Free Rad. Res., Vol. 25, No. 3, pp. 207–220
Reprints available directly from the publisher
Photocopying permitted by license only

Generation of Hydroxyl Radicals by Nucleohistone-Bound Metal—Adriamycin Complexes

SUBRATA CHAKRABARTI†, ASHFAQ MAHMOOD‡, AMIN I. KASSIS‡, EDWARD A. BUMP†, ALUN G. JONES‡ and G. MIKE MAKRIGIORGOS†[1]

†Joint Center for Radiation Therapy, Department of Radiation Oncology, and ‡Department of Radiology, Harvard Medical School, Boston, MA

Accepted by Prof B. Halliwell

(Received 13th February 1996; In revised form 12th March 1996)

A recently developed method has been utilized to demonstrate the generation of hydroxyl radicals ($HO^{\bullet}$) in the immediate proximity of DNA by copper(II)/iron(III)—adriamycin in the presence of ascorbate and hydrogen peroxide. SECCA, a succinylated derivative of coumarin, generates the fluorescent 7-hydroxy-SECCA following reaction with $HO^{\bullet}$. SECCA was coupled to polylysine or to histone H1 and then complexed to DNA. When $HO^{\bullet}$ was generated in the proximity of DNA by polylysine-coupled iodine-125, which emits short range Auger electrons, 7-hydroxy-SECCA was produced. DMSO was only moderately efficient in reducing the fluorescence induction, demonstrating the "local" generation of $HO^{\bullet}$ in this system. Copper(II)/iron(III)—adriamycin in the presence of ascorbate and hydrogen peroxide generated the fluorescent 7-hydroxy-SECCA both when SECCA was free in solution and when SECCA was DNA-conjugated. With SECCA free in solution, the fluorescence induction was almost eliminated in the presence of $HO^{\bullet}$ scavengers (ethanol, tert-butanol or DMSO) and the relative efficiency of the scavengers in reducing the fluorescence followed their rate constant with $HO^{\bullet}$. Furthermore, SECCA incubated with a singlet oxygen-generating compound demonstrated no fluorescence induction. When SECCA was positioned in close proximity to DNA as a SECCA-histone-H1—DNA complex, the relative efficiency of the scavengers in reducing the fluorescence still followed their rate constant with $HO^{\bullet}$; overall however the scavengers were much less effective in reducing the fluorescence, due presumably to the formation of $HO^{\bullet}$ radical in the immediate vicinity of DNA. These data suggest that copper(II)/iron(III)—adriamycin produces $HO^{\bullet}$ in the presence of ascorbate and hydrogen peroxide whether unbound or bound to DNA and suggest that in the latter case scavengers would not prevent $HO^{\bullet}$ from attacking chromatin. In addition, the ability of DMSO to trap $HO^{\bullet}$ was shown to decrease as the conformation of the H1—DNA complex becomes more compact indicating the strong dependence of the trapping ability on chromatin conformation.

Keywords: Hydroxyl radical, adriamycin, metals, DNA, histone, iodine-125

INTRODUCTION

Adriamycin belongs to a group of anthracycline antitumor antibiotics widely used in the treat-

[1]Corresponding author: G. M. Makrigiorgos, Ph.D.
Present address: Joint Center for Radiation Therapy, Department of Radiation Oncology, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA.

ment of different type of cancers. Its antitumor action is related to the blockage of replication and transcription via topoisomerase inhibition and the generation of DNA strand scissions[1–3]. A major limitation to the use of the drug is a dose-dependent cardiotoxicity which is related to its redox activity[4–6]. The formation of reactive oxygen species by adriamycin has been shown in human breast tumor cells and also in other cancer cells by using spin traps, and it has been demonstrated that catalase and superoxide dismutase have significant protective effects[7–9]. The high affinity of adriamycin for transition metals (e.g. Fe(III)[10]: $k = 2.5 \times 10^{28}$ Cu(II)[11]: $k = 4.6 \times 10^{16}$ [2:1], $k = 1.8 \times 10^{12}$ [1:1]) that easily catalyze redox reactions enhances its ability to form reactive oxygen species[12–15]. Among the redox-action-related damaging effects of adriamycin and its metal complexes are interference with electron transport in cardiac mitochondria[16], the peroxidation of cardiac lipids[17], the destruction of erythrocyte ghost membranes[18], and the generation of DNA damage[19–21]. To explain the mechanism of DNA damage, it has been suggested that the quinone moiety of the drug can undergo metabolic activation to a free radical state which may react with DNA either directly or through the generation of reactive oxygen species, e.g. hydroxyl radicals (HO•), superoxide ($O_2^{\bullet -}$)[22-24]. One problem in resolving the question of the generation of HO• in the immediate vicinity of DNA has been the difficulty in detecting this short-lived radical which can react almost immediately with the DNA moiety[25]. Thus, indirect approaches utilizing detection of the HO• fingerprint of damage on DNA have been utilized[24].

We have recently developed a direct approach that can be used to detect such *DNA-associated HO•*[26–28]. The method is based on the presence of an HO•-detecting molecule (SECCA) in the immediate vicinity of DNA. SECCA, a succinylated derivative of coumarin-3-carboxylic acid which under normal conditions is nonfluorescent, generates the highly fluorescent compound 7-hydroxy-SECCA following interaction with HO•[26]. Thus, when SECCA is complexed to DNA via polylysine (SECCA-polylysine—DNA complexes) and incubated with copper sulfate, ascorbate, and hydrogen peroxide, the generation of 7-hydroxy-SECCA reveals the presence of DNA-associated HO•[28]. In the current work we provide further verification of the ability of the system to detect DNA-associated HO•, by positioning close to DNA both the detector molecule SECCA and a radioactive nuclide (^{125}I) that emits low-energy Auger electrons. We then apply the method to examine whether HO• is generated by metal-complexed adriamycin bound to histone-H1—DNA (nucleohistone) when in the presence of ascorbate and hydrogen peroxide.

MATERIALS AND METHODS

Adriamycin, double-stranded calf thymus DNA (type XV, high molecular weight), histone (type III-S, lysine-rich fraction, mainly histone H1, mean formula weight = 21,000), copper(II) sulfate pentahydrate, hydrogen peroxide (H_2O_2), catalase, NaCl, Bolton—Hunter reagent, heavy water (D_2O), methylene blue and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Company (St. Louis, MO). Ammonium iron(III) sulfate dodecahydrate, ascorbic acid (sodium salt), DMSO, ethanol, tert-butanol, and EDTA were purchased from Aldrich Chemical Company, Incorporated (Milwaukee, WI). $Na^{125}I$ was purchased from NEN Research Products, Du Pont Company (Boston, MA). Anthracene-dipropionic acid (ADPA), a singlet oxygen probe, and 7-hydroxy-SECCA (succinimidyl ester of 7-hydroxycoumarin-3-carboxylic acid), the expected fluorescent product following hydroxylation of SECCA, were purchased from Molecular Probes (Eugene, OR). Ultrapure water (1820 MOhm m^{-1} resistivity) delivered by an Alpha-Q system (Millipore Corporation, Bedford, MA) was used throughout the work.

Labeling of Histone H1 and Polylysine with SECCA and 7-Hydroxy-SECCA and Formation of Complexes with DNA

The synthesis of the succinimidyl ester of coumarin-3-carboxylic acid (SECCA) and its conjugation with histone H1 or with polylysine (average molecular weight = 26,000) has been described previously[26]. SECCA-histone-H1—DNA complex (nucleohistone) was prepared by mixing calf thymus DNA with SECCA-histone H1 at high salt concentrations (1 mol dm^{-3} NaCl) and gradually reducing the NaCl content by dialysis[27]. Polylysine—DNA complex was prepared by the direct mixing of polylysine and DNA (1:4 ratio by weight)[28]. For fluorescence control studies, a nucleohistone sample containing SECCA plus a small amount (1%) of 7-hydroxy-SECCA was prepared as reported[27].

Synthesis of Bolton—Hunter—Polylysine Conjugate (BHPL)

To a vial containing 40 mg (1.49 µmol) poly D-lysine hydrobromide (average molecular weight = 26,900) was added 1.5 ml 10 mmol dm^{-3} phosphate-buffered saline (PBS), pH 8, followed by 2.35 mg (8.92 µmol) succinimidyl 3-(p-hydroxyphenyl)propionate (Bolton—Hunter reagent, BH) dissolved in 200 µl chilled methanol. The reaction was allowed to proceed for three hours, after which the solvent was evaporated to dryness under high vacuum at room temperature. The reaction mixture was redissolved in PBS (pH 7.4), and the modified polylysine was purified by centrifugal filtration using Centricon-10 molecular weight cutoff filters (Amicon, Incorporated, Beverly, MA). The filters were subsequently washed with 3 × 500 µl PBS (pH 7.4), followed by 3 × 500 µl distilled deionized water. Isolation of BHPL from the filters and estimation of the number of BH groups present by UV absorbance at 280 nm indicated 6 mol BH/mol polylysine.

Iodination of BHPL

To a vial containing 200 µg Bolton—Hunter-modified polylysine in 100 µl PBS (pH 9) was added 300 µCi $Na^{125}I$. To this solution was added 10 µl of a freshly prepared solution of Chloramine-T (1.5 mg/ml). The sample was vortexed and the reaction allowed to continue for 15 min, after which 10 µl sodium metabisulfite (2 mg/ml) and 10 µl KI (100 µmol dm^{-3}) were added. The reaction mixture was then carefully transferred with three separate washings to a Centricon-10 molecular weight cutoff filter. The ^{125}I-BHPL was purified by centrifugal filtration and washing with 3 × 500 µl 1 mmol dm^{-3} PBS (pH 7.0). Subsequent radioactivity measurement of the filtrate (including the three washings) and the ^{125}I-BHPL retained on the filter indicated >95% labeling efficiency.

Complexation of Adriamycin with Cu(II) and Fe(III)

Copper complexes of adriamycin were made by mixing adriamycin (6 mmol dm^{-3} dissolved in 4 mmol dm^{-3} HCl) with various concentrations of Cu(II) (10 mmol dm^{-3} copper sulfate pentahydrate dissolved in 4 mmol dm^{-3} HCl). After a 30-min incubation of the mixture, the pH of the metal—adriamycin solution was adjusted to 7.0 by the addition of 1 mol dm^{-3} NaOH. Similarly, the iron complexes of adriamycin were made by mixing adriamycin (6 mmol dm^{-3} dissolved in 4 mmol dm^{-3} HCl) with various amounts of Fe(III) (2 mmol dm^{-3} ammonium ferric sulfate dissolved in 4 mmol dm^{-3} HCl), incubating the mixture, and adjusting the pH as above.

Exposure of SECCA and SECCA-Labeled Biomolecules to Hydroxyl-Radical-Generating Agents and Fluorescence Measurements

For experiments involving ^{125}I irradiation, polylysine—DNA samples containing DNA hybridized simultaneously with SECCA-polyly-

sine and ^{125}I-polylysine (1:4 and 1:100 ratios of polylysine to DNA by weight, respectively) were dispensed in a cuvette. Irradiation of SECCA-polylysine—DNA complexes that did not contain ^{125}I was accomplished with Na^{125}I free in solution. Fluorescence measurements were obtained in a computer controlled Perkin-Elmer LS50B fluorometer (fluorescence emission at 450 nm; excitation at 400 nm, slit width 5). The fluorescence induction was followed as a function of exposure time of the reactants. The results were plotted as arbitrary units, "net relative fluorescence," the overall fluorescence detected at various time points during incubation of the reaction mixture minus the fluorescence detected at zero time point. Reproducibility was checked by repeating the experiments three times.

For experiments with metal—adriamycin complexes, 10 µl Cu(II)/Fe(III)—adriamycin complex was allowed to react for 60 min with SECCA (10 µmol dm^{-3}) or SECCA-conjugated biomolecules in the presence of ascorbate (500 µmol dm^{-3}) and hydrogen peroxide (1 mmol dm^{-3}) in 5 mmol dm^{-3} phosphate buffer (pH 7.4) and 10 mmol dm^{-3} NaCl. Immediately after adding hydrogen peroxide as the last reagent, fluorescence induction was followed as above. For experiments with EDTA and catalase, these reagents were added to the solution immediately prior to addition of ascorbate and hydrogen peroxide.

Exposure of SECCA and of ADPA to a Singlet Oxygen-Generating Agent

The system employed to generate $^1O_2$ was visible light exposure of the dye methylene blue (50 µmol dm^{-3}) dissolved in 5 mmol dm^{-3} phosphate buffer, prepared in heavy water (D_2O) under oxygen-saturated conditions. To verify production of $^1O_2$ under our experimental conditions, anthracenedipropionic acid (ADPA), a fluorescent dye, was used for the detection of $^1O_2$ (10^{-4} mol dm^{-3} dissolved in a 50-µmol dm^{-3} methylene blue solution in D_2O, in 5 mmol dm^{-3} phosphate, pH 7.3) following illumination by a 150 W halogen lamp. Singlet oxygen production was followed by the disappearance of ADPA fluorescence (excitation at 365nm, emission at 430nm). Next, the experiment was performed with SECCA replacing ADPA under the same experimental conditions. The concentration of SECCA in this experiment was 10^{-5} mol dm^{-3}. Finally, irradiation of these SECCA solutions with gamma rays was performed with a ^{137}Cs gamma cell (Atomic Energy of Canada Limited) that delivers 1.0 Gy min^{-1}.

RESULTS

Induction of 7-Hydroxy-SECCA Fluorescence by ^{125}I

The generation of 7-hydroxy-SECCA via ^{125}I-generated HO• on SECCA-polylysine—DNA complexes is plotted versus the integrated total ^{125}I decays/ml in Figure 1A. Irradiation by Na^{125}I or by DNA-bound ^{125}I-polylysine both result in linear increases in the 7-hydroxy-SECCA fluorescence. When increasing DMSO concentrations (0–0.1 mol dm^{-3}) are added during irradiation, the resulting 7-hydroxy-SECCA fluorescence is decreased relative to the absence of DMSO (Figure 4B), presumably due to the scavenging of the ^{125}I-generated-HO•. However, as shown in Figure 1B, DMSO is much less effective in decreasing fluorescence induction caused by irradiation via DNA-bound ^{125}I-polylysine than by irradiation with Na^{125}I, which is expected to distribute homogeneously in the solution.

Binding of Metals to Adriamycin and of Adriamycin to Nucleohistone

The reported quenching of adriamycin fluorescence following its complexation with metals or DNA was used to verify the formation of adriamycin complexes[29]. Figure 2A shows the fluorescence emission spectrum (excitation at 480 nm) of free adriamycin and the effect on adri-

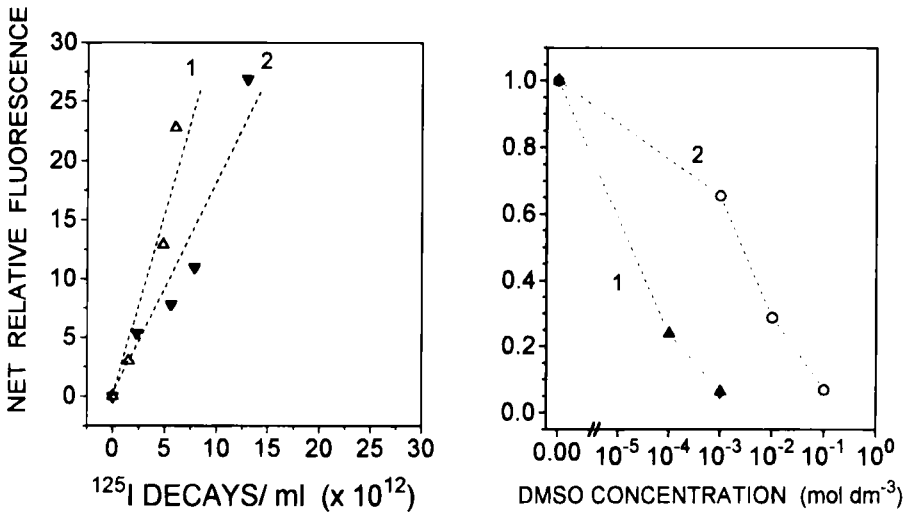

FIGURE 1 A. Induction of 7-hydroxy-SECCA fluorescence in solution containing SECCA-polylysine—DNA during decay of ^{125}I. B. Effect of [DMSO] on 7-hydroxy-SECCA fluorescence induction from ^{125}I radiation of SECCA-polylysine—DNA. Iodine is either free in solution (1) or bound to SECCA-polylysine—DNA (2).

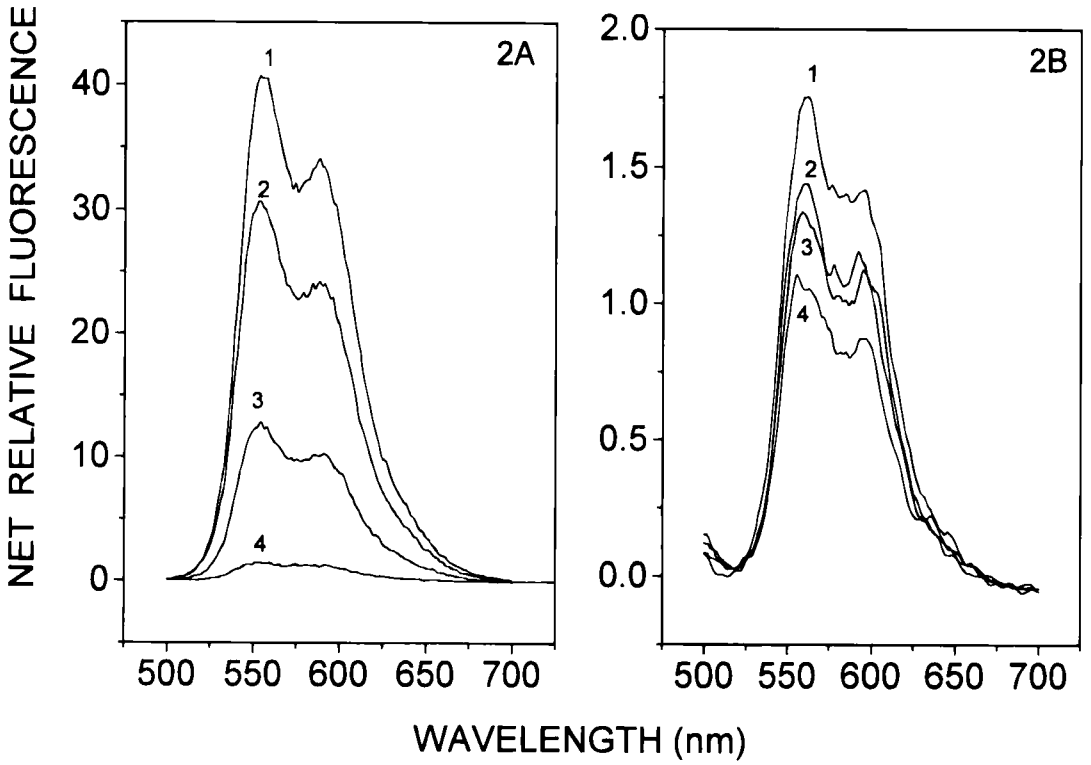

FIGURE 2 Fluorescence emission spectra (excitation = 480 nm) of adriamycin (20 μmol dm^{-3} in 5 mmol dm^{-3} phosphate buffer and 10 mmol dm^{-3} NaCl, pH 7.4). A. Complexed with increasing amounts of Cu(II): 1, 0 μmol dm^{-3}; 2, 5 μmol dm^{-3}; 3, 10 μmol dm^{-3}; 4, 20 μmol dm^{-3}. B. Complexed with Cu(II) as in A + H1—DNA.

amycin fluorescence of adding increasing concentrations of copper. Figure 2B demonstrates that the fluorescence intensity of adriamycin or its copper complexes is reduced in the presence of the H1—DNA complex. These results are in agreement with earlier fluorescence studies.[29]

Quenching of adriamycin fluorescence similar to that obtained with copper was also observed in the presence of iron at a Fe(III):adriamycin molar ratio of 1:3 (Figure 3). On the other hand the native fluorescence of adriamycin or its complexes was found to be little affected following variation of NaCl concentration (0–150 mmol dm^{-3}), whether in the presence or absence of H1—DNA complex (data not shown). This indicates the relative stability of the metal—adriamycin and adriamycin—DNA complexes at different salt concentrations.

Inability of Singlet Oxygen to Convert SECCA to 7-Hydroxy-SECCA

Because Cu has been reported to catalyze singlet oxygen ($^1O_2$) in addition to HO• in the presence of H_2O_2 and ascorbate[30] we investigated whether $^1O_2$ may also convert SECCA to 7-hydroxy-SECCA. $^1O_2$ has been shown to result in hydroxylation of aromatic rings at specific positions, although with a lower efficiency than HO• and with a different regioselectivity[31,32]. The system employed to generate $^1O_2$ was visible light exposure of methylene blue in phosphate buffer prepared in heavy water (D_2O) under oxygen-saturated conditions. D_2O allows $^1O_2$ a much longer lifetime than in H_2O and permits examination of $^1O_2$ reactions[33]. Upon exposure to visible light, methylene blue populates the triplet state and subsequently reacts with O_2 to produce $^1O_2$[33]. To verify production of $^1O_2$, anthracenedipropionic acid (ADPA) was used. ADPA reacts rapidly with $^1O_2$ to form a nonfluorescent endoperoxide[33] and the reaction is followed by the disappearance of ADPA fluorescence. Figure 4A demonstrates the rapid destruction of ADPA upon exposure to halogen lamp illumination. Consistent with the production of $^1O_2$, the destruction rate of ADPA decreased when the experiment was performed in H_2O instead of D_2O. ADPA remained intact when oxygen was excluded by continous infusion of nitrogen during the experiment (Figure 4A). Next, the experiment was performed with SECCA replacing ADPA, under the same experimental conditions (Figure 4B). Under these conditions, halogen-lamp illumination for up to 800 min showed negligible induction of fluorescence. For comparison, when a similar solution of SECCA and methylene blue in D_2O was irradiated with 0–10 Gy gamma rays, a rapid induction of fluorescence was observed (Figure 4B).

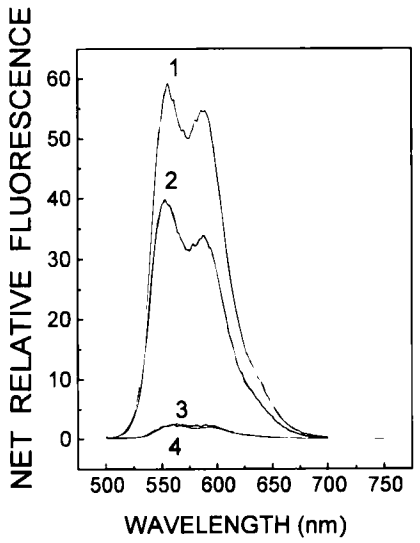

FIGURE 3 Fluorescence emission spectra (excitation = 480 nm) of: 1, adriamycin (30 μmol dm^{-3}); 2, Fe(III)—adriamycin complex (1:3); 3, adriamycin + H1—DNA; 4, Fe(III)—adriamycin + H1—DNA.

Induction of 7-Hydroxy-SECCA Fluorescence by Metal—Adriamycin Complexes

Figure 5A shows the time-dependent induction of 7-hydroxy-SECCA fluorescence following hydroxylation of free SECCA by various copper:adriamycin ratios in the presence of ascor-

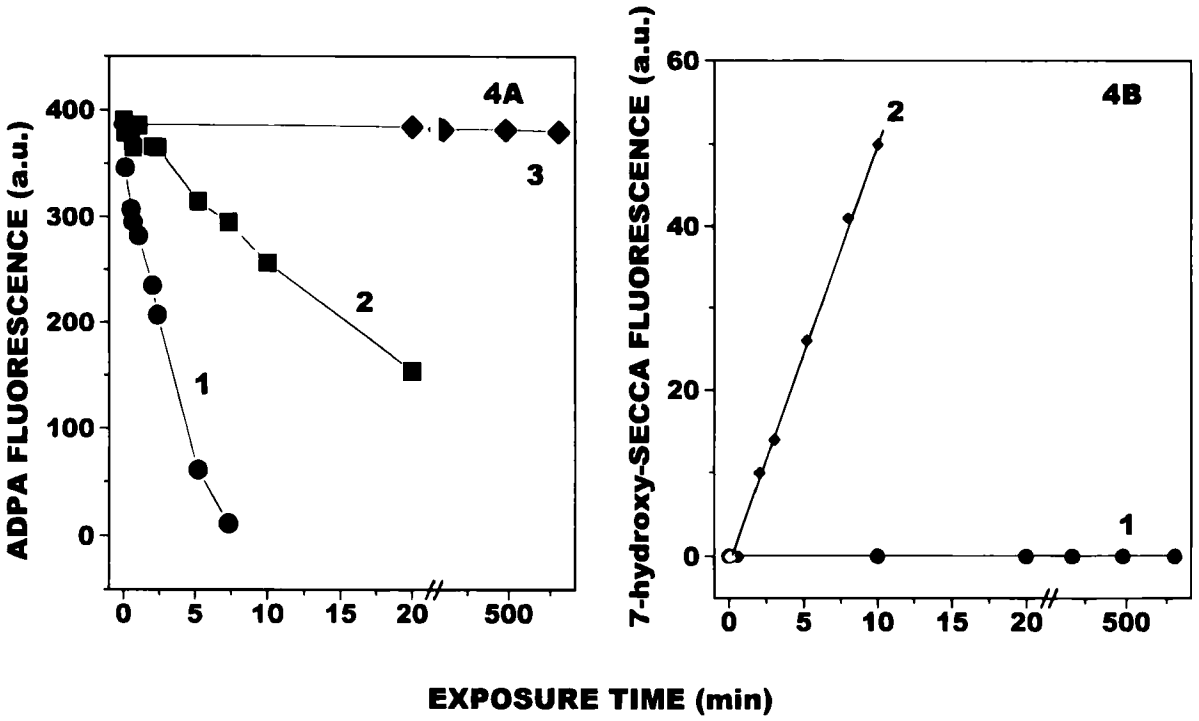

FIGURE 4 Exposure of ADPA and of SECCA to singlet oxygen generated by halogen light illumination of methylene blue. A. ADPA in D_2O, oxygen saturated (curve 1); in H_2O, oxygen saturated (curve 2); and in H_2O, nitrogen saturated (curve 3). B. SECCA in H_2O, oxygen saturated (curve 1); SECCA in same solution as for curve 1, but exposed to gamma rays (0–10 Gy) instead of halogen light (curve 2).

bate and hydrogen peroxide. In the absence of copper, no significant fluorescence induction is observed. Similar results are shown in the presence of iron—adriamycin complexes (Figure 5B). The relative ability of DMSO, ethanol, and tert-butanol to reduce the 7-hydroxy-SECCA fluorescence generated by a copper—adriamycin complex is depicted in Figure 6A. Among the three HO• scavengers, DMSO is the most efficient and tert-butanol the least. These observations are also valid for the iron—adriamycin system (Figure 6B).

The time-dependent generation of 7-hydroxy-SECCA fluorescence on a SECCA-labeled H1—DNA complex exposed to copper—adriamycin complexes (1:1 molar ratio) or iron—adriamycin complexes (1:3 molar ratio) for various ascorbate—hydrogen peroxide combinations is shown in Figures 7A and 7B. Depending on the relative amounts of ascorbate and hydrogen peroxide, the fluorescence induction can either follow a linear increase or reach a saturation level with little further increase. Figures 8A and 8B show that the excitation spectra (emission = 450 nm) following the exposure of SECCA-labeled H1—DNA to the same reagents are very similar to those obtained with a 7-hydroxy-SECCA-labeled H1—DNA complex, with or without Cu(II)/Fe(III)—adriamycin. This is an indirect verification of the generation of 7-hydroxy-SECCA from exposure of a SECCA-labeled H1—DNA complex to the reagents.

The relative ability of several modifiers—HO• scavengers (100 mmol dm^{-3}, DMSO, ethanol, and tert-butanol), catalase (8.3×10^{-5} mmol dm^{-3}), and EDTA (0.1 mmol dm^{-3})—to affect the amount of

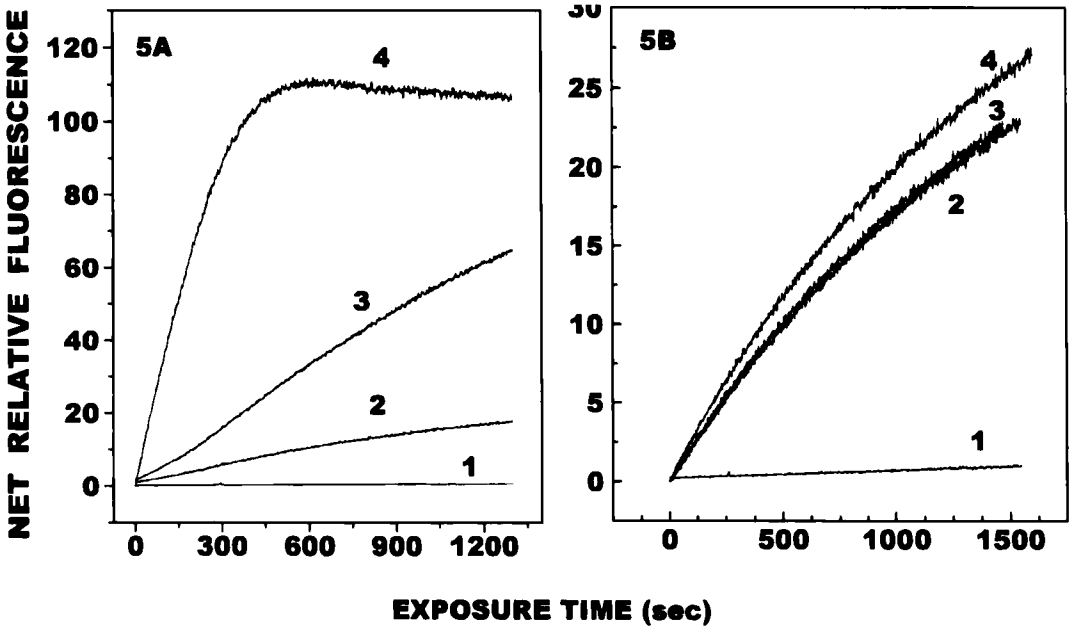

FIGURE 5 Induction of 7-hydroxy-SECCA fluorescence as function of time in incubation mixture containing 10 μmol dm^{-3} SECCA, 500 μmol dm^{-3} ascorbate, and 1 mmol dm^{-3} hydrogen peroxide. A. Adriamycin (20 μmol dm^{-3}) with increasing concentrations of Cu(II): 1, 0 μmol dm^{-3}; 2, 5 μmol dm^{-3}; 3, 10 μmol dm^{-3}; 4, 20 μmol dm^{-3}. B. Adriamycin (30 μmol dm^{-3}) with increasing concentrations of Fe(III): 1, 0 μmol dm^{-3}; 2, 2.5 μmol dm^{-3}; 3, 5 μmol dm^{-3}; 4, 10 μmol dm^{-3}.

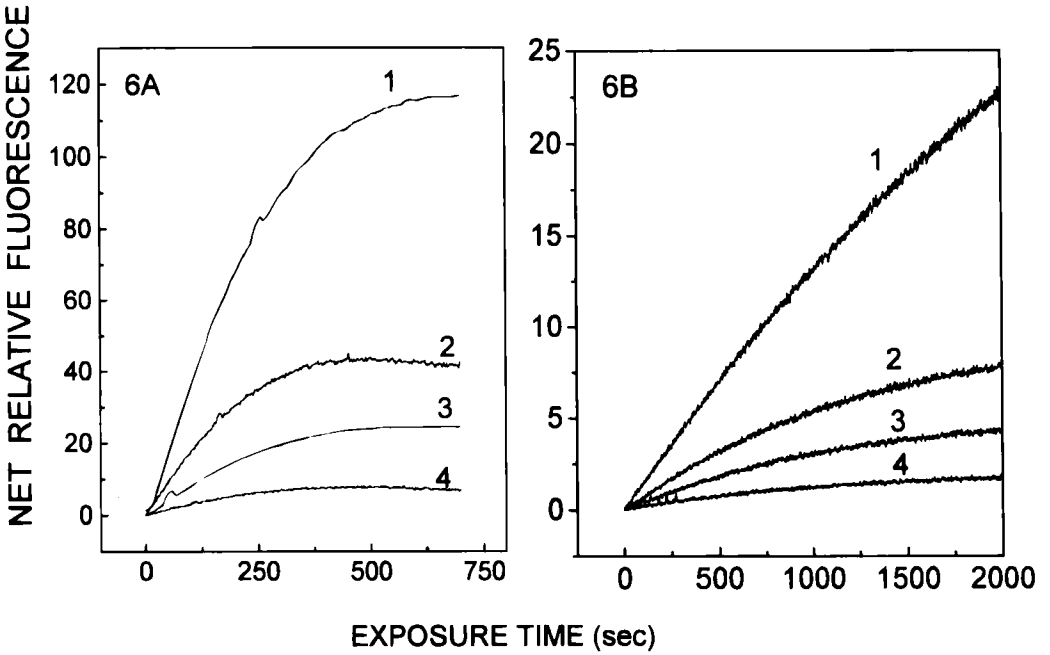

FIGURE 6 Relative effect of scavengers on fluorescence induction in incubation mixture containing 10 μmol dm^{-3} SECCA, 500 μmol dm^{-3} ascorbate, and 1 mmol dm^{-3} hydrogen peroxide. A. Cu(II)—adriamycin complex (1:1) with: 1, no scavenger; 2, 5 mmol dm^{-3} tert-butanol; 3, 5 mmol dm^{-3} ethanol; 4, 5 mmol dm^{-3} DMSO. B. Fe(III)—adriamycin complex (1:3) with: 1, no scavenger; 2, 10 mmol dm^{-3} tert-butanol; 3, 10 mmol dm^{-3} ethanol; 4, 10 mmol dm^{-3} DMSO.

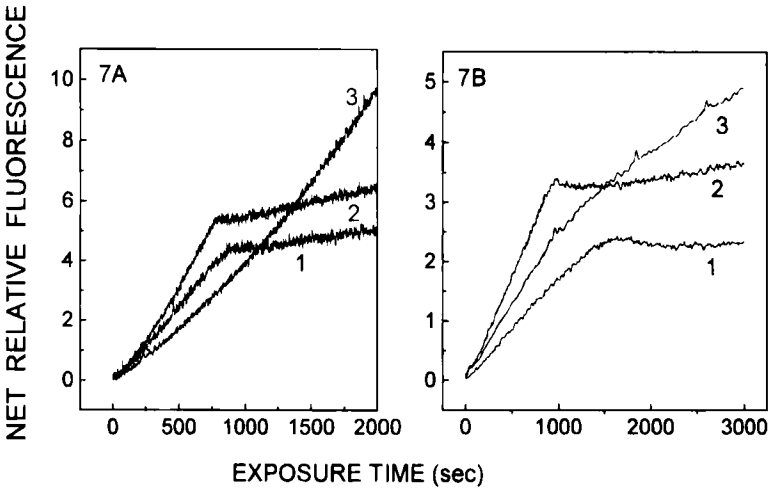

FIGURE 7 Induction of 7-hydroxy-SECCA fluorescence as function of time in incubation mixture containing H1—DNA, 10 μmol dm^{-3} SECCA, metal—adriamycin complex, and various concentrations of ascorbate and hydrogen peroxide. A. Cu(II)—adriamycin complex (1:1) with: 1, 100 μmol dm^{-3} ascorbate and 1 mmol dm^{-3} hydrogen peroxide; 2, 100 μmol dm^{-3} ascorbate and 2 mmol dm^{-3} hydrogen peroxide; 3, 500 μmol dm^{-3} ascorbate and 1 mmol dm^{-3} hydrogen peroxide. B. Fe(III)—adriamycin complex (1:3) with: 1, 100 μmol dm^{-3} ascorbate and 1 mmol dm^{-3} hydrogen peroxide; 2, 100 μmol dm^{-3} ascorbate and 5 mmol dm^{-3} hydrogen peroxide; 3, 500 μmol dm^{-3} ascorbate and 1 mmol dm^{-3} hydrogen peroxide.

7-hydroxy-SECCA generated on either free SECCA or a SECCA-labeled H1—DNA system is shown in Figure 9. While the fluorescence is diminished by all three HO• scavengers when SECCA is present free in solution, none of the scavengers can eliminate the fluorescence when SECCA is positioned close to DNA (Figure 9, III and IV). Catalase, on the other hand, can eliminate the fluorescence induction in both situations, while EDTA does so in the presence of

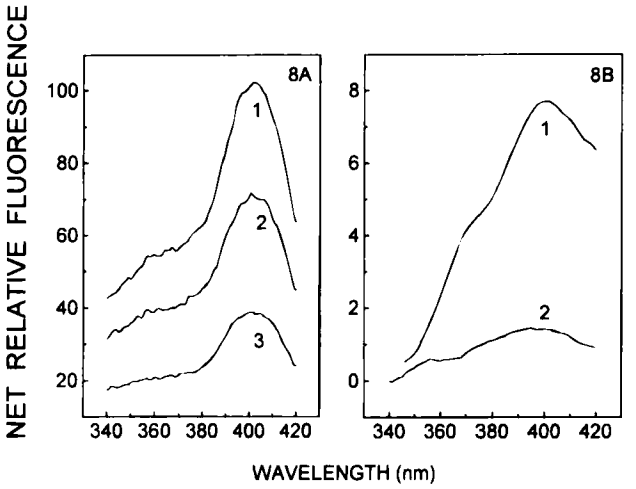

FIGURE 8 A. Fluorescence excitation spectra (emission = 450 nm) of 7-hydroxy-SECCA-labeled H1—DNA in presence or absence of Cu(II)— and Fe(III)—adriamycin complexes: 1, no metal complex; 2, Cu(II)—adriamycin (1:1); 3, Fe(III)—adriamycin (1:3). B. Fluorescence excitation spectra (emission = 450 nm, slit width 5) of SECCA-labeled H1—DNA in presence of metal—adriamycin complexes, 500 μmol dm^{-3} ascorbate, and 1 mmol dm^{-3} hydrogen peroxide: 1, Cu(II)—adriamycin (1:1); 2, Fe(III)—adriamycin (1:3).

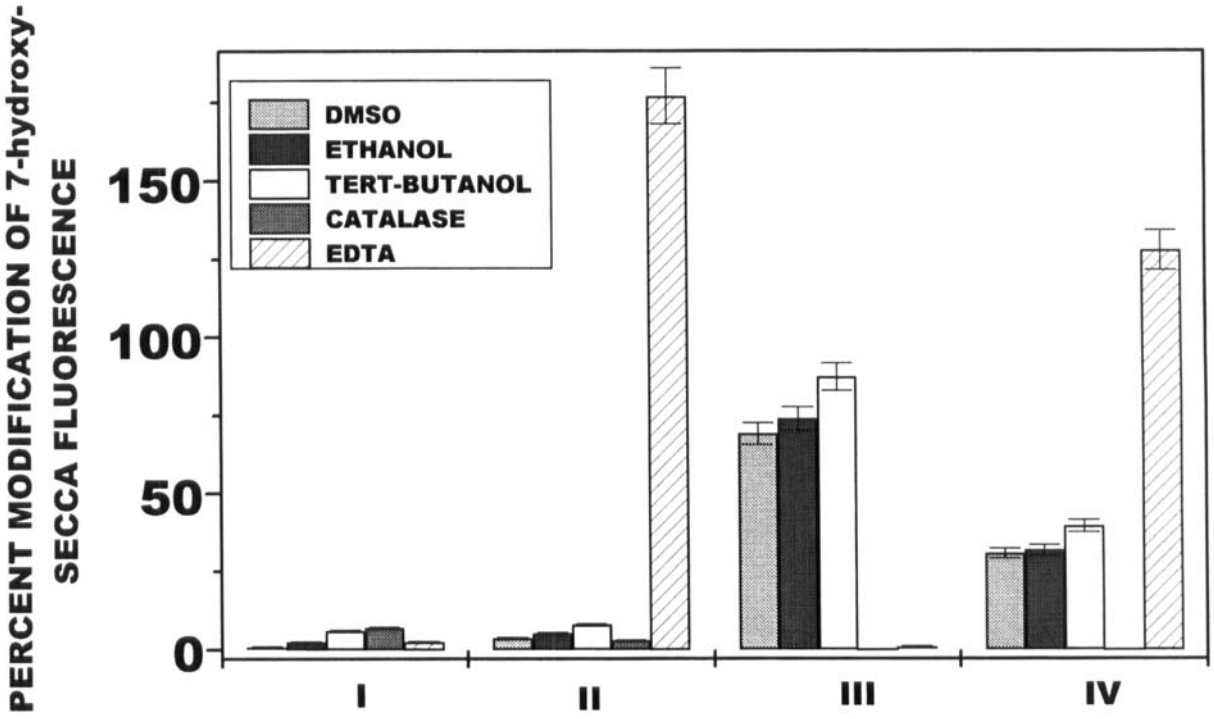

FIGURE 9 Modification of 7-hydroxy-SECCA fluorescence induced following exposure to Cu(II)— or Fe(III)—adriamycin complex in presence of 500 μmol dm^{-3} ascorbate and mmol dm^{-3} hydrogen peroxide and several modifiers. *Percent modification* is percent ratio of induced fluorescence in the presence versus absence of each modifier. I. Cu(II)—adriamycin complex (1:1), free SECCA; II. Fe(III)—adriamycin (1:3), free SECCA; III. Cu(II)—adriamycin complex (1:1), SECCA-H1—DNA; IV. Fe(III)—adriamycin (1:3), SECCA-H1—DNA.

Cu(II) but potentiates fluorescence in the presence of Fe(III).

Finally, the ability of 100 mmol dm^{-3} DMSO to reduce the amount of 7-hydroxy-SECCA generated on SECCA-labeled H1—DNA by a 60-min exposure to Cu(II)—adriamycin—ascorbate—hydrogen peroxide is shown as a function of NaCl in Figure 10. As the ionic strength of the salt increases to 50–100 mmol dm^{-3}, the scavenging ability of DMSO passes through a minimum.

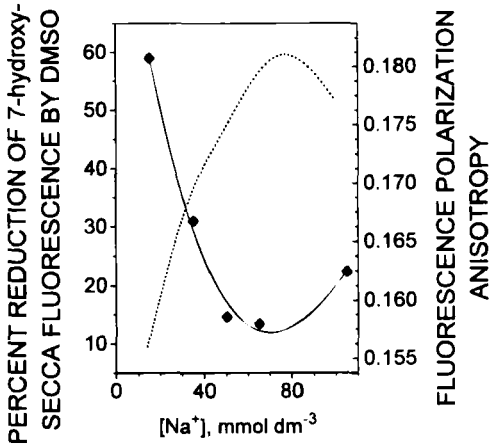

FIGURE 10 Effect of Na^+ on reduction of induced 7-hydroxy-SECCA fluorescence by 100 mmol dm^{-3} DMSO. SECCA-H1—DNA is incubated with Cu(II)—adriamycin complex (1:1) with 500 μmol dm^{-3} ascorbate and 1 mmol dm^{-3} hydrogen peroxide in presence of DMSO. Dotted line is variation in anisotropy of histone-H1—DNA complex as function of ionic strength[41].

DISCUSSION

Detection of hydroxyl radicals (HO•) generated in the microenvironment of DNA (*DNA-associated HO•*) has been a notoriously difficult problem, as these radicals are very short-lived and highly reactive toward DNA[25]. Due in part to

the difficulty of detecting DNA-associated HO•, the ability of several DNA-binding agents to generate HO• when bound to DNA has remained controversial. Such generation of DNA-associated HO• does not exclude the simultaneous presence of other mechanisms of DNA damage[24] whose relative contribution to the overall DNA damage may be larger than that of HO•. However, the carcinogenicity of various types of DNA damage is dependent not only on the quantity of DNA damage but also on its quality. For example, depending also on the reaction conditions, HO•-attack on DNA produces a characteristic fingerprint of base modifications[24,34] which could result in an HO•-specific mutation spectrum. As such, the ability of an agent to produce DNA-associated HO• is significant in its own right, since it may be indicative of the carcinogenic and other toxic effects one might expect under similar conditions in cells.

Adriamycin is a DNA-binding chemotherapeutic drug whose cardiotoxicity has been associated with its redox activity[5,6]. Although the drug is known to produce DNA damage, generation of DNA-associated HO• by DNA-bound metal—adriamycin complexes has been controversial[14,20]. Our earlier study with copper-catalyzed HO• had demonstrated that by associating DNA with an HO•-detecting molecule (SECCA) one can detect HO• within a few angstroms of DNA[28] (i.e. DNA-associated HO•). A similar approach was used in this study with metal—adriamycin complexes. To directly demonstrate that the system can detect HO• generated close to DNA, we first tested our method with molecules labeled with ^{125}I, a radionuclide whose decay is known to produce several low-energy Auger electrons with ranges of only a few nanometers[35,36]. Accordingly, ^{125}I positioned in close proximity to DNA in aqueous solution would be expected to generate a high yield of DNA-associated HO• via ionization of water molecules within the immediate vicinity of the decaying atom. Figure 1A demonstrates that SECCA bound in close proximity to DNA via SECCA-polylysine—DNA complexes can readily detect HO• via the generation of 7-hydroxy-SECCA. As was shown earlier[26,27], neither direct radiation action on the detector molecule SECCA, nor radiolytically-produced e_{aq}^-, $H^•$, $HO_2^•$, $O_2^{•-}$ can induce the fluorescence; thus, the observed 7-hydroxy-SECCA fluorescence induction is a specific indicator of HO•[26,27]. That a significant percentage of HO• is indeed generated close to DNA in these experiments is demonstrated in Figure 1B, where the addition of DMSO results in a different curve depending on whether ^{125}I is bound to DNA (^{125}I-polylysine) or free in solution ($Na^{125}I$). In the former case DMSO is much less effective in scavenging HO•, presumably beccause of the generation of HO• close to DNA and to the detector molecule SECCA.

Subsequently experiments were carried out with either free SECCA or SECCA-labeled histone-H1—DNA complexes exposed to metal—adriamycin complexes in the presence of ascorbate and hydrogen peroxide (Figures 5–7). In both cases, a similar time-dependent induction of 7-hydroxy-SECCA fluorescence was observed. Although oxidizing species other than HO• may also be produced during the applied reactions (e.g. ferryl/copper(III) species, or singlet oxygen) the present data point towards HO• as being the main species responsible for generation of the fluorescent 7-hydroxy-SECCA. Thus, DMSO, ethanol or tert-butanol reduced the fluorescence induction in the order DMSO > ethanol > tert-butanol, in accordance with their rate constants with hydroxyl radical (7×10^9, 1.8×10^9, 0.52×10^9 mol^{-1} dm^3 s^{-1} respectively[37]). In fact, in earlier experiments using gamma rays, where fluorescence induction can be attributed to HO• unequivocally[26,27,38], the three scavengers reduced fluorescence in the same order as shown in the adriamycin data in Figure 6. Although the possibility cannot be ruled out, it is unlikely that ferryl/copper(III) species produce 7-hydroxy-SECCA whose fluorescence reduction in the presence of three scavengers follows the same pattern obtained with radiolytically generated

"pure" HO•, both in the *presence* and in the *absence* of DNA. Our studies with light exposure of methylene blue (Figure 4) also show that singlet oxygen cannot induce significant amounts of 7-hydroxy-SECCA. Furthermore, since an 8-min exposure of methylene blue to halogen light is sufficient to destroy 10^{-4} mol dm^{-3} ADPA by reaction with an equimolar amount of $^1O_2$, it can be estimated that at least 10^{-2} mol dm^{-3} $^1O_2$ is generated during the 800-min illumination experiment (Figure 4B). On the other hand, 10 Gy of gamma rays (Figure 4B) produces approximately 10^{-5} mol dm^{-3} HO• in the SECCA-containing solution[39], much of which should be scavenged by methylene blue. Therefore, under similar conditions, HO• is many orders of magnitude more efficient than $^1O_2$ in generating 7-hydroxy-SECCA.

These results show that metal—adriamycin complexes in the presence of ascorbate and hydrogen peroxide probably produce HO• whether adriamycin is DNA-bound or unbound. However, as demonstrated with HO• scavengers (Figure 9), a major difference between experiments with free SECCA and with SECCA attached to H1—DNA complexes lies in *the extent* to which the HO• scavenger can reduce the 7-hydroxy-SECCA fluorescence generated. Figure 9 indicates that for free SECCA the fluorescence is diminished by 100 mmol dm^{-3} of all three scavengers, while only a moderate reduction of fluorescence is observed with SECCA-labeled H1—DNA. These data are consistent with the generation of HO• in the immediate vicinity of DNA and the inability of HO• scavengers to prevent HO• from accessing the sites on which SECCA is bound. On the other hand, agents that interfere in the metal-catalysis of HO• can have a significant effect. For example, when catalase, which is efficient in degrading hydrogen peroxide, is added, practically no difference was observed between free and DNA-bound SECCA (Figure 9). Furthermore, EDTA, a metal chelator known to inhibit copper redox reactions[28] and to often potentiate iron reactions[40], demonstrated both these effects (Figure 9). The data, therefore, suggest that copper(II)/iron(III)—adriamycin produces HO• in the presence of ascorbate and hydrogen peroxide whether unbound or DNA-bound and that in the latter case scavengers would not prevent HO• from attacking chromatin.

The influence of H1—DNA conformation on the scavenging efficiency of DMSO was also studied (Figure 10). As an earlier fluorescence polarization spectroscopy study demonstrated[41], an NaCl content in the region 50–150 mmol dm^{-3} causes the H1—DNA complex to attain a maximum anisotropy value (see dotted line in Figure 10) and a most compact conformation which reduces the accessibility of gamma-ray-generated HO• to the SECCA-labeled sites. These results indicate that increasing concentrations of Na^+ also influence the ability of DMSO to scavenge Cu(II)—adriamycin-catalyzed HO•. Since NaCl did not affect the binding of copper to adriamycin or of adriamycin to DNA (also reported by others[42]), this effect is not attributable to changes in the amount of copper—adriamycin complex bound to DNA. It seems, therefore, that the H1—DNA conformation may not only have an effect on the accessibility of HO• to chromatin sites[41], but also prevent scavengers from efficiently removing DNA-associated HO•.

Acknowledgements

This work was supported in part by USPHS grants RO1 CA72046 (GMM), R29 CA 63334 (GMM), RO1 CA 15523 (AIK) and R37 CA 34970 (AGJ).

References

[1] R. C. Young, R. F. Ozols and C. E. Myers (1981) The anthracycline antineoplastic drugs. *New England Journal of Medicine*, **305**, 139–153.

[2] C. Cullinane and D. R. Phillips (1990) Induction of stable transcriptional blockage sites by adriamycin: G_pC specificity of apparent adriamycin-DNA adducts and dependence on iron(III) ions. *Biochemistry*, **29**, 5638–5646.

[3] M. Delvaeye, V. Verovski, W. De Neve and G. Storme (1993) DNA breakage, cytotoxicity, drug accumulation

and retention in two human ovarian tumor cell lines AZ224 and AZ364 treated with adriamycin, modulated by verapamil. *Anticancer Research*, **13(5A)**, 1533–1538.
[4] K. Ollinger and A. Brunmark (1994) Effect of different oxygen pressures and N,N-diphenyl-p-phenylenediamine on Adriamycin toxicity to cultured neonatal rat heart myocytes. *Biochemical Pharmacology*, **48**, 1707–1715.
[5] V. J. Ferrans (1978) Overview of cardiac pathology in relation to anthracycline cardiotoxicity. *Cancer Treatment Reports*, **62**, 955–961.
[6] E. A. Lefrak, J. Pitha, S. Rosenheim and J. A. Gottlieb (1973) A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer*, **32**, 302–314.
[7] J. H. Doroshow (1986) Role of hydrogen peroxide and hydroxyl radical formation in the killing of Ehrlich tumor cells by anticancer quinones. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 4514–4518.
[8] J. H. Doroshow (1986) Prevention of doxorubicin-induced killing of MCF-7 human breast cancer cells by oxygen radical scavengers and iron chelating agents. *Biochemical and Biophysical Research Communications*, **135**, 330–335.
[9] B. K. Sinha, A. G. Katki, G. Batist, K. H. Cowan and C. E. Myers (1987) Differential formation of hydroxyl radicals by adriamycin in sensitive and resistant MCF-7 human breast tumor cells: implications for the mechanism of action. *Biochemistry*, **26**, 3776–3781.
[10] H. Beraldo, A. Garnier-Suillerot, L. Tosi and F. Lavelle (1985) Iron(III)—adriamycin and iron(III)—daunorubicin complexes: physicochemical characteristics, interaction with DNA, and antitumor activity. *Biochemistry*, **24**, 284–289.
[11] H. Beraldo, A. Garnier-Suillerot and L. Tosi (1983) Copper(II)—adriamycin complexes. A circular dichroism and resonance Raman study. *Inorganic Chemistry*, **22**, 4117–4124.
[12] K. Sugioka, H. Nakano, M. Nakano, S. Tero-Kubota and Y. Ikegami (1983) Generation of hydroxyl radicals during the enzymatic reductions of the Fe^{3+}-ADP-phosphate-adriamycin and Fe^{3+}-ADP-EDTA systems. *Biochimica et Biophysica Acta*, **753**, 411–421.
[13] K. Sugioka and M. Nakano (1982) Mechanism of phospholipid peroxidation induced by ferric ion-ADP-adriamycin-co-ordination complex. *Biochimica et Biophysica Acta*, **713**, 333–343.
[14] J. W. Lown, S.-K. Sim, K. C. Majumdar and R.-Y. Chang (1977) Strand scission of DNA by bound adriamycin and daunorubicin in the presence of reducing agents. *Biochemical and Biophysical Research Communications*, **76**, 705–710.
[15] A. Someya and N. Tanaka (1979) DNA strand scission induced by adriamycin and aclacinomycin A. *Journal of Antibiotics*, **32**, 839–845.
[16] J. Azuma, N. Sperelakis, H. Hasegawa, T. Tanimoto, S. Vogel, K. Ogura, N. Awata, A. Sawamura, H. Harada, T. Ishiyama, Y. Morita and Y. Yamamura (1981) Adriamycin cardiotoxicity: possible pathogenic mechanisms. *Journal of Molecular and Cellular Cardiology*, **13**, 381–397.
[17] C. E. Myers, W. P. McGuire, R. H. Liss, I. Ifrim, K. Grotzinger and R. C. Young (1977) Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science*, **197**, 165–167.
[18] C. E. Myers, L. Gianni, C. B. Simone, R. Klecker and R. Greene (1982) Oxidative destruction of erythrocyte ghost membranes catalyzed by the doxorubicin—iron complex. *Biochemistry*, **21**, 1707–1713.
[19] H. Eliot, L. Gianni and C. Myers (1984) Oxidative destruction of DNA by the adriamycin—iron complex. *Biochemistry*, **23**, 928–936.
[20] J. R. F. Muindi, B. K. Sinha, L. Gianni and C. E. Myers (1984) Hydroxyl radical production and DNA damage induced by anthracycline—iron complex. *FEBS Letters*, **172**, 226–230.
[21] J. Muindi, B. K. Sinha, L. Gianni and C. Myers (1985) Thiol-dependent DNA damage produced by anthracycline-iron complexes. The structure-activity relationships and molecular mechanisms. *Molecular Pharmacology*, **27**, 356–365.
[22] N. R. Bachur, S. L. Gordon and M. V. Gee (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Research*, **38**, 1745–1750.
[23] N. R. Bachur, M. V. Gee and R. D. Friedman (1982) Nuclear catalyzed antibiotic free radical formation. *Cancer Research*, **42**, 1078–1081.
[24] S. A. Akman, J. H. Doroshow, T. G. Burke and M. Dizdaroglu (1992) DNA base modifications induced in isolated human chromatin by NADH dehydrogenase-catalyzed reduction of doxorubicin. *Biochemistry*, **31**, 3500–3506.
[25] W. A. Pryor (1988) Why is the hydroxyl radical the only radical that commonly adds to DNA? Hypothesis: it has a rare combination of high electrophilicity, high thermochemical reactivity, and a mode of production that can occur near DNA. *Free Radical Biology and Medicine*, **4**, 219–223.
[26] G. M. Makrigiorgos, J. Baranowska-Kortylewicz, E. Bump, S. K. Sahu, R. M. Berman and A. I. Kassis (1993) A method for detection of hydroxyl radicals in the vicinity of biomolecules using radiation-induced fluorescence of coumarin. *International Journal of Radiation Biology*, **63**, 445–458.
[27] G. M. Makrigiorgos, M. Folkard, C. Huang, E. Bump, J. Baranowska-Kortylewicz, S. K. Sahu, B. D. Michael and A. I. Kassis (1994) Quantification of radiation-induced hydroxyl radicals within nucleohistones using a molecular fluorescent probe. *Radiation Research*, **138**, 177–185.
[28] G. M. Makrigiorgos, E. Bump, C. Huang, J. Baranowska-Kortylewicz and A. I. Kassis (1995) A fluorimetric method for the detection of copper-mediated hydroxyl free radicals in the immediate proximity of DNA. *Free Radical Biology and Medicine*, **18**, 669–678.
[29] J. B. Chaires, N. Dattagupta and D. M. Crothers (1982) Studies on interaction of anthracycline antibiotics and deoxyribonucleic acid: equilibrium binding studies on interaction of daunomycin with deoxyribonucleic acid. *Biochemistry*, **21**, 3933–3940.
[30] S. Kawanishi, S. Inoue and K. Yamamoto (1989) Hydroxyl radical and singlet oxygen production and DNA damage induced by carcinogenic metal compounds and hydrogen peroxide. *Biological and Trace Element Research*, **21**, 367–372.
[31] B. Kalyanaraman, S. Ramanujam, R. J. Singh, J. Joseph and J. B. Feix (1993) Formation of 2,5-dihydroxybenzoic acid during the reaction between $^1O_2$ and salicylic acid: analysis by ESR oximetry and HPLC with electrochemi-

cal detection. *Journal of American Chemical Society*, **115**, 4007–4012.

[32] K. Briviba, T. P. Devasagayam, H. Sies and S. Steenken (1993) Selective para hydroxylation of phenol and aniline by singlet molecular oxygen. *Chemical Research in Toxicology*, **6**, 548–553.

[33] B. A. Lindig, M. A. J. Rodgers and A. P. Schaap (1980) Determination of the lifetime of singlet oxygen in D_2O using 9,10-anthracenedipropionic acid, a water-soluble probe. *Journal of American Chemical Society*, **102**, 5590–5593.

[34] B. Halliwell and J. M. C. Gutteridge (1986) Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Archives of Biochemistry and Biophysics*, **246**, 501–514.

[35] A. I. Kassis, K. S. R. Sastry and S. J. Adelstein (1987) Kinetics of uptake, retention, and radiotoxicity of ^{125}IUdR in mammalian cells: implications of localized energy deposition by Auger processes. *Radiation Research*, **109**, 78–89.

[36] G. M. Makrigiorgos, A. I. Kassis, J. Baranowska-Kortylewicz, K. D. McElvany, M. J. Welch, K. S. R. Sastry and S. J. Adelstein (1989) Radiotoxicity of 5-[^{123}I]iodo-2′-deoxyuridine in V79 cells: a comparison with 5-[^{125}I]iodo-2′-deoxyuridine. *Radiation Research*, **118**, 532–544.

[37] L. M. Dorfman and G. E. Adams (1973) Reactivity of the hydroxyl radical in aqueous solutions. US Department of Commerce, National Bureau of Standards Report NSRDS-NBS 46. Washington, DC: US Government Printing Office.

[38] S. Chakrabarti, G. M. Makrigiorgos, K. O'Brien, E. Bump and A. I. Kassis (1996) Measurement of hydroxyl radicals catalyzed in the immediate vicinity of DNA by metal-bleomycin complexes. *Free Radical Biology and Medicine* **20**, 777–783.

[39] A. K. Collins, G. M. Makrigiorgos and G. K. Svensson (1994) Coumarin chemical dosimeter for radiation therapy. *Medical Physics*, **21**, 1741–1747.

[40] H. U. Enright, W. J. Miller and R. P. Hebbel (1992) Nucleosomal histone protein protects DNA from iron-mediated damage. *Nucleic Acids Research*, **20**, 3341–3346.

[41] G. M. Makrigiorgos, E. Bump, C. Huang, J. Baranowska-Kortylewicz and A. I. Kassis (1994) Accessibility of nucleic acid-complexed biomolecules to hydroxyl radicals correlates with their conformation: a fluorescence polarization spectroscopy study. *International Journal of Radiation Biology*, **66**, 247–257.

[42] S. Ando, K. Kamiya, T. Yoshimura, H. Tsutani, T. Ueda, M. Uchida, T. Nakamura and H. Uchino (1988) DNA-binding characteristics of aclarubicin as compared with daunorubicin and doxorubicin. *Anticancer Research*, **8**, 409–415.