Free Rad. Res. Comms., Vol. 5, No. 3, pp. 169–176
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A COMPARISON OF THE FREE RADICAL PROPERTIES OF SEVERAL ANTHRACYCLINE ANTI-TUMOUR DRUGS AND SOME OF THEIR ANALOGUES

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(Received May 10th 1988; in revised form May 25th 1988)

NADPH consumption and esr spectroscopy have been used to study the rate of formation and signal intensity of free radicals produced by various anthracycline anti-tumour drugs in rat liver microsomal extract. The drugs investigated were Adriamycin, 4'Deoxyadriamycin, Daunorubicin, 4 Demethoxydaunorubicin and Carminomycin. Pulse radiolysis was also used to determine the ease of reduction of each of the analogues to its semiquinone radical and some kinetic properties of the radicals produced. It is believed that the occurrence of reactions other than dismutation could be responsible for the shortened lifetimes of the semiquinone radicals observed in biological systems.

KEY WORDS: Anthracyclines, anti-tumour agents, esr, NADPH consumption, radiation chemistry.

INTRODUCTION

The action of the anthracycline antitumour agents now encompasses not only intercalation into DNA but also membranal disruptions, free radical production and the generation of activated oxygen species as well as the formation of iron-anthracycline complexes. The impressive clinical success of the compounds, Adriamycin and Daunorubicin, has been moderated by the realization that, in some instances, the antitumour effect can only be achieved at the high cost of dose-limiting side effects including bone-marrow suppression, stomatitis and cardiotoxicity.[1]

Several workers have suggested that the cardiotoxicity observed results from the production of free radicals and their ensuing atack on cellular components, (e.g. 2–4). No specific enzyme has been proposed but certain systems including NADPH-cytochrome P-450 reductase, mitochondrial NADH dehydrogenase and cytosolic xanthine oxidase can all function as quinone reductase; e.g.

$$2Q + NAD(P)H \rightarrow 2Q^{\cdot -} + NAD(P)^{+} + H^{+} \quad (1)$$

The resulting semiquinone radical ($Q^{\cdot -}$) can react with oxygen producing superoxide radicals and subsequently more active oxygen species. The production of these free radicals is believed to account for the damage to membranes and proteins observed in anthracycline-treated systems.

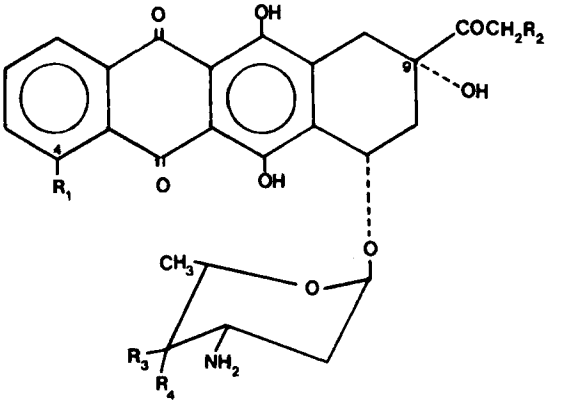
With these problems in mind, great effort has been expended in the search for an analogue which could modify the distressing toxicities of these agents. Structural

modifications have been introduced into the parent anthracycline compound on the planar ring section and also on the amino-sugar group resulting in some of these anthracycline analogues undergoing clinical trials.[5,6]

In this study, six anthracycline analogues were chosen which have different substituents at positions C-4 and C-9 or on the amino sugar moiety and/or are currently undergoing clinical trials. Comparisons have been made between the esr free radical signals observed in rat liver microsomal extract and the ability of each analogue to consume NADPH in this system. Pulse radiolysis has been used to study the ease of reduction of each of the analogues to its semiquinone radical as well as some kinetic properties of the radicals produced.

MATERIALS AND METHODS

The anthracyclines used were Adriamycin, Daunorubicin, 4'Epiadriamycin, Carminomycin, 4 Demethoxydaunorubicin and 4'Deoxyadriamycin (Figure 1). Adriamy-



Anthracycline	R_1	R_2	R_3	R_4
Adriamycin	OCH_3	OH	H	OH
4'Epiadriamycin	OCH_3	OH	OH	H
4'Deoxyadriamycin	OCH_3	OH	H	H
Daunorubicin	OCH_3	H	H	OH
4 Demethoxydaunorubicin	H	H	H	OH
Carminomycin	OH	H	H	OH

FIGURE 1 The structures of the anthracycline analogues.

cin, 4'Epiadriamycin and 4 Demethoxydaunorubicin were purchased from Farmatalia Carlo Erba Ltd while Daunorubicin was obtained from May & Baker Ltd. Carminomycin was a generous gift from Dr C Franks, Bristol-Myers Int. Corp., Belgium and 4'Deoxyadriamycin was a gift from Farmitalia Carlo Erba Ltd.

Rat liver microsomal extract was prepared by manual homogenisation of tissue in KCl/Tris buffer at pH 7.5, centrifugation for 30 minutes at 25,000 × g and the resulting supernatant subjected to further centrifugation at 100,000 × g for 1 hour. The protein concentration determined using the Bio-Rad assay.

NADPH consumption was measured at ambient temperature by monitoring the rate of NADPH oxidation at 340 nm ($\varepsilon = 6220\,M^{-1}cm^{-1}$). The incubation mixture (3.0 ml) consisted of KCl/Tris buffer, pH 7.5, NADPH-generating system[7] (0.20 ml), microsomal extract (0.20 mg protein) in the absence or presence of anthracycline drug (50 μM).

Electron spin resonance studies were conducted at ambient temperature using an incubation mixture consisting of drug (50 μM), NADPH-generating mixture (0.20 ml) and microsomal extract (6 mg protein) and were purged with nitrogen. Studies were also carried out using a deaerated solution of hypoxanthine (0.1 mM), xanthine oxidase (1 unit, (1 unit mg^{-1} protein)) and drug (50 μM) in phosphate buffer (10 mM), pH 7.0, containing desferal (0.1 mM) in a total volume of 1.0 ml. The esr spectra were scanned using a Varian E-9 X-band spectrometer with 100 kHz modulation.

The pulse radiolysis experiments were conducted using the Paterson Institute 10 MeV Linear Accelerator facility.[8] On pulsing solutions which contain sodium formate (100 mM), phosphate buffer (10 mM, pH 7.0) and anthracycline (5–30 μM) the following reactions are initiated:

$$H_2O \rightarrow e_{aq}^-, \cdot OH, \cdot H, H_2O_2, H_2 \ldots \quad (2)$$

$$e_{aq}^- + N_2O + H_2O \rightarrow \cdot OH + OH^- + N_2 \quad (3)$$

$$\cdot OH(\cdot H) + HCOO^- \rightarrow H_2O(H_2) + CO_2^{\cdot -} \quad (4)$$

$$e_{aq}^- (CO_2^{\cdot -}) + Q \rightarrow CO_2 + Q^{\cdot -} \quad (5)$$

$$Q^{\cdot -} + Q^{\cdot -} \rightarrow Q^{2-} + Q \quad (6)$$

The rate constants for reaction (6) were measured for the decay of the semiquinones at their λ_{max}. The total dose given to the solutions was changed such that the concentration of semiquinone varied between 0.2 and 3 μM.

One electron reduction potentials were measured by setting up an equilibrium between the anthracycline/anthracycline semiquinone and the corresponding reduced and oxidised forms of 1,1 dimethyl-4,4 bipyridylium chloride, (MV^{2+}), $E_7^1 = -450$ mV.[9] By measuring the absorbance changes at equilibrium for various drug concentrations, it is possible to calculate the differences in one electron reduction potential between the anthracycline and the standard compound (e.g. 10, 11).

In the presence of N_2O/O_2 mixtures, the anthracycline semiquinone radicals do not undergo reaction (6) but rather react with oxygen:

$$Q^{\cdot -} + O_2 \rightleftharpoons Q + O_2^{\cdot -} \quad (7)$$

The rate constants for the above reaction were determined at the λ_{max} of the semiquinone radicals. The concentration of oxygen was varied between 1% and 2.5%.

RESULTS

Table I shows the properties of the various semiquinone radicals as measured by pulse-radiolysis. The values for the one-electron reduction potentials show little variation and within experimental error could be considered to be approximately the same, $E_7^1(Q/Q^{\cdot-}) = -(347) \pm 20\,mV$. Similarly, the rate constants for the reactions of the semiquinone radicals with oxygen, k_7, are all around $5.1 \pm 0.5 \times 10^8\,M^{-1}\,s^{-1}$ with Carminomycin being the exception, $k_7 = 1.5 \pm 0.2 \times 10^8\,M^{-1}\,s^{-1}$. The rate constants for the decay of the semiquinone radicals are greatly different in that they span six orders of magnitude from 4'Epiadriamycin ($2k_6 = 3.0 \pm 0.5 \times 10^9\,M^{-1}\,s^{-1}$) to Carminomycin ($2k_6 = 2.3 \times 1.0 \times 10^3\,M^{-1}\,s^{-1}$).

The increase in the basal rate of NADPH consumption in the rat liver microsomal extracts for each of the anthracyclines is given in Table II as a ratio of the values obtained for Adriamycin. Interestingly, only Carminomycin shows any significant increase in NADPH consumption compared to that for Adriamycin.

The ability of the anthracyclines to produce semiquinone radicals was also assessed from the intensity of the esr signals (see Figure 2). It can be seen that the traces are similar to those observed for anthracycline semiquinone radicals produced in a purified enzyme system in that the spectra are broad unresolved singlets centred on free spin.[12] The ratios of the signal intensities (rat liver microsomal extract (a–c) and

TABLE I

Properties of the semiquinone radical ($Q^{\cdot-}$) from the anthracyclines investigated.

Anthracycline (Q)	E_7^1 /mV	$k(Q^{\cdot-} + O_2)$ $/10^8\,M^{-1}\,s^{-1}$	$2k(Q^{\cdot-} + Q^{\cdot-})$ $/10^9\,M^{-1}\,s^{-1}$
Adriamycin	-347 ± 10*	4.6 ± 0.5	2.8 ± 1.0
4'Epiadriamycin	-351 ± 10	5.3 ± 0.5	3.0 ± 0.5
4'Deoxyadriamycin	-357 ± 10	5.5 ± 0.5	2.2 ± 0.5
Daunorubicin	-337 ± 15	5.3 ± 0.5	1.8 ± 0.2
4 Demethoxydaunorubicin	-345 ± 10	4.6 ± 0.5	$3.5 \pm 0.3 \times 10^{-5}$
Carminomycin	-341 ± 10	1.5 ± 0.2	$2.3 \pm 1.0 \times 10^{-6}$

*The previous value of $-328\,mV$[20] has since been superseded by $-341 \pm 15\,mV$ (Mukherjee, Land, Swallow and Bruce, in preparation).

TABLE II

The ratios of anthracycline NADPH consumption and esr signal intensities in rat liver microsomal extract to those measured for Adriamycin. The increase over the basal rate of NADPH consumption on addition of Adriamycin was found to be $7.7 \pm 4.7\,nmol\,min^{-1}\,mg^{-1}$ protein. (see Materials and Methods for further details).

Anthracycline (Q)	(n)	(Q) (Adriamycin)	
		NADPH	ESR[a]
Adriamycin	10	1.0	1.0
4'Epiadriamycin	2	1.2 ± 0.1	1.5 ± 0.1
4'Deoxyadriamycin	4	1.1 ± 0.4	1.15 ± 0.05
Daunorubicin	2	0.8 ± 0.2	1.3 ± 0.1
4 Demethoxydaunorubicin	6	1.0 ± 0.3	1.2 ± 0.1
Carminomycin	6	2.3 ± 0.6	2.3 ± 0.1

n = number of determinations performed in NADPH assay.
a = esr signals averaged over 12 scans.

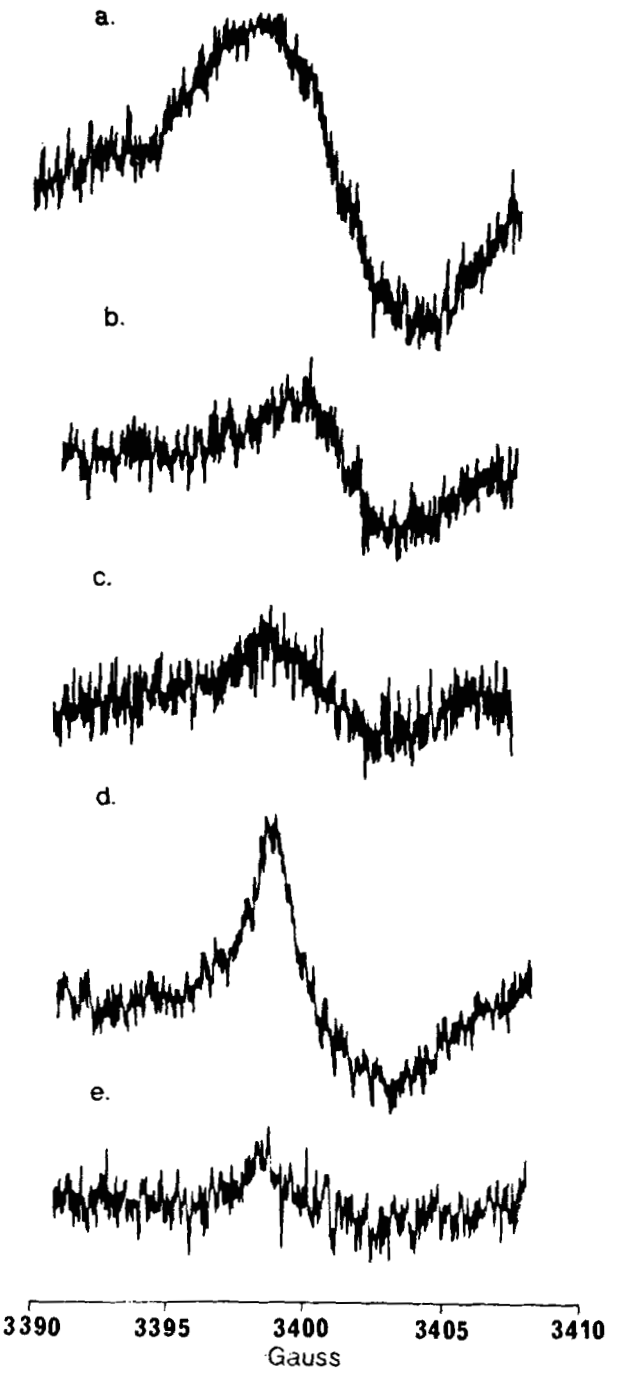


FIGURE 2 X-band esr spectra showing a) Carminomycin free radical, b) 4′Deoxyadriamycin and c) Adriamycin in NADPH-supplemented rat liver microsomal extract. d) Carminomycin and e) Adriamycin free radicals in a Xanthine oxidase/hypoxanthine system (see text for details). The scan range was 20G, modulation amplitude = 1G, microwave frequency = 9.47 GHz, incident microwave power = 10 mW, gain = 4×10^4.

xanthine oxidase/hypoxanthine (d,e)) at their peak heights were compared to that of Adriamycin and are included in Table II. Although the spectra do display some line broadening, approximate comparisons can be made to indicate the ease of free radical production. While most of the anthracyclines produced similar intensities to Adriamycin, it was observed that Carminomycin showed a two-fold increase over Adriamycin. The increase was found to rise to a factor of five when the xanthine oxidase/hypoxanthine system was used (Figure 2).

DISCUSSION

The values for the one-electron reduction potentials of the six anthracyclines are all centred around -347 ± 20 mV. This is not surprising considering the relative structures of the drugs and the fact that in general the anthracyclines tend to have relatively negative potentials. In a previous study on the reduction of various quinones and 1,1 dimethyl-4,4 bipyridylium chloride by the xanthine oxidase/hypoxanthine system,[13] it was observed that the rate of reduction varied in a non-linear manner by over one hundred-fold when the reduction potential of the substrate was between -70 and -450 mV. It can be seen from the results in Table II that within the experimental errors, the rate of NADPH consumption in the microsomal extracts are all very similar and is consistent with the hypothesis that the ease at which electrons are donated depends upon the reduction potential of the quinone/semiquinone couple. Carminomycin is possibly an exception in that the rate of NADPH consumption was consistently higher than those of the other anthracyclines.

It is also interesting to note that 4'Epiadriamycin is known to be less cardiotoxic than Adriamycin (e.g. 14) and references therein, and has been shown to enhance heart lipid peroxidation to a lesser extent than Adriamycin in mice.[15] However, it can be seen from the results in Tables I and II that the ability of the drugs to form free radicals and the reactivities of these radicals are practically the same. A similar conclusion was arrived at by comparing the relative amounts of TBA-reactive materials formed by the two drugs in NADPH stimulated liver microsomes.[17] The difference in the cardiotoxicities has to be due to other pharmokinetic parameters which could include differences in influx/efflux and cellular accumulation.

4 Demethoxydaunorubicin and Carminomycin semiquinone radicals are different to those of the other anthracyclines in that the second order rate constants for the decay of these radicals are relatively low (see Table I). All of the other anthracyclines have a methoxy group in the C-4 position of the A-ring whereas Carminomycin has a hydroxyl group and 4 Demethoxydaunorubicin has a hydrogen. Thus, the semiquinone radicals for these two drugs will be partially stabilised via hydrogen bonding from the carbonyl group on the quinone to the hydrogen on position 4. This is also demonstrated by the fact that the esr signals from the Carminomycin semiquinone radicals produced in the pure xanthine oxidase/hypoxanthine system are much more intense than those for Adriamycin (Figure 2). However, the esr signals intensities produced in the rat liver microsomes (Table II) are apparently more dependent on the rate of NADPH consumption i.e. the rate of semiquinone radical formation, than the rate of decay of the radicals.

In a previous study,[16] it was shown that ratio of Carminomycin to Adriamycin semiquinone yield in highly fractionated rat liver microsomes was greater than twenty. This would be more consistent with our purified xanthine oxidase/hypoxanth-

ine results. However, in the previous study the high ratios of the semiquinone yields in microsomes were obtained using 1 mM anthracycline (c.f. 50 μM in the present study) and the yields of radicals and the time course of this formation were strongly dependent on the total protein concentration. In an ideal environment, the formation and decay of semiquinone radical should be dependent on just two reactions:

$$Q \rightarrow Q^{\cdot-} \quad (8)$$

$$Q^{\cdot-} + Q^{\cdot-} \rightarrow Q^{2-} + Q \quad (6)$$

The steady state concentration of the semiquinone radical is equal to $(k_8[Q]/k_6)^{0.5}$. As $k_6 = 2.8 \pm 1.0 \times 10^9\ M^{-1}\ s^{-1}$ for Adriamycin and $2.3 \pm 1.0 \times 10^3\ M^{-1}\ s^{-1}$ for Carminomycin, it would be predicted that the steady state concentration of the Carminomycin semiquinone radials should be $> 10^3$ times more than that of the Adriamycin radicals. It is apparent from this study and the previous work that reactions (6) and (8) cannot fully explain the differences in the yields of radicals and at least one further reaction must occur. From the previous study[16] it is evident that by increasing the extract protein concentration, the initial yield of radicals is greater while the corresponding rate of decay of these radicals also increases. This implies that the semiquinone radicals can react with the constituents in the protein extract. These reactions could involve metalloproteins[17] or metal complexes.[18] However, a recent study has clearly demonstrated the formation of semiquinone/amino acid adducts[19] and reactions of this type could also explain the effect. It would appear that under more physiologically relevant conditions, i.e. low concentration of drug and high concentration of protein extract, that the lifetime of the semiquinone radicals is not very dependent on the rate of dismutation of the radicals but more dependent on the initial rate of formation.

Acknowledgments

This work has been supported by the Cancer Research Campaign.

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Accepted by Dr. B. Halliwell