Hydroxyl radical production and DNA damage induced by anthracycline–iron complex

Josephia R.F. Muindi, Birandra K. Sinha, Luca Gianni and Charles E. Myers

Clinical Pharmacology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, USA

Received 27 April 1984; revised version received 10 May 1984

Adriamycin–Fe^{3+} complex catalyzes the formation of hydroxyl radical from hydrogen peroxide but the DNA–adriamycin–iron ternary complex is much more effective. 11-Deoxyadriamycin, which shows no spectral evidence of complex formation with iron, was ineffective. The generation of hydroxyl radical by adriamycin–Fe^{3+} complex in the presence of DNA correlates with its ability to cleave DNA. Hydroxyl radicals are thus implicated as the reactive oxygen species involved in the DNA damage caused by the adriamycin–Fe^{3+} complex.

Anthracycline–iron complex *DNA damage* *Spin trapping* *Hydroxyl radical*

1. INTRODUCTION

While it has been known for some time that the anthracycline antibiotics, daunomycin and adriamycin, bind transition metal ions, it is only recently that association constants for this interaction have been published [1]. These studies revealed that adriamycin possesses a high affinity for Fe^{3+}, with step association constants of 10^{18}, 10^{11}, and $10^{4.4}$. These values are high enough that adriamycin may be able to chelate iron in vivo. For this reason we have examined the biochemistry of adriamycin–iron complexes. We have shown that the adriamycin–iron complex can bind to erythrocyte ghost membranes and in the presence of glutathione catalyze the oxidative destruction of these membranes [2]. In a separate study, we have been able to show that the adriamycin–iron chelate binds to DNA to form a ternary complex. As with the erythrocyte ghost, oxidative destruction of the DNA is initiated in the presence of glutathione [3]. In both situations, the destruction of the macromolecular target first involves thiol-mediated reduction of oxygen to hydrogen peroxide followed by subsequent reaction of hydrogen peroxide with the drug–metal complex which is responsible for the damage. In both cases, the hydroxyl radical scavengers, mannitol and DMSO, lessened the damage, albeit inefficiently, requiring much higher concentrations than normal. For these reasons, we have used the spin trapping of $^{\cdot}OH$ to reexamine more definitively the role of hydroxyl radical in hydrogen peroxide-dependent DNA damage catalyzed by adriamycin–iron complex. The results show that while the adriamycin–Fe^{3+} complex can catalyze the production of hydroxyl radical, the DNA–adriamycin–iron ternary complex is more effective.

2. MATERIALS AND METHODS

Adriamycin HCl was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD and 11-deoxyadriamycin HCl was a gift from Dr F.F. Arcamone, Farmitalia C. Erba, Milan. $FeCl_3 \cdot 6H_2O$ (99.5% pure, Allied Chemicals) was the source of Fe^{3+}. H_2O_2 (30%) was obtained from Fisher. Diethylenetriaminepentaacetic acid (DETAPAC) and the spin trap 5,5-dimethyl-1-pyrroline oxide (DMPO) were from Aldrich. DMPO was purified by two distillations before use.

Published by Elsevier Science Publishers B.V.

Desferrioxamine mesylate (Desferal) was purchased from Ciba. All reaction solutions and buffers were passed over Chelex-100 resin (Bio-Rad) before use. SV 40 [^{3}H]DNA was supplied by Bethesda Research Laboratories and used without further purification. Highly polymerized calf thymus DNA (Sigma) for ESR studies was freed of metal ions by dialysis for 24 h against 0.1% DETAPAC followed by 48 h dialysis against metal ion-free buffer at 4°C [4].

2.1. *Spectrophotometric studies*

One μl aliquots of freshly prepared 2.5 mM aqueous $FeCl_3$ was added to 6 μl of 5 mM adriamycin or 11-deoxyadriamycin at ~pH 2 to form a complex. The complex was then adjusted to pH 7.5 with 1 ml of 20 mM NaCl, 10 mM Tris–HCl buffer immediately before recording the spectrum. The spectrum was recorded in 1 cm path-length cells on a Hewlett Packard 8450A diode array spectrophotometer equipped with a Hewlett Packard 7470A plotter and 89100A temperature controller. All spectrophotometric titrations were performed at 24°C.

2.2. *DNA cleavage reaction*

DNA degradation assay mixture consisted of 0–1 mM H_2O_2, 0.25 μg (~5000 cpm) SV 40 [^{3}H]DNA (>95% form I) and 10 μM 1:1 anthracycline–Fe^{3+} complex in a final volume of 100 μl. The assay was performed in 20 mM NaCl, 10 mM Tris–HCl buffer (pH 7.5) and was initiated by addition of the complex. Following 10 min incubation at room temperature, the reaction was terminated by adding 0.25 mM Desferal, 1% SDS and adjusting the pH to 5.0 with HCl. Desferal and SDS stop the reaction by dissociating the ternary complex of DNA–drug–iron. Desferal chelates iron (affinity constant ~10^{33}) and prevents its involvement in additional redox catalysis while SDS prevents the freed anthracycline from intercalating into DNA. At pH 5.0, the dissociation of the ternary complex is completed in less than 1 min.

2.3. *Separation of DNA from the reaction components*

Gel filtration was used to separate DNA from the rest of the assay reactants. This step is essential because the presence of anthracyclines interferes with the estimation of nicked DNA by the nitrocellulose filters. The assay mixture was applied to a prepacked Sephadex G-25 column (PD10, Pharmacia) previously washed with SSC buffer (pH 7.1). DNA was eluted with 4 ml of the same buffer. DNA recovery from the column was routinely greater than 95%.

2.4. *Estimation of nicked DNA*

The percentage of nicked DNA was quantitated after a denaturation–renaturation cycle using Schuller and Schneider BA 85 nitrocellulose filters [5]. These filters allow unnicked double-stranded DNA (form I) to pass through while retaining nicked (linear and circular) single-stranded DNA. Alkaline denaturation was achieved with 0.9 M NaCl, 0.1 M K_2HPO_4 and 0.025 M EDTA buffer adjusted to pH 12.15 with NaOH. HCl (2 M) was used for renaturation. Filters were dried at 60°C and then allowed to dissolve in hydrofluor (National Diagnostics) scintillation liquid prior to counting. Filter counts (nicked DNA) were expressed as a percentage of total counts in the assay.

2.5. *Hydroxyl radical assay*

Hydroxyl radical formation was assayed by ESR spectrometry using DMPO as a spin-trapping agent. A Varian E-104 spectrometer operating at X-band (9.5 GHz) and employing 100 kHz modulation was used for ESR measurements. The assay mixture in 20 mM NaCl, 10 mM Tris–HCl buffer (pH 7.5) contained 10 μM 1:1 anthracycline–Fe^{3+} complex, 100 μg/ml calf thymus DNA and 100 mM DMPO. The reaction was started by adding 90 μM H_2O_2 and followed for 15 min at room temperature (24°C).

3. RESULTS AND DISCUSSION

Spectral evidence for adriamycin–Fe^{3+} complex formation was reported in [2]. This was characterized by a marked loss in the absorption at 480 nm and the appearance of a new band between 580 and 610 nm in association with two clearly defined isosbestic points at 430 and 535 nm. Whereas the addition of $FeCl_3$ to adriamycin produced spectral changes indicative of adriamycin– Fe^{3+} complex formation as reported, no spectral evidence of complex formation was observed with 11-deox-

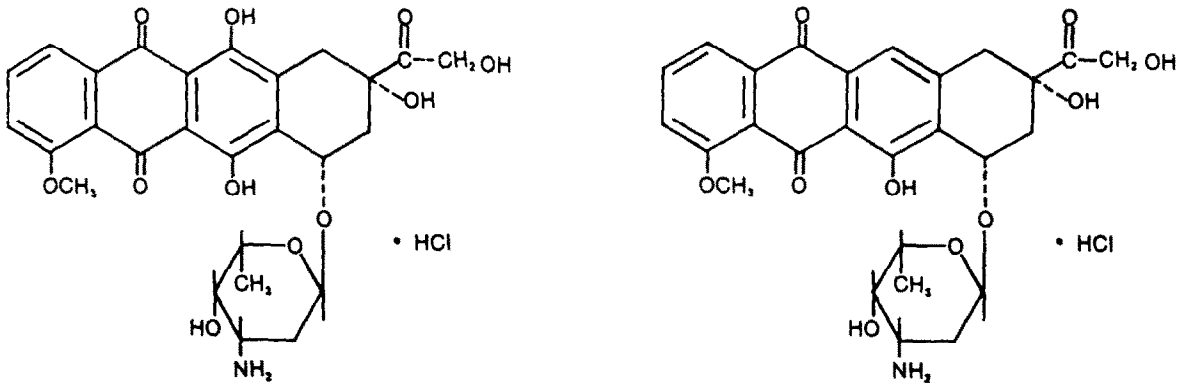

Fig.1. Structures of adriamycin and 11-deoxyadriamycin.

yadriamycin (fig.2A,B). The removal of the C_{11}-hydroxy group of adriamycin is thus associated with complete loss of iron binding to the drug chromophore. Because of this observation we have used 11-deoxyadriamycin as a control to study the chemistry of the H_2O_2-dependent DNA cleavage reaction catalyzed by adriamycin–Fe^{3+} complex.

As apparent from fig.3, the 1:1 adriamycin–Fe^{3+} complex causes H_2O_2-dependent DNA degradation. The concentration of H_2O_2 required to cleave 50% of the DNA was 1 μM. In contrast, the 11-deoxyadriamycin–Fe^{3+} mixture was much less effective. Fe^{3+} even in the presence of 1 mM H_2O_2 caused less than 5% damage of the DNA. Adriamycin and 11-deoxyadriamycin in the absence of iron did not cause H_2O_2-dependent DNA damage. H_2O_2 alone, even at 1 mM, was also without effect.

Because hydroxyl radical is known to be a product of the reaction of either Fe^{2+} or, less efficient-

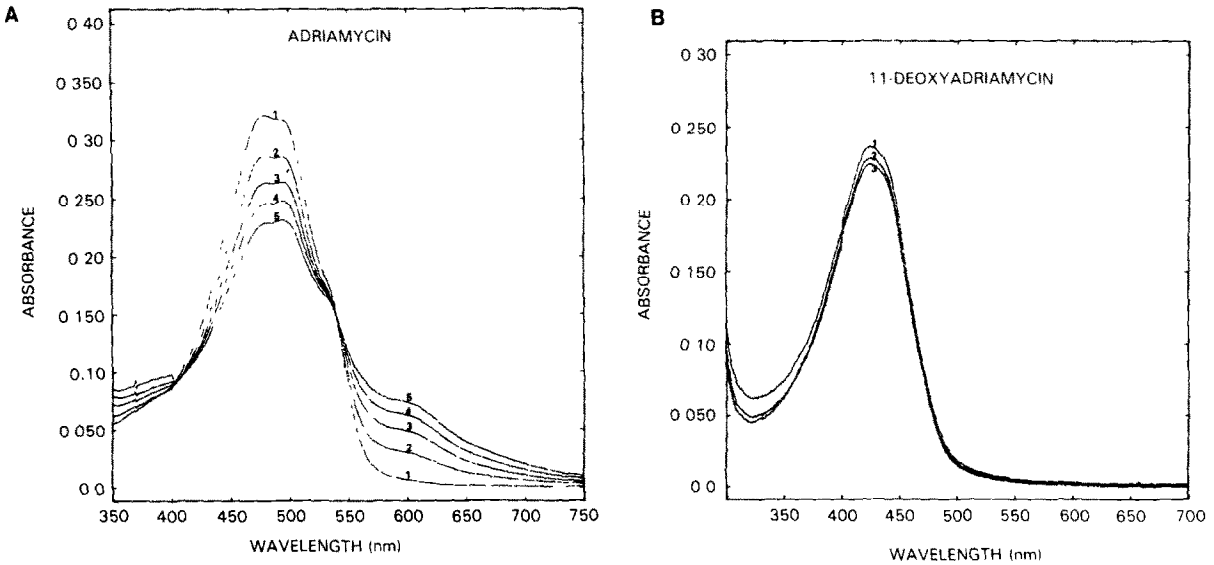

Fig.2. Titration of adriamycin and 11-deoxyadriamycin with $FeCl_3$. The final concentration of the anthracycline antibiotic in the assay was 30 μM. (A) Curve 1, adriamycin alone, curves 2,3,4 and 5, 12:1, 6:1, 4:1 and 3:1 anthracycline/metal ion ratios, respectively. (B) Curve 1, 11-deoxyadriamycin alone; curves 2 and 3, 6:1 and 3:1 drug/iron ratios.

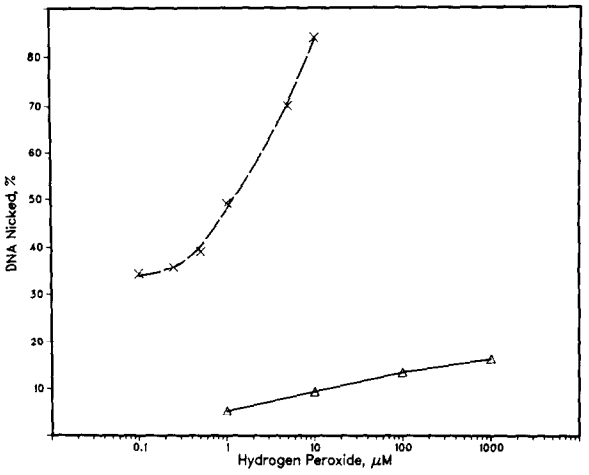

Fig.3. H_2O_2-dependent DNA damage induced by anthracycline–iron complexes. (×---×) Adriamycin–Fe^{3+}, (△—△) 11-deoxyadriamycin and Fe^{3+}.

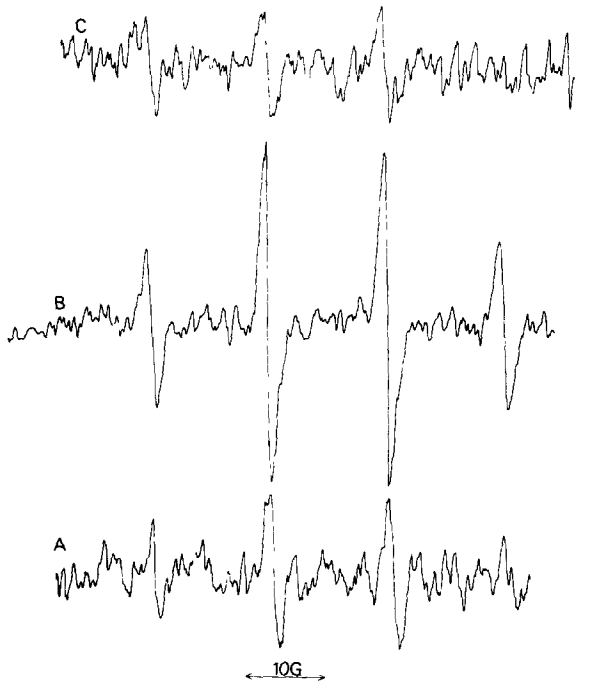

Fig.4. ESR spectrum of the DMPO-OH spin adduct formed in the presence of 100 μg/ml calf thymus DNA and 90 μM H_2O_2 from (A) Fe^{3+} (10 μM), (B) adriamycin (10 μM)–Fe^{3+} (10 μM) and (C) 11-deoxyadriamycin (10 μM)–Fe^{3+} (10 μM). ESR settings: field, 3390 G; field scan, 100 G; modulation frequency, 100 kHz; modulation amplitude, 2.0 G; microwave power, 20 MW; and the receiver gain, 1.25×10^5.

ly, Fe^{3+} with H_2O_2, spin-trapping studies were carried out to detect this species. Addition of H_2O_2 to adriamycin solutions in the presence of DMPO produced no detectable ESR signal. However, when H_2O_2 was added to the adriamycin–Fe^{3+} complex, a small but detectable ESR spectrum consisting of a 1:2:2:1 quartet was observed. The hyperfine splitting constants for this DMPO adduct were $a^N = a^H = 14.9$ G, which are identical to those of a DMPO-OH adduct resulting from the trapping of ·OH [6]. Moreover, formation of the DMPO-OH adduct was significantly decreased in the presence of either ethanol (0.22 mM) or DMSO (0.14 mM). These observations suggest that the adriamycin–Fe^{3+} complex catalyzed the formation of ·OH in the presence of H_2O_2 and that the DMPO-OH adducts observed did not arise from the decomposition of DMPO-OOH as shown in [7]. In the presence of DNA, the adriamycin–Fe^{3+} complex significantly stimulated the formation of hydroxyl radicals from H_2O_2 (fig.4). Moreover, the concentration of ·OH radicals as measured by monitoring the signal intensity of the low-field line of the DMPO-OH adduct spectrum increased with time and reached a maximum in 15 min. The ability of DNA to stimulate hydroxyl radical formation by the adriamycin–iron complex correlates with the ability of the adriamycin–iron chelate to form a stable ternary complex with DNA which is itself an active redox catalyst. This is the opposite of the effect that DNA addition has on the microsomal reduction of adriamycin to a semiquinone radical, where DNA addition quenches radical formation. In contrast to the adriamycin–Fe^{3+} complex, the 11-deoxyadriamycin–Fe^{3+} mixture, in the presence of DNA, was ineffective in stimulating the formation of ·OH from H_2O_2 (fig.4). Actually the 11-deoxyadriamycin–Fe^{3+} mixture was no better than Fe^{3+} alone in catalyzing ·OH formation from H_2O_2 in the presence of DNA. Thus, both in terms of H_2O_2-catalyzed DNA destruction and hydroxyl radical production 11-deoxyadriamycin is markedly less efficient than adriamycin.

Our results reveal that the DNA–iron–adriamycin complex can catalyze the production of hydroxyl radicals from H_2O_2 under the same conditions in which this complex also causes DNA cleavage.

Table 1

Hydroxyl radical formation and DNA damage catalyzed by anthracycline–Fe^{3+} complexes

Reactant	Relative OH formation	% DNA damage[a]
DNA–Fe^{3+}	100	3.0
11-Deoxyadriamycin + Fe^{3+} + DNA	90	9.4
Adriamycin + Fe^{3+} + DNA	533	84.0

[a] In each case the reaction mixture contained 10 μM H_2O_2

For details see section 2. Relative OH formation is given in arbitrary units based upon peak heights with the signal of DNA–Fe^{3+} set to 100

Furthermore, the relative efficiency of adriamycin and 11-deoxyadriamycin–iron complexes as catalysts of hydroxyl radical production correlates with their effectiveness in the H_2O_2-dependent DNA cleavage reaction (table 1). When taken together with our observation that hydroxyl radical scavengers lessen the DNA damage [3], these results strongly suggest that the DNA damage caused by adriamycin–Fe^{3+} complex in the presence of H_2O_2 is mediated by the hydroxyl radical. The relative inefficiency of the hydroxyl radical scavengers, mannitol and DMSO, can now be explained by the fact that the site of hydroxyl radical generation is the DNA-bound drug–metal complex: the close proximity of source and DNA target makes any reaction of hydroxyl radical with the scavenger less likely.

Finally, these results point to the critical role of the C_{11}-hydroxyl group in iron binding. The lack of spectral shift on iron addition and the fact that DNA damage and hydroxyl radical production are equivalent to iron alone all suggests that 11-deoxyadriamycin does not chelate iron. This is consistent with a prediction we had made earlier based upon the X-ray crystal structure of daunomycin [8]. Briefly, the distance between the oxygens at C_{11} and C_{12}, but not between those at C_5 and C_6, is ideal for iron chelation. Based upon this, we predicted that the absence of the C_{11} hydroxyl would have drastic effects on the iron-chelating properties of adriamycin. Thus, the present results support the hypothesis that the iron-chelation site on adriamycin is between the hydroxyl at C_{11} and the carbonyl at C_{12}. We are now attempting to verify this proposed structure via X-ray crystallography.

REFERENCES

[1] May, P.M., Williams, G.K. and William, V.R. (1980) Eur. J. Cancer 16, 1275–1276.
[2] Myers, C.E., Gianni, L., Simone, C.B., Klecker, R. and Green, R. (1982) Biochemistry 21, 1707–1713.
[3] Eliot, H., Gianni, L. and Myers, C.E. (1984) Biochemistry 23, 928–936.
[4] Sinha, B.K. and Patterson, M.A. (1983) Biochem. Pharmacol. 32, 3279–3284.
[5] Braun, A.G. (1981) in: DNA Repair – A Laboratory Manual of Research Procedures (Friedberg, E.C. and Hanawall, P.M. eds) vol.1, part B, pp.447–455, Marcel Dekker, New York.
[6] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1980) J. Am. Chem. Soc. 102, 4994–4999.
[7] Finkelstein, E., Rosen, C. and Rauckman, E.J. (1980) Arch. Biochem. Biophys. 202, 1–16.
[8] Gianni, L., Corden, B.J. and Myers, C.E. (1983) in: Reviews in Biochemical Toxicology (Hodgson, E. et al. eds) pp.1–82, Elsevier, Amsterdam, New York.