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ORIGINAL ARTICLE

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The inactivation of doxorubicin by long ultraviolet light

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Abstract *Purpose*: Irradiation of doxorubicin (DOX) dissolved in RPMI medium 1640 by long ultraviolet (UVA) light results in a rapid decline in the cytotoxic activity of the drug. The present study was designed to sort out which component(s) of this medium are associated with the UVA inactivation of DOX. *Methods*: The effects of UVA irradiation of DOX in solutions of various compositions were evaluated by measuring the changes in the drug growth inhibitory activity in P388 cells and in the DOX absorbance spectrum. *Results*: Riboflavin seemed to be the major photosensitizing component in the medium and the effect was enhanced by the presence of histidine, methionine, tryptophan and tyrosine but not by other amino acids. The changes in DOX resulting from UVA irradiation in the presence of riboflavin, were not blocked by 1,4-diazabicyclo [2.2.2]octane (5 m*M*), superoxide dismutase (300 units/ml), catalase (150 units/ml) or sodium benzoate (50 m*M*). The effects of UVA light on doxorubicin could be prevented by excess ascorbic acid. *Conclusions*: The effects of UVA on DOX are mediated by riboflavin. The photoexcited riboflavin apparently interacts directly with DOX rather than by first forming reactive oxygen species. The results suggest that the photoinactivation of DOX may involve an oxidation step. The mechanism by which certain amino acids facilitate the photoinactivation of DOX is not known. It is suggested that patient intake of riboflavin and exposure to the sun and fluorescent light could affect the outcome of anthracycline treatment.

Key words Doxorubicin · UVA · Riboflavin

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Introduction

A few studies on the effects of visible and ultraviolet light on anticancer drugs have been reported [6, 7, 11–13, 16, 19, 23–26]. Most of the reports regarding anthracycline drugs suggest that light enhances the binding of anthracyclines to DNA and membranes. However, in one study, irradiation of doxorubicin (DOX) by long ultraviolet (UVA) light resulted in reduced cytotoxic activity. The half-time for inactivation of DOX by two 15-W tubes emitting 365 nm light was reported to be 9 h [24]. In preliminary experiments, we found that when P388 murine leukemia cells cultured in the presence of DOX were exposed to UVA with a comparable intensity, the decline in the growth inhibitory activity of DOX was much faster than the previously reported rate. As these effects were observed with light intensities similar to those used to treat psoriatic patients (in combination with psoralen) and as UVA is an important component of radiation from the sun and fluorescent lamps, it seemed that a detailed study of the enhanced photodestruction of DOX may have relevant consequences. The purpose of the present study was to determine the conditions that lead to the UVA inactivation of DOX in cell culture.

Materials and methods

Chemicals and reagents

Riboflavin, ascorbic acid, sodium benzoate, 1,4-diazabicyclo-[2.2.2]octane (DABCO), superoxide dismutase (5800 units/mg protein, SOD) and catalase (2200 units/mg protein) were purchased from Sigma (St. Louis, Mo.). RPMI medium 1640, RPMI medium 1640 without riboflavin, RPMI medium 1640 without phenol red,

Dulbecco's phosphate-buffered saline (PBS) and HEPES buffer solution were purchased from GibcoBRL (Gaithersburg, Md.). RPMI medium 1640 Select-Amine Kit, which contained (a) 4 × concentrate of the medium's inorganic salt mixture, (b) 100 × concentrate of the medium's vitamin mixture and (c) 100 × concentrates of each of the medium's amino acids, was purchased from GibcoBRL. Doxorubicin HCl (Adriamycin RDF) was purchased from Farmitalia Carlo Erba (Milan, Italy).

Cell culture and measurements of growth

Cell culture was carried out as previously described [17]. Briefly, P388 murine leukemia cells were maintained in RPMI medium 1640 supplemented with 10% fetal calf serum, penicillin base (50 units/ml) and streptomycin (50 μg/ml) (all from GibcoBRL) and 10 μ*M* 2-mercaptoethanol (Sigma). An inoculum of cells was transferred to fresh medium once every 4 days to maintain exponential growth. Cell growth was assessed 4 days after seeding of 1×10^5 viable cells/ml by measuring cell density in a Coulter Counter with multisizer (Coulter Electronics, Luton, UK). The sensitivity to DOX was assessed by culturing the cells in 24-well cell culture clusters (Costar, Cambridge, Mass.) in a volume of 1 ml/well with various drug concentrations and by measuring cell density 4 days later.

Long ultraviolet light exposure

Various concentrations of DOX were dissolved in RPMI medium 1640 or other solutions as indicated below. Drug solutions, 0.2 ml/well in 24-well culture clusters without the lid, were irradiated 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 DOX solutions, measured with a Cole-Parmer 97503-00 Radiometer (Niles, Ill.) with a 365 nm sensor, was 5–6 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 exposure of the DOX solutions to UVA, 1×10^5 viable cells in 0.8 ml of the complete culture medium were added to each well, and the cell density was measured after 4 days of culture.

Spectral measurements

DOX solutions (3 ml, 20 μ*M*) were placed in 35 mm open cell-culture dishes and irradiated with the same source of UVA in a laminar flow hood for up to 20 min. After the irradiation the 350 to 600 nm absorbance spectra were measured in a double beam UV-VIS scanning spectrometer (Shimadzu Scientific Instruments, Columbia, Md.). The DOX absorbance (measured at 480 nm) decayed exponentially over the UVA irradiation time. The first-order rate constant of the decrease in DOX absorbance (K_{ADR}) was calculated. In repeated experiments, the standard deviation of this parameter was consistently < 17% of the mean value.

Results

Long ultraviolet light effect on doxorubicin growth-inhibitory activity

Figure 1 shows the effect of length of UVA irradiation of DOX, dissolved in RPMI medium 1640 or in PBS, on the growth-inhibitory effect of the drug on P388 cells added after the irradiation of the drug solution. Irradiating DOX in PBS for up to 30 min did not affect the growth-inhibitory effect of the drug, but irradiating it in RPMI medium 1640 resulted in a decrease of the drug activity that was proportional to the length of irradiation. When RPMI medium 1640 was irradiated prior to adding the drug, a reduced growth-inhibitory effect was not observed (data not shown). These findings suggested that if the UVA activated one or more component(s) of the RPMI medium 1640 that could inactivate the drug, this component decayed rapidly and therefore did not affect the DOX that was added later. Replacing the standard RPMI medium 1640 with RPMI medium 1640 without phenol red did not affect the outcome of the experiment (data not shown), indicating that the dye did not participate in the drug inactivation process.

Long ultraviolet light effect on the absorbance spectrum of doxorubicin

Because the reduction in the DOX growth-inhibitory activity after UVA irradiation may reflect structural

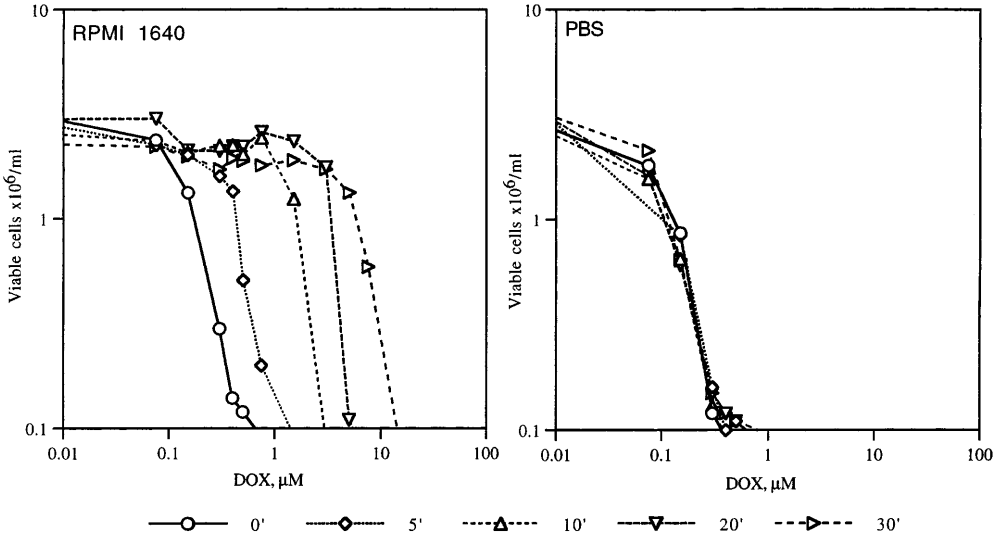

Fig. 1 The effect of length of UVA irradiation (0, 5, 10, 20 or 30 min) of DOX dissolved in 0.2 ml RPMI medium 1640 or in PBS on the growth inhibitory effect of the drug on P388 cells that were added after the irradiation in 0.8 ml growth medium

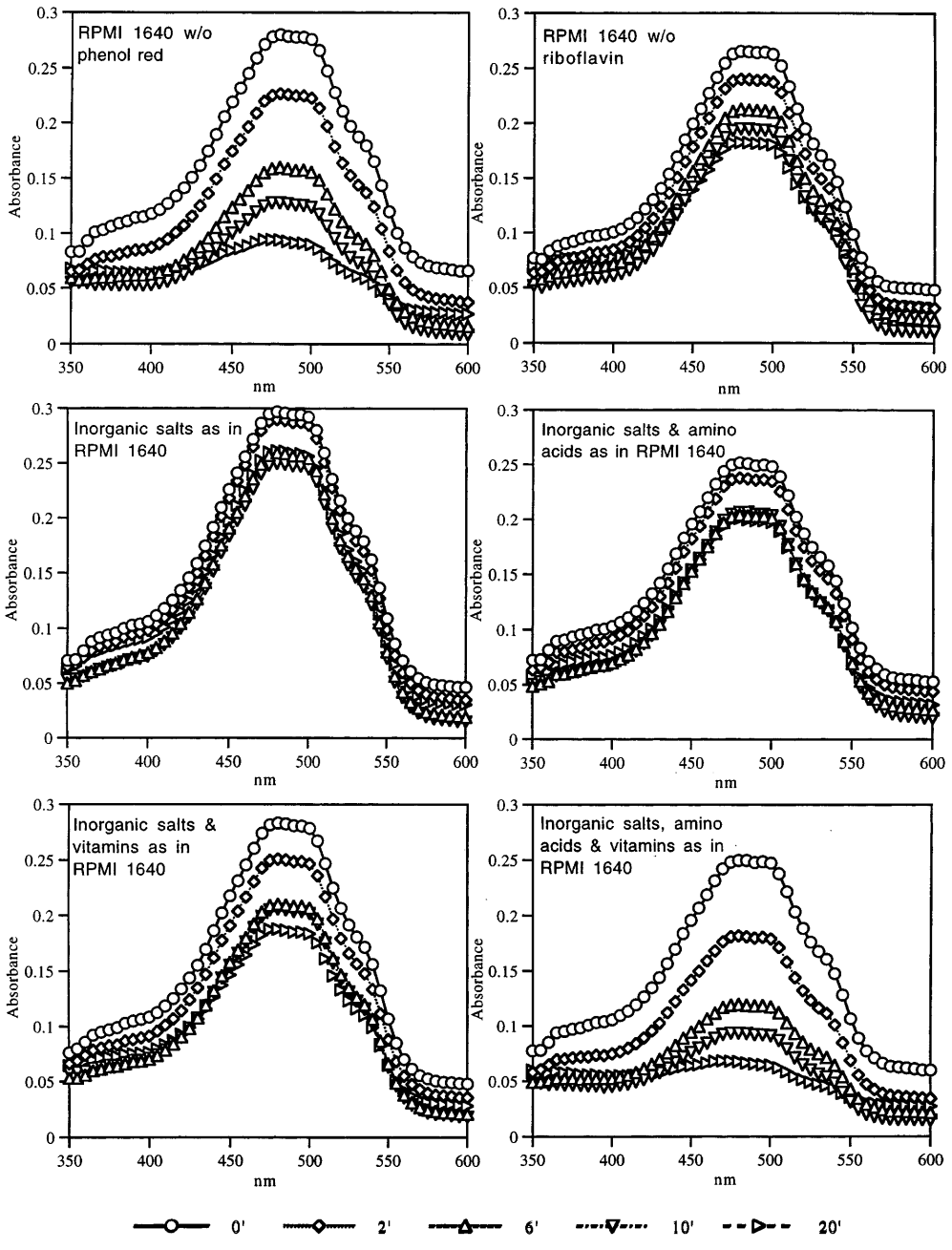

Fig. 2 The effect of length of UVA irradiation (0, 2, 6, 10 or 20 min) of 20 μ*M* DOX in different media with 50 m*M* HEPES (pH 7.2) on the absorbance spectrum of DOX

changes in the DOX molecule, the effect of UVA irradiation on the absorbance spectrum of DOX was also studied. As shown in Fig. 2, irradiating the drug in RPMI medium 1640 without phenol red containing 50 m*M* HEPES (to maintain pH 7.2) resulted in a decrease in absorbance in the 425–550 nm range. This decrease was an exponential function of the irradiation time with a first-order rate constant of 0.025/min (Fig. 3). When DOX was dissolved in a solution containing only the inorganic salts of RPMI medium 1640, the effect of UVA irradiation on the absorbance spectrum of DOX was tenfold slower (K_{ADR} = 0.002/min). Adding the 20 amino acids that are present in RPMI medium 1640 to its inorganic salts at the same concentrations as in the medium accelerated somewhat the decrease in DOX absorbance resulting from UVA irradiation (K_{ADR} = 0.004/min). Adding the vitamin mixture to the inorganic salt solution also accelerated the reaction (K_{ADR} = 0.007/min). However, when the amino acid and vitamin mixtures were added together to the inorganic salt solution, the reaction rate (K_{ADR} = 0.029/min) reached a level comparable to that obtained in the standard RPMI medium 1640.

As shown in Fig. 4, to maintain the reaction at a high rate (K_{ADR} = 0.026/min), it was sufficient to add only the following four amino acids: histidine, methionine, tryptophan and tyrosine. Furthermore, the other 16 amino acids did not accelerate the effect of UVA and

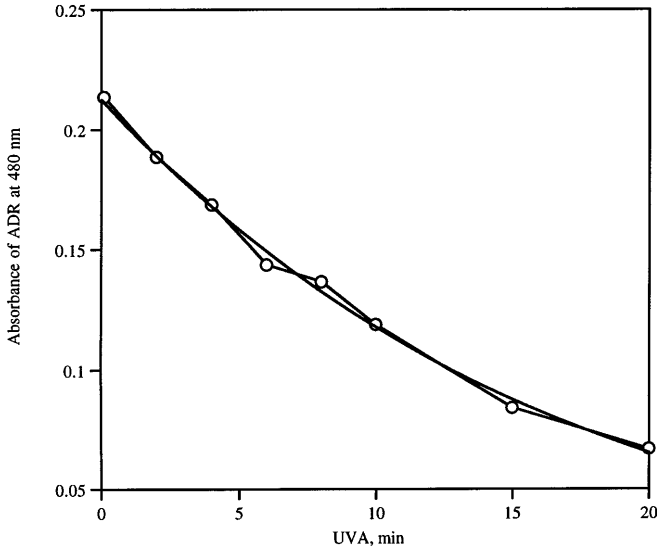

Fig. 3 The effect of UVA irradiation of 20 μ*M* DOX in RPMI medium 1640 without indicator but with 50 m*M* HEPES (pH 7.2), on the absorbance of DOX at 480 nm (equation of the curve: $y = 0.213 \times 10^{-0.026x}$)

vitamins on the absorbance spectrum of DOX (K_{ADR} = 0.003/min). Without the vitamin mixture, histidine, methionine, tryptophan and tyrosine did not enhance the UVA irradiation effect on DOX (K_{ADR} = 0.002/min, data not shown). These results indicate that the effect of UVA on DOX in RPMI medium 1640 is carried out through some component(s) of the vitamin mixture serving as a photosensitizer and that the photosensitizer effect is enhanced by at least one of the amino acids histidine, methionine, tryptophan or tyrosine.

Riboflavin is a well-known photosensitizer [9]. Adding riboflavin at the same concentration as in RPMI medium 1640 (0.5 μ*M*) to the inorganic salt mixture somewhat accelerated the reaction (K_{ADR} = 0.006/min). However, if histidine, methionine, tryptophan and tyrosine were also added, the reaction was highly accelerated (K_{ADR} = 0.022/min). The effect of UVA irradiation on DOX dissolved in RPMI medium 1640 without riboflavin (Fig. 2) proceeded at a rate (K_{ADR} = 0.007/min) significantly slower than that observed in the standard RPMI 1640. Similarly, the addition of riboflavin lacking vitamin mixture to the inorganic salt mixture (Fig. 4) resulted in marginal acceleration of the reaction (K_{ADR} = 0.004/min), which was not enhanced by adding histidine, methionine, tryptophan and tyrosine (K_{ADR} = 0.005/min).

Mediation of long ultraviolet light effect on doxorubicin by riboflavin

Although many compounds have been shown to be modified by UVA and riboflavin [9], DOX has never been identified as such a compound. The effect of UVA irradiation of 20 μ*M* DOX, 20 μ*M* riboflavin or 20 μ*M* DOX with 20 μ*M* riboflavin in PBS on their absorbance spectra was also measured (Fig. 5). In the presence of riboflavin, the UVA irradiation caused a much larger decrease in the absorbance of DOX than in its absence.

Effects of ascorbic acid, DABCO, SOD, catalase and sodium benzoate

When the experiment described above was repeated after the addition of 2 m*M* ascorbic acid, the absorbance spectrum of DOX irradiated in the presence of riboflavin did not decline (data not shown), indicating that ascorbate protected DOX from UVA. The effect of ascorbate in protecting DOX from UVA is further demonstrated in Fig. 6, which shows that UVA irradiation of 20 μ*M* DOX in RPMI medium 1640, which contains no ascorbic acid, resulted in decreased DOX absorbance. However, in the presence of 0.16 m*M* ascorbate this decrease was smaller and in the presence of 1.6 m*M* ascorbate, even after 20 min irradiation, there were no changes in the absorbance spectrum of DOX.

Reactive oxygen species participate in certain photosensitizing reactions [1, 8–10, 15]. Therefore, the effects of 5 mM DABCO (quenches singlet oxygen), 300 units/ml SOD (removes superoxide anion radicals), 150 units/ml catalase (decomposes hydrogen peroxide), or 50 m*M* sodium benzoate (quenches hydroxyl radicals) on the rate of destruction of DOX (20 μ*M*), dissolved in RPMI medium 1640 or in PBS (with 20 μ*M* riboflavin) by the UVA irradiation were tested. None of these compounds reduced the rate of destruction of DOX by UVA irradiation (data not shown).

Discussion

The present studies demonstrate that the rate of change caused by UVA light in DOX cell growth-inhibitory activity and absorbance spectrum is dependent on the composition of the solution. When the drug was dissolved in nanomolar quantities in a 1-mm-thick PBS solution, its cell growth-inhibitory activity was not reduced by exposure to 20 J of UVA energy. However, in RPMI medium 1640 this irradiation energy was sufficient to destroy about 90% of the drug activity (Fig. 1). When 60 nmol of DOX dissolved in 3 ml (3.75 mm thick, 962.1 mm^3, 0.84 mW/nmol DOX) RPMI medium 1640 was exposed to 80 J UVA, the drug absorbance between 425 and 550 nm was reduced to background level. When DOX was dissolved in a solution that contained only the inorganic salts of RPMI medium 1640 or in PBS, this UVA energy had only a minor effect on the drug absorbance spectrum.

To determine which of the 38 components of the RPMI medium 1640 mediate the effect of UVA irradiation on DOX, studies were carried out in solutions that contained only some of the medium components. These studies revealed that the major mediator of the effect of

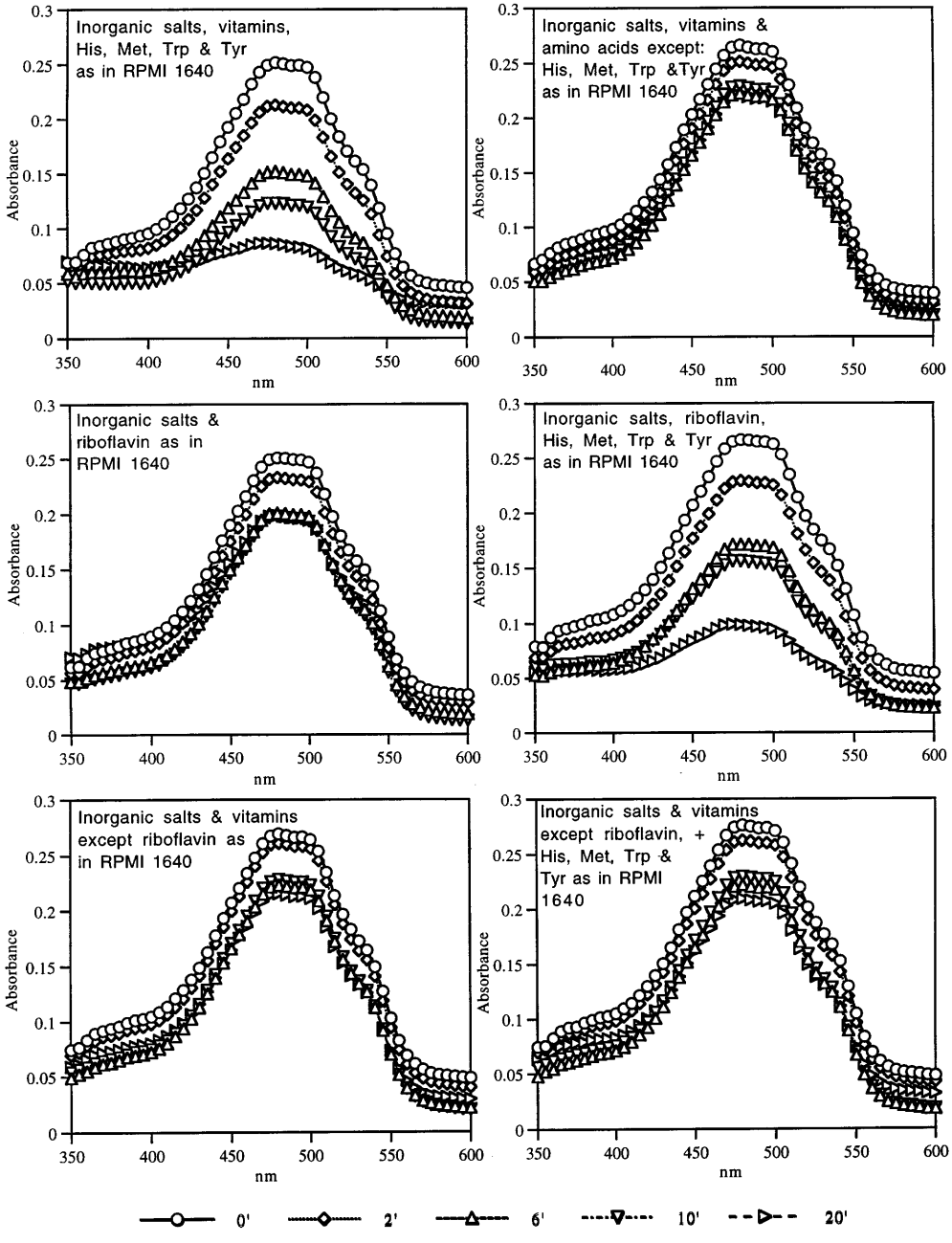

Fig. 4 The effect of length of UVA irradiation (0, 2, 6, 10 or 20 min) of 20 μ*M* DOX in different media with 50 m*M* HEPES (pH 7.2) on the absorbance spectrum of DOX

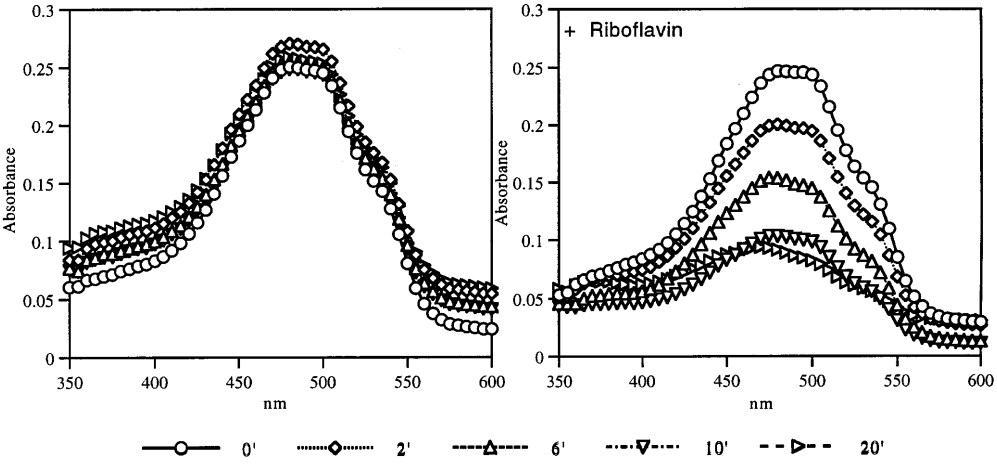

Fig. 5 The effect of length of UVA irradiation (0, 2, 6, 10 or 20 min) of 20 μ*M* DOX dissolved in PBS, in the absence or presence of 20 μ*M* riboflavin, on the absorbance spectrum of DOX

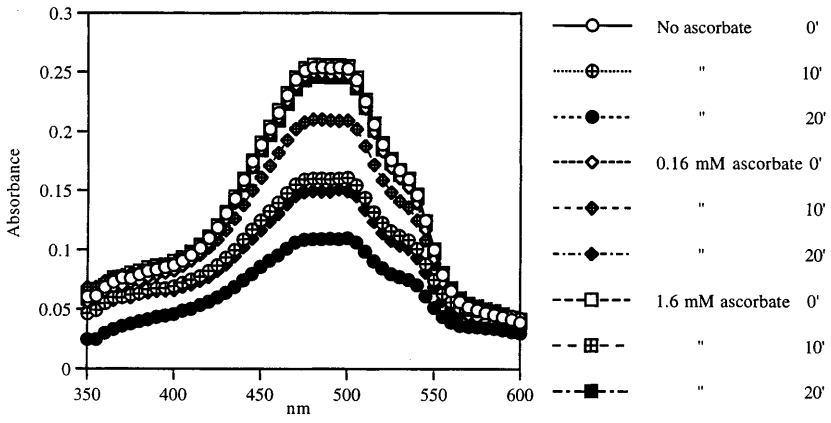

Fig. 6 The effect of the presence of ascorbic acid on the changes that UVA irradiation induce in the absorbance spectrum of 20 μ*M* DOX irradiated in RPMI medium 1640 without phenol red

UVA on DOX was riboflavin, a well-known photosensitizer [8, 9]. Excited riboflavin, like other photosensitizers, can interact directly with a large variety of molecules (reaction type I) or indirectly after converting ground-state molecular oxygen to a variety of reactive oxygen species (reaction type II) [10]. As DABCO, SOD, catalase or sodium benzoate did not reduce the effects of UVA irradiation on DOX in the presence of riboflavin, it seems unlikely that the destruction of DOX is mediated by a type II reaction. It has been long known that riboflavin, excited by 365 nm light, can undergo photoreduction while oxidizing certain substrates [2]. Therefore, the changes in the cell growth-inhibitory activity and in the absorbance spectrum of DOX after UVA irradiation in the presence of riboflavin could reflect oxidation of the drug. The observation that a reducing agent, ascorbic acid (1.6 m*M*), blocked the changes in the absorbance spectrum of DOX (20 μ*M*) irradiated in the presence of 20 μ*M* riboflavin (Fig. 6) could be interpreted as a competition between oxidizable substrates for the limited availability of an oxidizing agent (e.g. the UVA excited riboflavin).

The high ascorbate/DOX concentration ratio necessary for blocking the effect of UVA on DOX could be related to the tendency of DOX to form complexes with riboflavin via π–π ring stacking interaction [14], Although it is not yet certain whether DOX is indeed oxidized by UVA and riboflavin, it has been previously shown that DOX can undergo oxidation at its 6-hydroxy residue [3]. However, such a change would be expected to cause some shift in the DOX absorbance spectrum and not a reduction without a shift, as was observed in the present study. Alternatively, the effect of UVA and riboflavin on DOX may involve other chemical reactions, e.g. polymerization. DOX polymers have been obtained, with a very low quantum efficiency, by irradiating DOX with UVA in the absence of a photosensitizer [24].

In the presence of a photosensitizer, the only amino acids that are known to be affected by UVA irradiation at an appreciable rate are histidine, methionine, tryptophan and tyrosine [10]. The photosensitizing effect of riboflavin was indeed greatly enhanced in the presence of these (and not the other) amino acids at concentrations the same as in RPMI medium 1640 (Fig. 3). Light-excited riboflavin has been shown to interact with certain amino acids, such as tyrosine, through type I and type II interactions [20]. However, the mechanism by which histidine, methionine, tryptophan and tyrosine enhance the UVA and riboflavin effect on DOX remains unknown.

Plasma levels of riboflavin tend to be variable and reflect current intake [4, 22]. Furthermore, many cancer patients treated with chemotherapy take also megadose vitamins [5]. In addition, it seems that the degree of exposure to natural and artificial light could also affect the riboflavin blood level [18]. Of the total electromagnetic energy that is emitted by the sun and reaching sea level, 7% is in the UVA range (320–400 nm). Furthermore, UVA is also a significant component of light emitted by cool-white fluorescent tubes [21]. To what extent riboflavin intake and light exposure of a patient affects the outcome of treatment with anthracycline and other anticancer agents, is therefore an open question.

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