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Oxidative DNA damage following photoexcitation of daunomycin: Direct role of oxygen

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ABSTRACT

The chemistry of the photoactivation of daunomycin–DNA complexes is reported and the mechanism is elucidated. We quantitatively assessed the type of DNA damage, such as strand breaks, oxidized bases, and abasic sites, that arise using a plasmid relaxation assay coupled with DNA repair endonucleases. Photoexcitation of daunomycin leads to oxidative DNA damage in a dose- and irradiation time-dependent manner and guanine-specific oxidized purines are substantially produced under these conditions. Oxidative DNA base damage was also inhibited by argon degassing, indicating that guanine-specific damage arises from an oxygen-dependent mechanism. In addition, photoexcitation of daunomycin–DNA complexes leads to superoxide anion radical formation. From these studies of the actual product formed, we conclude that a charge transfer is a main driving force of the mechanism.

1. Introduction

The extensively used anthracycline antibiotics adriamycin and daunomycin (DM) are effective anti-cancer drugs used to treat a wide spectrum of cancers including solid tumors and leukemia. Their effectiveness is believed to stem from their direct interaction with DNA and these interactions have been well-characterized [1–4]. The planar anthraquinone chromophore of DM strongly intercalates DNA through the minor groove with a preferred binding site for guanine–cytosine base pairs [5,6]. Although the precise basis for its anti-tumor activity remains unclear, DNA intercalation inhibits DNA processing enzymes and interferes with topoisomerase II leading to DNA double strand breaks [7,8]. In addition, in the presence of formaldehyde, reductively active DM and adriamycin can form covalent DNA adducts which may also contribute to their cytotoxicity [9,10]. The quinone moiety of the anthracycline antibiotics and other anthraquinone derivatives have been shown to be redox active under reducing and photoirradiation conditions leading to the formation of reactive oxygen species (ROS) [11–14].

The cytotoxicity of DM is known to be enhanced when exposed to visible light [15,16]. Recently, the timescale of photoactivation and charge transfer between DM and the DNA base guanine was determined to be through a charge transfer mechanism [17]. Time resolved dynamic experiments revealed that photoexcitation leads to an ultrafast electron transfer from guanine to DM on the femtosecond to picosecond timescale (Scheme 1). In the absence of molecular oxygen, guanine and DM return to their initial uncharged state. However, under aerobic conditions, the electron from the reduced form of DM is presumably transferred to oxygen forming a superoxide radical anion. Overall, there is a net transfer of an electron to molecular oxygen forming a guanine radical cation and a superoxide radical anion. Based on this mechanism, oxidative DNA damage would be expected to occur in an oxygen-dependent manner.

To our knowledge, there is no report of the DNA damage profile that results from photoexcitation of DM and DNA or direct identification of the oxygen species involved. Using a plasmid relaxation assay coupled with DNA repair endonucleases, we were able to identify the major type of DNA damage that arises under these conditions. Here, we show that UVA photoexcitation of DM–DNA complexes leads to the oxidation of DNA with high levels of guanine-specific oxidative damage that depend on the presence of molecular oxygen. In addition, we demonstrate that irradiation leads to the formation of the reduced oxygen species superoxide radical.

Abbreviations: DM, Daunomycin; DNA, deoxyribonucleic acid; hOGG1, human 8-oxoguanine DNA glycosylase 1; Endo III, endonuclease III; APE1, human apurinic endonuclease 1; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ROS, reactive oxygen species; CT, calf thymus; SOD, superoxide dismutase.

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$$\text{Daunomycin (DM)} \xrightarrow{h\nu} \text{DM}^*$$

$$\text{DM}^* + \text{dG} \longrightarrow \text{DM}^{\bullet-} + \text{dG}^{\bullet+}$$

$$\text{DM}^{\bullet-} + \text{O}_2 \longrightarrow \text{DM} + \text{O}_2^{\bullet-}$$

Scheme 1. Schematic representing the electron transfer mechanism from the DNA base guanine to photoexcited DM and the role of oxygen in this process.

2. Materials and methods

2.1. Materials

Daunomycin was purchased from Sigma Chemical Co. (USA) or Molecular Technology, Inc. (Moltox, NC, USA). Cytochrome *c* from horse heart, and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma Chemical Co. Calf thymus (CT) deoxyribonucleic acid, sodium salt was supplied from Invitrogen. Supercoiled ΦX174 RF I phage and pBR322 plasmid DNA, hOGG1, endonuclease III, and APE1 were obtained from New England Biolabs.

2.2. The plasmid relaxation assay to monitor DM-induced DNA strand breaks

Samples containing 600 ng of ΦX174 RF I phage DNA, 2 mM phosphate buffer (pH 7.4), and up to 100 μM of daunomycin were irradiated for the indicated amount of time on ice in a Rayonet Photochemical Reactor (The Southern New England Ultraviolet Company) equipped with 350 nm lamps. Aqueous DNA samples were ethanol precipitated upon addition of sodium acetate (pH 5.0) and then resuspended in 2 mM phosphate buffer (pH 7.4). The DNA concentration of each reaction mixture was determined on a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) to ensure that equal amounts of DNA were loaded into each well. Samples were mixed with 10 μL of loading buffer (1% sodium dodecyl sulfate, 50% glycerol, and 0.1% bromophenol blue) and loaded onto a 1.2% (w/v) agarose gel in TAE buffer (0.04 M Tris base, 0.02 M acetate, and 1 mM EDTA; pH 8.0). After running the gel at a constant voltage of 35 V for 3 h, the gel was stained in an ethidium bromide solution (1 μg/mL) for 30 min and visualized with a UV transilluminator. The gel was photographed on Polaroid 665 film and the ratio of open circular DNA to the total amount of DNA was quantified using the ImageJ (NIH) software. The values for supercoiled DNA were multiplied by 1.66 to correct for the decreased intercalating ability of ethidium bromide. The graphical data represent the average from at least three experiments and error bars are standard deviations.

2.3. Using DNA repair endonucleases to characterize the DNA damage profile

Irradiated daunomycin-treated DNA samples each received 2 units of the repair enzymes hOGG1, APE1, or endonuclease III (Endo III) and appropriate reaction buffer recommended by the manufacturer. After incubation at 37 °C for 30 min, the samples were loaded onto a 1.2% agarose gel. For anaerobic conditions, pBR322 plasmid DNA samples containing 50 μM DM were purged with argon gas and irradiated for 20 min. The supercoiled and open circular DNA were separated using agarose gel electrophoresis. In the experiment to test the involvement of singlet oxygen, water in the buffer was replaced by D_2O such that the final concentration (v/v) was 45%.

2.4. Spectroscopic measurements to detect superoxide radical formation using cytochrome c

The concentration of CT DNA was measured per base pair by measuring the absorbance at 260 nm and using a molar extinction coefficient of 13,200 M^{-1} cm^{-1}. Solutions contained CT DNA (100 μM), 10 μM DM, 50 μM cyt *c*, 0.010 mM EDTA, and 2 mM phosphate buffer (pH 7.4) were irradiated for 1 h in the presence or absence of SOD (500 units/mL). After irradiation, a Jasco V-660 UV–vis spectrophotometer was used to record the absorption spectra in a cell with a path length of 1 cm.

3. Results and discussion

3.1. Photoexcited DM induces oxidative DNA damage that is dose- and irradiation time-dependent

The objective of this work was to examine the types of damage induced by photoactivated DM in order to gain insight into its mechanism of action. We first examined if irradiated DM can cleave purified DNA. The plasmid relaxation assay was used to assess the level of single strand breaks by monitoring the conversion of open circular DNA from supercoiled plasmid DNA, which can be separated by agarose gel electrophoresis. Commercially supplied plasmid DNA irradiated with UV light showed about 20% open circular DNA (Fig. 1). Upon irradiation in the presence of DM, there was a slight increase in the level of damage with increasing DM concentration. However, with increasing irradiation time, there is a distinct effect in which spontaneous cleavage of supercoiled plasmid DNA occurs in an irradiation time-dependent manner. By 90 min of irradiation, most (80%) of the plasmid DNA is in the open circular form. It should be noted that there was no observed change in the amount

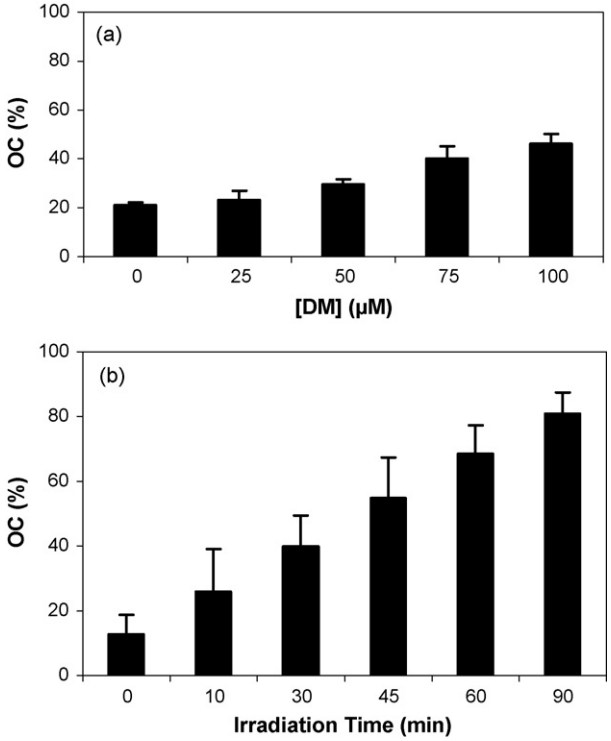


Fig. 1. Histogram showing photoinduced cleavage of ΦX174 phage DNA by DM. The percent of open circular (OC) DNA was plotted as a function of (a) increasing concentrations of DM irradiated for 30 min and (b) increasing photoirradiation time in the presence of 100 μM DM.

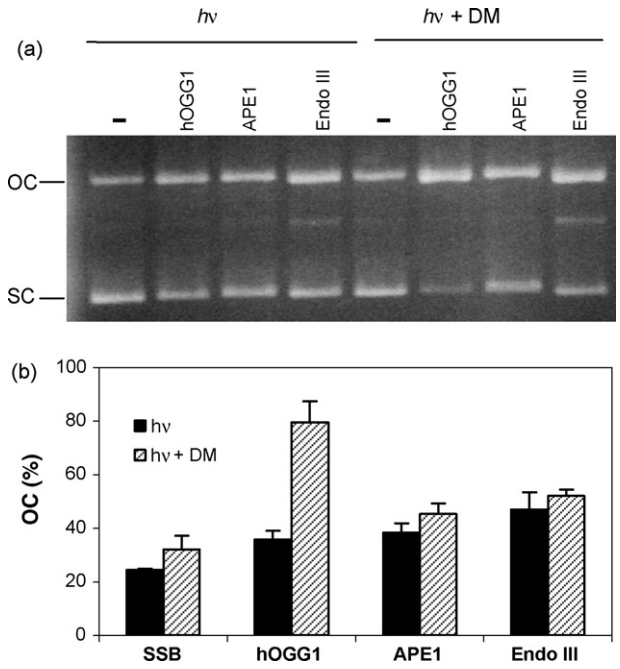


Fig. 2. Characterizing the DNA damage profile induced by photoactivated DM using DNA repair endonucleases. Supercoiled DNA was irradiated in the presence or absence of 50 μM DM following treatment with the indicated repair enzymes hOGG1, APE1, and Endo III. (a) Photograph of the agarose gel showing the separation of supercoiled (SC) and OC forms of plasmid DNA and (b) histogram of percent OC DNA. Samples not treated with an endonuclease are representative of single strand breaks (SSBs).

of supercoiled DNA when the plasmid was incubated with DM alone and a slight increase (5%) in DNA strand breaks when the plasmid was irradiated alone. The results of photocleavage of supercoiled plasmid DNA are consistent with other quinone containing derivatives [18].

To further characterize the DNA damage profile, we used DNA repair endonucleases to convert oxidized purine or pyrimidine bases, and abasic sites into DNA strand breaks. Employing repair enzymes with the plasmid relaxation assay has been a successful method to fingerprint damage induced by numerous DNA damaging compounds and reactive oxygen species [19,20]. The DNA glycosylase hOGG1 is specific for 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and other damaged guanine derivatives while Endo III and APE1 specifically recognize a variety of oxidatively damaged pyrimidine bases and abasic sites (AP sites), respectively. The DNA damage profile shows that enzyme treatment of irradiated plasmid DNA slightly increased the level of strand scission over that of single strand breaks (SSBs), with Endo III treatment showing the largest increase, indicating that UV light induces some DNA base oxidation (Fig. 2). In the presence of photoactivated DM, there are no major changes in the amount of base modifications sensitive to Endo III and APE1. However, treatment of photoactivated DM–DNA complexes with hOGG1 results in a large increase in open circular DNA compared to UV light alone. These results indicate that oxidized purine residues were the major lesions produced under cell-free conditions while oxidized pyrimidine bases and abasic sites were not a prominent form of damage.

3.2. The role of oxygen in DNA damage mediated by irradiated DM–DNA complexes

To confirm that oxygen is involved in the DNA damage mechanism, the level of strand breaks and guanine-specific oxidative base damage were measured in solutions degassed with argon. Argon saturation of irradiated DM led to the formation of both open circular and linear DNA (Fig. 3A). While the level of strand scission was enhanced when the solution was depleted with oxygen, hOGG1 treatment of the irradiated complexes showed an overall reduction of open circular DNA as compared to the air saturated oxygen sample and a return to similar levels as the control (Fig. 3B). These results suggest that while the strand breaks can occur via an oxygen-independent mechanism, oxidative base damage is dependent on the presence of oxygen. Excited DM could generate strand breaks directly by abstracting a hydrogen atom from the deoxyribose moiety or from some other oxygen-independent mechanism. On the other hand, oxygen-dependent, DM mediated damage of DNA could be the result of triplet energy transfer generating the highly reactive singlet oxygen, or a charge transfer mechanism whereby the one electron oxidation of guanine leads to the formation of the DM anion radical.

Singlet oxygen has been shown to primarily produce 8-oxodG [21,22]. To determine if singlet oxygen is responsible for the observed DNA damage, reaction mixtures were incubated in D_2O, which extends the lifetime of singlet oxygen [23]. Incubating the reaction mixtures in D_2O did not lead to an increase in oxidized guanine formation or single strand breaks, which would be expected if singlet oxygen was involved. These results suggest that singlet oxygen is not the primary oxygen species involved in the damage mechanism. In addition, the diffusion process would be on a longer timescale than what was previously observed [17].

A charge transfer mechanism, where activated DM can react with DNA and abstract an electron from guanine producing a guanine cation radical, can also account for the substantial levels of photooxidative guanine base damage. Hydration of the resulting guanine cation radical followed by one electron oxidation produces 8-oxodG, the major product produced from a guanine cation radical [24]. Although the repair endonuclease hOGG1 recognizes a variety of guanine base damage, the formation of the guanine oxidation product 8-oxodG in CT DNA photoexcited with DM was confirmed using HPLC with electrochemical detection.

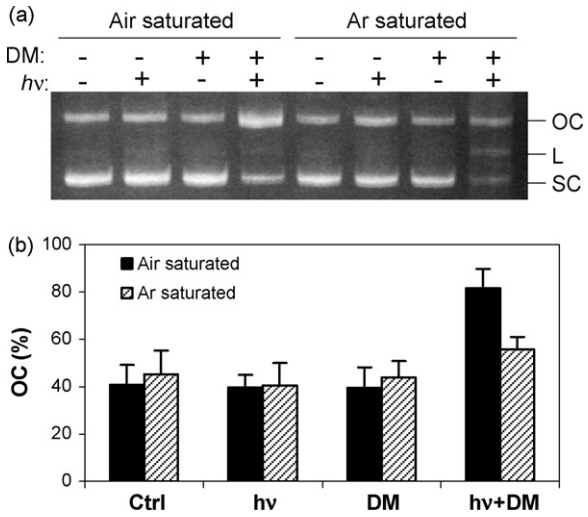
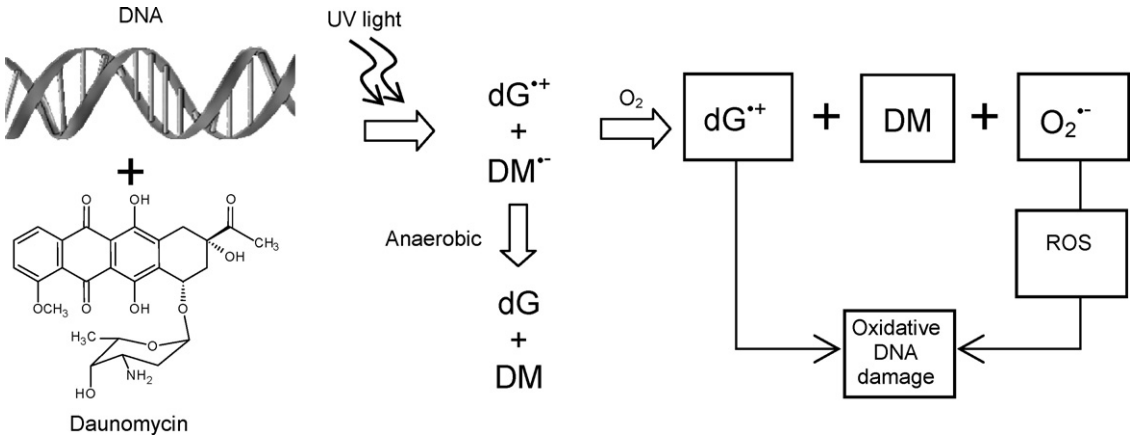


Fig. 3. The effect of oxygen on strand scission and guanine-specific oxidative DNA damage. (a) Photoinduced cleavage of pBR322 plasmid DNA under air or argon saturated aqueous conditions. OC, L, and SC refer to open circular, linear, and supercoiled forms of the plasmid DNA, respectively. (b) After irradiation, air and argon saturated reaction mixtures were treated with hOGG1. Ctrl represents control samples containing plasmid DNA alone.



Scheme 2. Proposed mechanism of DM mediated oxidative DNA damage in the presence of UV light.

3.3. Detection of superoxide radical generated from irradiated DM–DNA complexes

In a one electron transfer mechanism, the DM radical could then reduce molecular oxygen forming superoxide radical. The question of interest is can the superoxide radical be produced under these conditions. The reduction of the heme of ferricytochrome *c* produces a distinct spectral change which allows for the detection of electron transfer reactions. The production of superoxide radicals was indirectly measured by monitoring the formation of reduced cytochrome *c* at a wavelength of 550 nm in the absence and presence of SOD. Control experiments with irradiated cytochrome *c* alone or in the presence of 10 μM DM did not result in an increase in absorbance at 550 nm. However, in three independent experiments, irradiation of CT DNA–DM complexes produced a peak at 550 nm, which is characteristic of cytochrome *c* reduction. This absorption band is clearly pronounced in the difference spectra for all three measurements. Using the baseline at 550 nm, the increase in absorbance upon irradiation is measured to be $\Delta A_{550} = 0.03 \pm 0.01$, indicating a significant change in the concentration of the reduced form of cytochrome *c*. At higher irradiation dosages, the change in concentration due to the reduced form of cytochrome *c* may become even larger and its quantification as a function of irradiation dosage and DM concentration will allow us to examine the maximum yield of this reductive channel. To confirm the production of superoxide radicals, SOD, a scavenger that catalyzes the reduction of superoxide, was added to the reaction mixtures before irradiation. If the superoxide radical species is involved, the addition of SOD would inhibit the reduction of cytochrome *c*. The absorbance peak at 550 nm was suppressed when SOD was added, indicating that the one electron reduction of cyctochrome *c* is a result of the photogeneration of superoxide radicals that occur in the presence of DNA.

Evidence for a charge transfer mechanism stems from the fact that we are observing superoxide radical formation and primarily guanine-specific photooxidation that is dependent on molecular oxygen. Based on this mechanism, DM would be capable of redox cycling as it returns to its initial uncharged state after reducing molecular oxygen. Since the initial electron transfer to DM does not depend on oxygen [17], electron back transfer would occur under oxygen depleted conditions, reverting the guanine cation and DM anion radicals to their initial states. Under these conditions, the oxidation of guanine would not occur. Indeed, we observed a decrease in guanine-specific oxidation under argon saturation. However, the level of strand breaks increased, indicating that activated DM directly cleaves DNA in an oxygen-independent mechanism.

Although superoxide radicals are unreactive towards DNA, they can generate DNA damaging hydroxyl radicals through the Fenton reaction [25–27]. The question remains whether superoxide radicals play a role in DNA oxidative damage via hydroxyl radical formation. Ultimately, DNA damage could arise from two pathways: the formation of guanine radical cations and perhaps from the formation of highly reactive hydroxyl radical via superoxide radical formation (Scheme 2). Although the initial damage arises from the guanine radical cation, the production of ROS such as hydroxyl radical could also contribute to the DNA damage pathway, although to a lesser extent, by inducing spontaneous strand scission, abasic sites, and oxidative base damage. We are currently evaluating the role of ROS in oxidative DNA damage. Our results provide an alternate mechanism of action of DM under irradiated conditions and can account for the enhanced photocytotoxicity of DM.

4. Conclusions

In summary, the findings reported here demonstrate that photoactivated DM oxidatively damages DNA leading to DNA strand scission and substantial levels of guanine-specific oxidation. We have successfully confirmed that superoxide is generated under our photoirradiation conditions and that oxidative guanine damage, which was reduced by argon degassing, arises from an oxygen-dependent mechanism. The femtosecond dynamic experiments provided the timescale for charge separation, which is much shorter than any diffusion process. Our results which identify the reactive species involved and the final products are consistent with the charge transfer mechanism being involved in the initial photoactivation of DM.

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