Reduction of daunorubicin aqueous solutions by $COO^{\cdot -}$ free radicals

Reactions of reduced transients with H_2O_2

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Daunorubicin aqueous solutions were reduced by COO^- free radicals produced by γ-radiolysis. This reaction leads to 7-deoxyaglycon daunomycinone. Added before irradiation, H_2O_2 oxidizes hydroquinone daunorubicin giving back the drug directly and thus preventing C-O bond cleavage. The implications of this reaction on the mechanism of the reductive cleavage are discussed.

Daunorubicin *H_2O_2* *γ-Radiolysis* *Glycosidic cleavage*

1. INTRODUCTION

Daunorubicin (daunomycin) (fig.1), one of the anthracycline antitumour antibiotics, is widely used in cancer chemotherapy. It is generally believed that this antibiotic is activated by electron transfer to provide semiquinone and/or hydroquinone reduced states [1]. One of these forms or both is able to lose its sugar moiety under anaerobic conditions [2]. The resulting aglycon has been isolated after in vivo or in vitro reduction (e.g., [3]).

This study concerns two main points: (i) the reductive glycosidic bond cleavage mechanism and (ii) H_2O_2 reactions with the transient reduced forms of daunorubicin. These reactions are poorly understood although they might play a role in daunorubicin toxicity during lipid peroxidation in vivo.

2. MATERIALS AND METHODS

Daunorubicin–HCl was purchased from Sigma. Daunomycinone was donated by Rhône-Poulenc. Reagents (NaH_2PO_4, $NaHCO_3$, NaOH) were Rhône-Poulenc Normapur products. N_2O was a CFPO product. Water was triply distilled and its purity controlled by conductivity measurements ($\leqslant 10^{-6}\ \Omega^{-1}\cdot cm^{-1}$). All the vessels for irradiation were cleaned after washing by heating at 400°C for 3–4 h.

γ-Irradiations were made in a ^{60}Co irradiator.

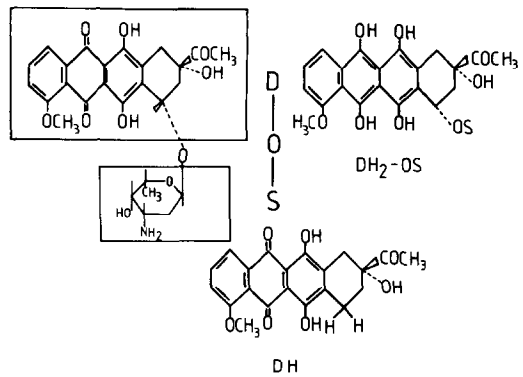

Fig.1. Structure of daunorubicin (DOS), hydroquinone daunorubicin (DH_2OS) and 7-deoxyaglycon daunomycinone (DH). D denotes the anthraquinone part and S the amino sugar. These symbols have no acido-basic significance.

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Its dosimetry was determined by Fricke's method taking G = 15.6 molecules/100 eV and ϵ_{304} = 2160 $mol^{-1} \cdot l \cdot cm^{-1}$. The doses were delivered at a rate of ~3 × 10^{17} $eV \cdot cm^{-3} \cdot h^{-1}$ and varied between 10^{17} and 3 × 10^{18} $eV \cdot cm^{-3}$. Irradiated solutions were transferred to the absorption cell under deaerated atmosphere to avoid eventual reoxidation.

To identify the precipitate, the irradiated solutions were centrifuged. The precipitate was washed several times with distilled water and dried. The NMR analysis was made in the ESCOM NMR service using a Brücker WP 80 device.

3. RESULTS

3.1. *Irradiations*

Daunorubicin (DOS) aqueous solutions (pH 7, phosphate buffer 6 × 10^{-2} $mol \cdot l^{-1}$, $[DOS]_o$ between 2 × 10^{-5} and 3.6 × 10^{-4} $mol \cdot l^{-1}$) were irradiated in the presence of sodium formate (0.1 $mol \cdot l^{-1}$) and under an atmosphere of N_2O to transform e^-_{aq} into $OH^\cdot$ radicals. $H^\cdot$ and $OH^\cdot$ radicals reacted with $HCOO^-$ to produce $COO^{\cdot -}$ free radicals. Thus the only species which could react with DOS were COO^- reducing ions and their yield of formation was equal to:

$$G(COO^{\cdot -}) = G_H + G_{OH} + G_{e^-_{aq}} \approx 6 \text{ molecules/100 eV}$$

Fig.2 shows the absorption spectra of some irradiated solutions for $[DOS]_o$ = 1.52 × 10^{-4} $mol \cdot l^{-1}$. When the dose increases the maximum at 480–500 nm decreases and the absorption at 56–750 nm increases slightly. For doses < 1 × 10^{18} $eV \cdot cm^{-3}$ these spectra can be normalized to the DOS spectrum. For higher doses there is a difference of 10% between the experimental and normalized spectra (fig.2d) at λ < 440 nm and λ > 540 nm. These spectra are not modified by air entry after irradiation.

For doses higher than ~1 × 10^{18} $eV \cdot cm^{-3}$, a red powder precipitates. NMR analysis of this precipitate was performed. The whole spectrum was compared to the daunomycinone NMR spectrum and could be attributed to 7-deoxyaglycon daunomycinone (DH) (see fig.1 for DH structure). DH solubility in water is ~10^{-5} $mol \cdot l^{-1}$, perhaps slightly higher in DOS solutions. For such concentrations, DH contribution to the absorption spectra is negligible. In particular, the absorption at 560–750 nm does not belong to DH and is not due to H_2O_2 reactions (see section 3.2). It may belong to another reduced form of the drug which would represent only a few percent of $[DOS]_o$. This was demonstrated by comparison of the weight of precipitate and the amount of disappeared DOS. This amount of disappeared drug $[DOS]_{dis}$ was calculated at 480 nm (ϵ_{480} = 10450 $mol^{-1} \cdot l \cdot cm^{-1}$) (e.g., see fig.3a). $[DOS]_{dis}$ is proportional to the dose. The slope of the straight line is equal to $G(-DOS)$ = 2.30 ± 0.10 molecules/100 eV and is independent of $[DOS]_o$ (inset, fig.3). $G(-DOS)$ is very close to

$$\frac{G(COO^{\cdot -})}{2} - G_{H_2O_2} = \frac{6}{2} - 0.7 =$$

2.3 molecules/100 eV

This means that DOS is reduced with two equivalents and that H_2O_2 produced radiolytically reoxidizes a reduced form of DOS giving back the initial drug.

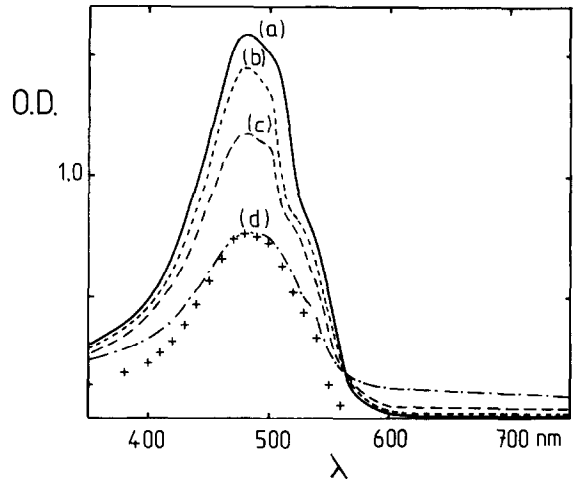

Fig.2. Absorption spectra of daunorubicin irradiated aqueous solutions. [DOS] = 1.52 × 10^{-4} M, $[HCOO^-]$ = 0.1 M, pH 7, phosphate buffer 6 × 10^{-2} M, N_2O. Curves: (a) no irradiation, initial solution; (b) 3.78 × 10^{17} $eV \cdot cm^{-3}$; (c) 1.026 × 10^{18} $eV \cdot cm^{-3}$; (d) 2.08 × 10^{18} $eV \cdot cm^{-3}$, (+) D-OS spectrum normalized at 480 nm.

3.2. *Action of H_2O_2*

To determine which compound(s) is/are attacked by H_2O_2 and the mechanism of this oxida-

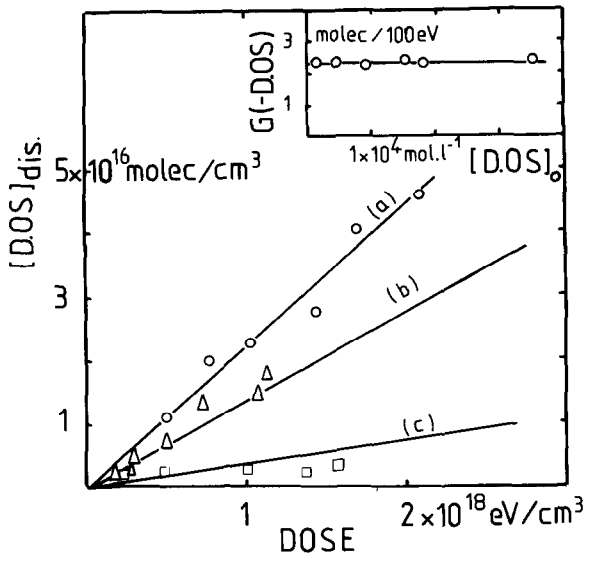

Fig.3. DOS disappearance on irradiation. $[DOS]_o$ = 8.85×10^{-5} M, $[HCOO^-]$ = 0.1 M, pH 7, phosphate buffer 6×10^{-2} M, N_2O, dose rate ~3×10^{17} eV·cm^{-3}. (a) No added H_2O_2, (b) $[H_2O_2]_o = 5 \times 10^{-5}$ M, (c) $[H_2O_2]_o = 2 \times 10^{-4}$ M. (inset) Variation of $G(-DOS)$ with $[DOS]_o$.

tion, the reactions of H_2O_2 during and after irradiation were studied. The initial antibiotic does not react with H_2O_2.

3.2.1. After irradiation

Addition of H_2O_2 after irradiation led to no change in the absorption spectrum, amount of precipitate and $G(-DOS)$, showing that H_2O_2 does not react after irradiation.

3.2.2. During irradiation

DOS aqueous solutions identical to the preceding ones were irradiated in the presence of various H_2O_2 initial concentrations from 5×10^{-5} to 2×10^{-4} mol·l^{-1} (see fig.3b,c). $[DOS]_{dis}$ is proportional to the dose but $G(-DOS)$ decreases when H_2O_2 increases. The minimal dose for the beginning of precipitation increases with $[H_2O_2]_o$. As an example, for $[H_2O_2]_o = 2 \times 10^{-4}$ mol·l^{-1}, precipitation begins at doses ~2×10^{18} eV·cm^{-3}. So it seems that H_2O_2 reacts with an intermediate reduced form of DOS, thus delaying precipitation.

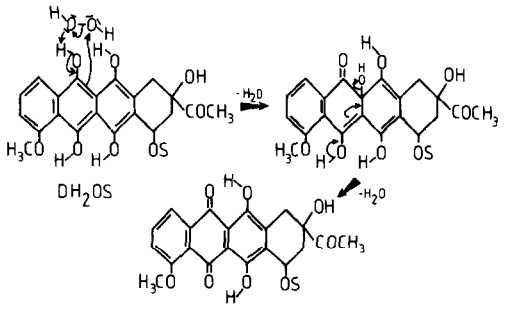

Fig.4. A proposed mechanism for hydroquinone daunorubicin oxidation by H_2O_2.

4. DISCUSSION

4.1. *Reactions of $COO^{\cdot -}$*

The kinetic scheme for $COO^{\cdot -}$ reactions may be written:

$$DOS + COO^{\cdot -} \longrightarrow DOS^{\cdot -} + CO_2 \quad (1)$$

$$DOS^{\cdot -} + CO_2^{\cdot -} \xrightarrow{2H^+} DH_2OS + CO_2 \text{ and/or} \quad (2)$$

$$2DOS^{\cdot -} \xrightarrow{2H^+} DOS + DH_2OS \quad (3)$$

$DOS^{\cdot -}$ and DH_2OS represent the semiquinone and the hydroquinone forms of daunorubicin, respectively. $COO^{\cdot -}$ disproportionation does not occur here since $G(-DOS)$ is independent of $[DOS]^o$, which means that all $COO^{\cdot -}$ react with the drug.

4.2. *DH precipitation*

According to the literature, this can occur by:

$$DOS^{\cdot -} \overset{H^+}{=} DH\downarrow + SO^{\cdot} \text{ or } D^{\cdot} + SOH \text{ and/or} \quad (4)$$

$$DH_2OS = DH\downarrow + SOH \quad (5)$$

It is known that the hydroquinone drug is unstable. Under the conditions of γ-radiolysis reaction 4 does not proceed because it would lead to $G(-DOS) \approx 6$ molecules/100 eV. So, during γ-radiolysis, C-O bond cleavage occurs on the hydroquinone form DH_2OS, the concentration of which is at a steady state.

4.3. *Reactions of H_2O_2*

It was seen that $G(-DOS) = [G(COO^{\cdot -})/2] - G_{H_2O_2}$ which indicated that H_2O_2 was responsible for a reverse reaction. As this reaction gives back DOS, it must concern a compound still possessing its sugar. It was proposed recently [4–6]:

$$DOS^{\cdot -} + H_2O_2 \longrightarrow DOS + OH^{\cdot} + OH^- \quad (6)$$

Similarly, one can suppose:

$$DH_2OS + H_2O_2 \longrightarrow DOS^{\cdot -} + OH^{\cdot} + OH^- + 2H^+ \quad (7)$$

However, with some hydroquinonic compounds, a direct two-electron oxidation by H_2O_2 may be encountered [7]:

$$DH_2OS + H_2O_2 \longrightarrow DOS + 2H_2O \quad (8)$$

Since $HCOO^-$ ions are in excess, reactions 6 and 7 would be followed by:

$$OH^{\cdot} + HCOO^- \longrightarrow H_2O + COO^{\cdot -} \quad (9)$$

giving a reducing $COO^{\cdot -}$ and $G(-DOS)$ would be equal to $G(COO^{\cdot -})/2 = 3$ molecules/100 eV. To explain the lower experimental value, reaction 8 leading directly from DH_2OS to DOS has to occur. It also means that under the condition of γ-radiolysis, reactions 6 and 7 do not occur. Fig.4 shows a proposed mechanism.

Calculation of $G(-DOS)$ from the kinetic scheme of reactions 1–3,5 and 8 leads to:

$$G(-DOS) = G(COO^{\cdot -})\left(1 - \frac{1}{1 + \frac{k_5}{k_8[H_2O_2]}}\right)$$

Knowing $G(-DOS)$ for different $[H_2O_2]_o$, k_5/k_8 was evaluated:

$$\frac{k_5}{k_8} = (3.3 \pm 0.7) \times 10^{-5}\ mol \cdot l^{-1}$$

4.4. *Mechanism of the glycosidic bond cleavage*

Reaction 5 can proceed by two different mechanisms, heterolytic or homolytic elimination [8]. Homolytic breakage would lead to hydroquinone 7-deoxydaunomycinone by radical disproportionation. This compound has never been found in our irradiated solutions. So our results seem to favour heterolytic C-O bond cleavage, at least for the major process.

In conclusion, this work demonstrates that when daunorubicin is reduced by $COO^{\cdot -}$ during γ-radiolysis: (i) the C-O bond cleavage occurs on the hydroquinone compound and probably has a heterolytic mechanism and (ii) H_2O_2 oxidizes hydroquinone daunorubicin (DH_2OS) giving in one step the quinone form. This reaction should be taken into account in the mechanism of cardiotoxicity and of lipid peroxidation induced by this antibiotic.

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