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The riboflavin-mediated photooxidation of doxorubicin

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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 spectromograms 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

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 hexachloro-ridate, 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 9750-00 radiometer (Niles, Ill., USA) with a 365 nm sensor, was 4-6 mW/cm². 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-36, 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 multisolvant 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 re-dissolved 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 T1/2) 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

![Graph](image-url)  
**Fig. 1** The effect of UVA irradiation (5 mW/cm²) 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 T1/2).