riboflavin and 1 m*M* L-histidine, imidazole and imidazole analogs in PBS (pH 7.2) on the rate of decrease in the ADR absorbance at 480 nm (expressed as $T_{1/2}$ in minutes)

	ADR $T_{1/2}$
–	6.14 ($r=0.984$)
Histidine	0.766 ($r=0.995$)
Imidazole	1.22 ($r=0.995$)
4-Imidazole carboxylic acid	2.47 ($r=0.984$)
4-Imidazole acetic acid	2.01 ($r=0.997$)
Urocanic acid	0.12 ($r=0.997$)

In further studies, with the experimental design described in Fig. 1 but with urocanic acid instead of histidine, the equation describing the exponential increase in the rate of bleaching of ADR as a function of the concentration of urocanic acid was $Y = 3.906 \times X^{-0.540}$ ($r=0.970$), where Y is ADR $T_{1/2}$ (minutes) and X is the concentration of urocanic acid (in μM). When the experiment was repeated with riboflavin at a concentration of 0.5 μ*M* rather than 20 μ*M*, the equation describing the exponential increase in the rate of ADR bleaching was $Y = 207.4 \times X^{-0.721}$ ($r=0.990$). These data indicate that urocanic acid was a much stronger enhancer of the riboflavin-mediated photobleaching of ADR than the amino acid histidine. In another experiment with 20 μ*M* ADR, 20 μ*M* riboflavin, 10 μ*M* RNO and increasing concentrations of urocanic acid, the equation describing the exponential increase in the rate of ADR bleaching was $Y = 8.468 \times X^{-0.314}$ ($r=0.936$). This indicates that increasing the concentration of urocanic acid resulted in reduced ability of RNO to block the enhancement of the riboflavin-mediated photobleaching of ADR. These data suggest that ADR and RNO competed on the same *trans*-annular peroxide of urocanic acid, as previously found for the *trans*-annular peroxide of histidine.

In another experiment, 10 μ*M* RNO with 20 μ*M* riboflavin and increasing concentrations of urocanic acid in PBS (pH 7.2) were UVA-irradiated (4.31–440 mW/cm^2) for periods up to 20 min, and then the absorbance of RNO at 440 nm was measured. In this experiment the equation describing the exponential increase in the rate of RNO bleaching as a function of the concentration of urocanic acid was $Y = 84.17 \times X^{-0.617}$ ($r=0.953$), where Y is RNO $T_{1/2}$ (minutes) and X is the concentration of urocanic acid (in μM). Comparison of these results with those obtained with ADR suggests that ADR might have been a better substrate than RNO for oxidation by the *trans*-annular peroxide of urocanic acid. Another possibility is that in addition to the formation of *trans*-annular peroxides, urocanic acid can also enhance the riboflavin-mediated photo-oxidation of ADR by some other mechanism.

We have previously shown that superoxide anion radicals inhibit the oxidation of ADR by the excited triplet riboflavin [12]. We investigated whether urocanic acid could quench superoxide anion radicals or block

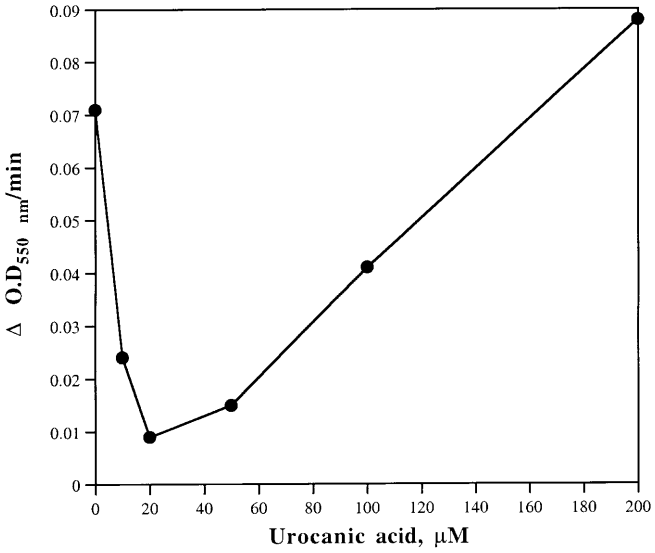

Fig. 3 The effect of UVA irradiation (4.53–4.78 mW/cm^2) of 20 μ*M* cytochrome *c* with 20 μ*M* riboflavin and increasing concentrations of urocanic acid in PBS (pH 7.2) on the initial rate of increase in the 550 nm absorbance

their formation. As shown in Fig. 3, at concentrations below 20 μ*M*, urocanic acid was found to block the increase in the absorbance of cytochrome *c* at 550 nm in response to UVA irradiation of riboflavin, but at higher concentrations, the blockade was gradually eased and at concentrations higher than 150 μ*M* urocanic acid actually accelerated the rate of increase in the 550 nm absorbance. These data suggest that at least at low concentrations, part of the urocanic acid enhancement of the riboflavin-mediated photo-oxidation of ADR could be due to its ability to quench superoxide anion radicals and/or prevent their formation.

We have previously shown that the photodegradation of ADR by UVA-excited riboflavin results in the formation of 3-methoxysalicylic acid. This is the outcome of direct oxidation of ADR by the excited triplet riboflavin [12]. As the enhancement of the photobleaching of ADR by imidazole compounds is mediated via the formation of their *trans*-annular peroxides, we investigated whether the imidazole-enhanced oxidation of ADR would also result in the formation of 3-methoxysalicylic acid. As shown in Fig. 4, the chromatogram of an ethyl acetate extract of a 10-min UVA-irradiated solution containing 20 μ*M* ADR, 2 μ*M* riboflavin and 5 m*M* imidazole had a peak with a retention time and an absorbance spectrum identical to those of 3-methoxysalicylic acid. Such a peak was not observed in extracts of the same solution prior to irradiation. This peak was also absent in extracts of UVA-irradiated solutions of riboflavin with imidazole or of ADR with imidazole. In the chromatograms of these solutions before and after UVA irradiation, none of the peaks could be identified with 6-methoxysalicylic acid. When compared to the results obtained in the presence of 20 μ*M* riboflavin [12], it is clear that the area under the 9.203-min peak of the chromatogram shown in Fig. 4e is much greater than would be expected to appear with a solution containing 20 μ*M* ADR and 2 μ*M* riboflavin. These results indicate that the imidazole enhancement of the riboflavin-mediated photo-oxidation of ADR was also associated with the enhanced formation of 3-methoxysalicylic acid.

In another experiment the effect of the length of the irradiation period of a solution of 20 μ*M* ADR, 2 μ*M* riboflavin and 5 m*M* imidazole on the formation of 3-methoxysalicylic acid was measured. In this experiment the retention time of 3-methoxysalicylic acid was 9.258 min. The gradual increase in the size of the 9.3-min peak (Fig. 5a) with the length of the UVA irradiation indicates that formation of 3-methoxysalicylic acid was a time-dependent process and, as shown in Fig. 5c, was directly proportional to the decrease in ADR absorbance at 480 nm. These data suggest that the process of oxidation of ADR by *trans*-annular peroxides of imidazoles is similar to that of the direct oxidation of the drug by the excited triplet of riboflavin.

Discussion

The results presented in Figs. 1 and 2 indicate that histidine enhanced the riboflavin-mediated photobleaching of ADR in a dose-dependent manner. Furthermore, in the presence of a sufficient concentration of histidine, the importance of the histidine-dependent mechanism grew (compared to the direct oxidation of ADR by the excited triplet riboflavin) with the increase in riboflavin concentration. Unlike DABCO, histidine did not quench superoxide anion radicals or prevent their formation, and therefore it seemed to enhance the riboflavin-mediated photobleaching of ADR by a different mechanism. Although, the $^1O_2$ generated by the UVA-excited riboflavin did not seem to affect the rate of ADR bleaching, it is possible that upon addition of histidine, this $^1O_2$ could have reacted with histidine to form a *trans*-annular peroxide of the histidine imidazole ring moiety and that this product might have oxidized ADR [14].

It has been reported that the bleaching of RNO by *trans*-annular peroxides of imidazole compounds proceeds at much faster rates than by the direct oxidation by light-excited dyes or by the $^1O_2$ generated by their interaction with dissolved oxygen [8, 10, 13]. As previously reported for other dyes, the riboflavin-mediated photobleaching of RNO was also found to be greatly enhanced by histidine (Table 1). It was therefore con-

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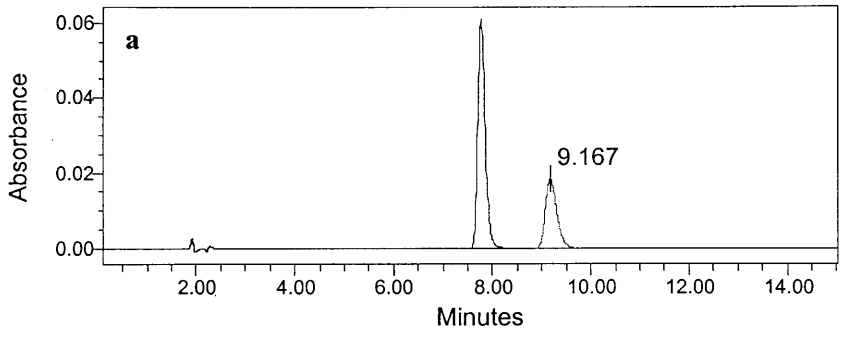
Fig. 4a–f Chromatograms (detection at 310 nm) of (**a**) 3-methoxysalicylic acid (peak at 9.167 min) and 6-methoxysalicylic acid (peak at 7.770 min), (**b**) 10-min UVA-irradiated solution of 2 μ*M* riboflavin with 5 m*M* imidazole, (**c**) 10-min UVA-irradiated solution of 20 μ*M* ADR with 5 m*M* imidazole, (**d**) nonirradiated solution of 20 μ*M* ADR, 2 μ*M* riboflavin and 5 m*M* imidazole, and (**e**) 10-min UVA-irradiated solution of 20 μ*M* ADR, 2 μ*M* riboflavin and 5 m*M* imidazole. The absorbance spectra of the 3-methoxysalicylic acid 9.167-min peak from **a** and of the 9.203-min peak of **e** are shown in **f**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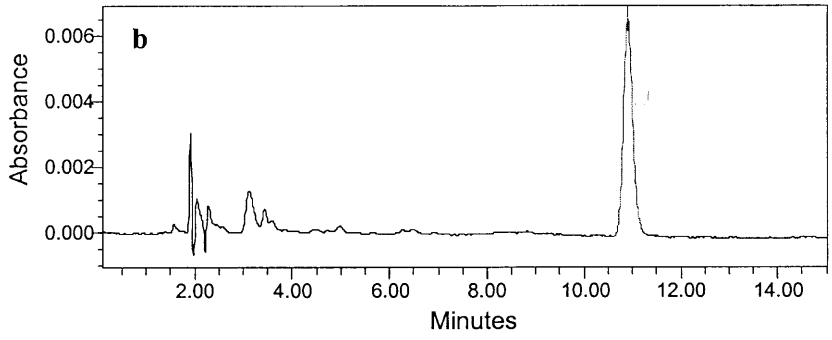
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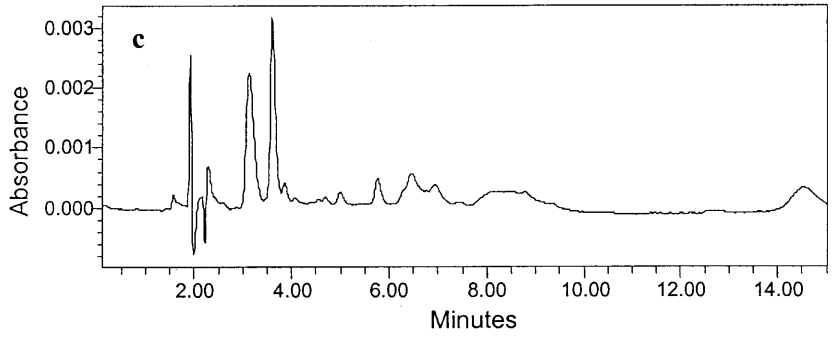
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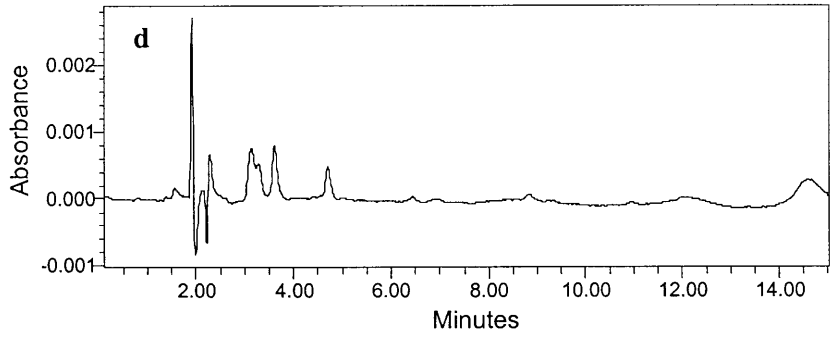
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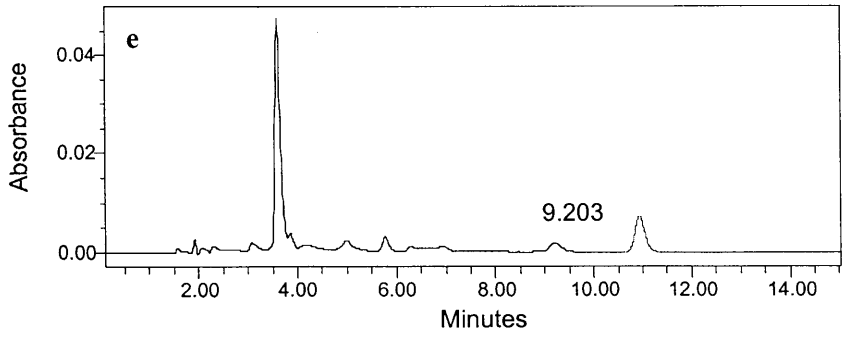
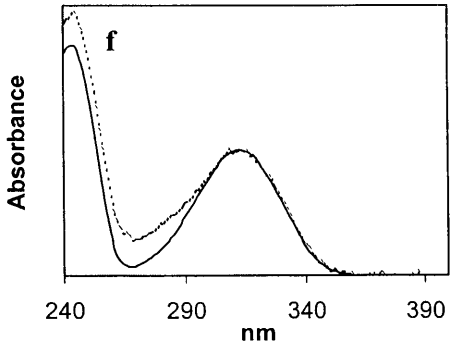

e
9.203
Absorbance
0.04
0.02
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Minutes


f
Absorbance
240
290
340
390
nm

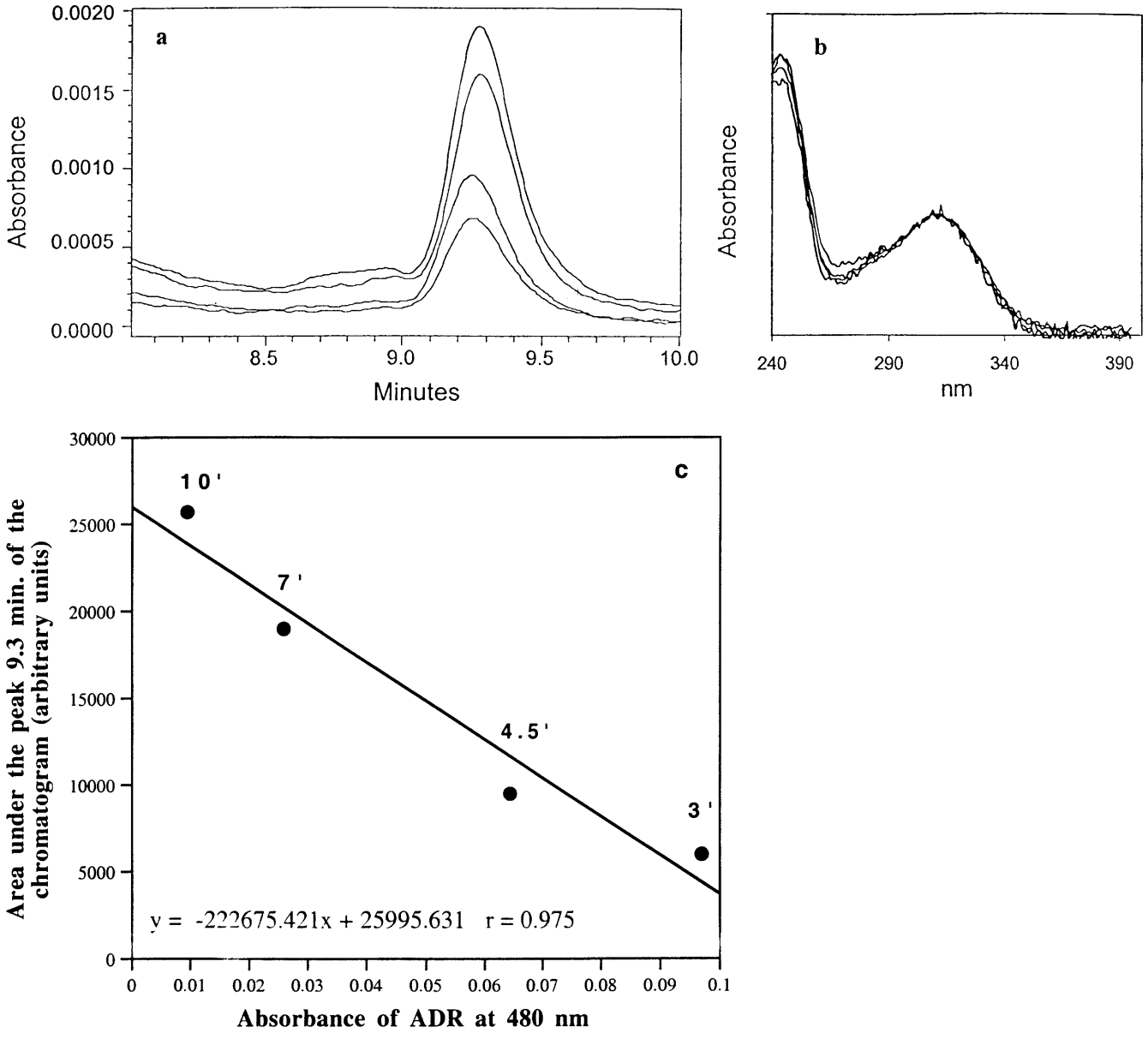


Fig. 5a–c The effects of UVA irradiation (4.50–4.52 mW/cm^2) of 20 μ*M* ADR with 2 μ*M* riboflavin and 5 m*M* imidazole in PBS (pH 7.2). **a** The size of the 9.3-min peak of the chromatogram (as described in Fig. 4) measured after 3, 4.5, 7 and 10 min of irradiation. **b** The relative absorbance spectra of the 9.3-min peaks obtained after these irradiation times. **c** The relationship between the absorbance of ADR at 480 nm in the water phase remaining after ethyl acetate extraction and the area under the 9.3-min peak of the chromatograms of the corresponding ethyl acetate extractables redissolved in methanol. The length of the UVA irradiation is shown above the corresponding data points. In this experiment the retention time of 3-methoxysalicylic acid was 9.258 min

cluded that the bleaching of RNO could be used to investigate whether *trans*-annular peroxides of histidine might play a role in the riboflavin-mediated photobleaching of ADR. As shown in Table 2, RNO specifically blocked the enhancement by histidine of the riboflavin-mediated photobleaching of ADR and the nature of this blockade indicated a competition between RNO and ADR for oxidation by the *trans*-annular peroxide of histidine (Table 3). These findings strongly suggest that although not oxidized to an apparent degree by $^1O_2$, ADR is readily oxidized by the product of the interaction between $^1O_2$ and histidine. We speculate that this might be due to differences in the time-lives of $^1O_2$ and of the *trans*-annular peroxides and/or in their binding affinities to ADR.

It has been reported by Hartman et al. that out of 30 imidazole compounds tested, 4-imidazole acetic acid is the strongest enhancer of the rose bengal-mediated photobleaching of RNO [8]. Among the imidazole analogs tested in the present study, it was also found to be the strongest enhancer of the riboflavin-mediated photobleaching of RNO. However, it was not the strongest enhancer of the riboflavin-mediated photobleaching of ADR (Table 4). These results suggest that the relative ability of *trans*-annular peroxides of

imidazole compounds to oxidize various substrates may also depend on their specific ability to interact with a particular substrate. This proposal is further supported by the finding that urocanic acid, although having a similar activity to that of histidine, imidazole or 4-imidazole carboxylic acid in enhancing the riboflavin-mediated photobleaching of RNO, was by far the strongest enhancer of the riboflavin-mediated photobleaching of ADR ($T_{1/2}$ 0.12 min; Table 4).

Urocanic acid was also found to inhibit the conversion of light-excited riboflavin to lumichrome. As the conversion of light-excited riboflavin to lumichrome was not affected by 4-imidazole carboxylic acid and accelerated by 4-imidazole acetic acid, while both had a similar enhancing activity in the riboflavin-mediated photobleaching of ADR, these effects seem unrelated. It is therefore also unlikely that the ability of urocanic acid to slow the production of lumichrome has an important role in its enhancement of the riboflavin-mediated photobleaching of ADR. Although we found that RNO was able to inhibit the urocanic acid enhancement of the riboflavin-mediated photobleaching of ADR, the results indicate that urocanic acid enhanced the bleaching of ADR to a larger extent than the bleaching of RNO. These findings suggest that ADR is a better substrate than RNO for oxidation by the *trans*-annular peroxides of urocanic acid and/or that the enhanced bleaching of ADR by urocanic acid might proceed also by additional pathways. As shown in Fig. 3, at concentrations below 20 μM, urocanic acid inhibited the riboflavin-mediated formation of superoxide anion radicals and/or quenched them. It can therefore be concluded that at least at low concentrations, the effects of urocanic acid on superoxide anion radicals may have mediated part of the enhancement of the riboflavin-mediated photobleaching of ADR. However, it cannot be ruled out that the oxidation of ADR by the *trans*-annular peroxides of urocanic acid is also more efficient than the drug's oxidation by *trans*-annular peroxide of histidine or of the other imidazole analogs.

The *trans* isomer of urocanic acid is an endogenous skin chromophore and is considered to be a major absorbent of ultraviolet light in the range 290–320 nm [7, 9]. The conversion of *trans*-urocanic acid to the *cis* isomer by ultraviolet light has been suggested to be involved in mediating the immune suppression effect of this light [15]. However, as indicated by these investigators the photoprotective role of *trans*-urocanic acid is not fully understood. As previously suggested by Morrison and Deibel, the present study showed that urocanic acid can also react with $^1O_2$ and with superoxide anion radicals [11]. Both of these effects could account for the enhancement of the riboflavin-mediated photo-oxidation of ADR by urocanic acid. In the presence of urocanic acid the photoactivation of riboflavin at biologically relevant concentrations (about 0.5 μM) resulted in rapid oxidation of ADR ($T_{1/2}$ in the range of minutes). The presence of urocanic acid in the skin suggests that such rates of ADR oxidation could occur if patients treated with this drug were exposed for even a short period of time to sunlight or for more extended periods to cool-white fluorescent light (for details see Discussion in reference 1).

Imidazoles are not an exclusive source of *trans*-annular peroxides. It has been shown that $^1O_2$ can undergo, a [4 + 2] cycloaddition with a large variety of 1,3-dienes to form the corresponding *trans*-annular peroxides in a process analogous to the Diels-Alder reaction [5]. It has been reported that in addition to photoreactions, $^1O_2$ can also be generated chemically in biological systems [2]. These findings suggest that ADR might be oxidized in vivo by a variety of *trans*-annular peroxides and that these processes are not necessarily dependent on light activation. In addition, *trans*-annular peroxides could also be synthesized by reactions not involving $^1O_2$, such as the reaction between ground-state triplet oxygen and radical cations or diradicals. A biologically relevant example for such a process are the intermediate endoperoxides formed in the bioconversion of polyunsaturated fatty acids into prostaglandins [4]. It is suggested that the light-dependent or -independent formation of *trans*-annular peroxides could result in ADR oxidation in vivo. However, as indicated by the formation of 3-methoxysalicylic acid (Figs. 4 and 5), the process of ADR degradation by *trans*-annular peroxides could be similar to that of the direct oxidation of ADR by the excited triplet riboflavin [12].

References

1. Bomgaars L, Gunawardena S, Kelly SE, Ramu A (1997) The inactivation of doxorubicin by long ultraviolet light. Cancer Chemother Pharmacol 40:506
2. Briviba K, Klotz L-O, Sies H (1997) Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems. Biol Chem 378: 1259
3. Chacon JN, McLearie J, Sinclair RS (1988) Singlet oxygen yields and radical contribution in the dye-sensitized photo-oxidation in methanol of esters of polyunsaturated fatty acids (oleic, linolenic and arachidonic). Photochem Photobiol 47: 647
4. Clennan EL, Foote CS (1992) Endoperoxides. In: Ando W (ed) Organic peroxides. John Wiley & Sons, Chichester, p 255
5. Foote CS, Clennan EL (1995) Properties and reactions of singlet dioxygen. In: Foote CS, Valentine JS, Greenberg A, Liebman JF (eds) Active oxygen in chemistry. Blackie Academic and Professional, Glasgow, p 105
6. Gollnik K (1975) Chemical aspects of photodynamic action in the presence of molecular oxygen. In: Nygaard OF, Adler HI, Sinclair WK (eds) Radiation research: biochemical, chemical and physical perspectives. Academic Press, New York, p 590
7. Hanson KM, Simon JD (1998) The origin of the wavelength-dependent photoreactivity of trans-urocanic acid. Photochem Photobiol 67: 538
8. Hartman PE, Hartman Z, Ault KT (1990) Scavenging of singlet molecular oxygen by imidazole compounds: high and sustained activity of carboxy terminal histidine dipeptides and exceptional activity of imidazole-4-acetic acid. Photochem Photobiol 51: 59
9. Kammeyer A, Pavel S, Asghar SS, Bos JD, Teunissen MBM (1997) Prolonged increase of cis urocanic acid levels in human skin and urine after single total-body ultraviolet exposures. Photochem Photobiol 65: 593
10. Kraljic I (1986) Detection of singlet oxygen and its role in dye-sensitized photooxidation in aqueous and micellar solutions. Biochimie 68: 807

11. Morrison H, Deibel RM (1988) Urocanic acid photobiology. Photooxidation and superoxide formation. Photochem Photobiol 48: 153
12. Ramu A, Mehta MM, Liu J, Turyan I, Aleksic A (2000) The riboflavin-mediated photo-oxidation of doxorubicin. Cancer Chemother Pharmacol 46: 449
13. Sharpatyi VA, Kraljic I (1978) Detection of singlet oxygen in radiolysis of aerated aqueous solutions. Photochem Photobiol 28: 587
14. Tomita M, Irie M, Ukita T (1969) Sensitized photooxidation of histidine and its derivatives. Products and mechanism of the reaction. Biochemistry 8: 5149
15. Weber LJ, Whang E, De-Fabo EC (1997) The effects of UVA-I (340–400 nm), UVA-II (320–340 nm) and UVA-I + II on the photoisomerization of urocanic acid in vivo. Photochem Photobiol 66: 484