Biochemical Pharmacology, Vol. 34, No. 23, pp. 4099–4103, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

FREE RADICAL DAMAGE TO DEOXYRIBOSE BY ANTHRACYCLINE, AUREOLIC ACID AND AMINOQUINONE ANTITUMOUR ANTIBIOTICS

AN ESSENTIAL REQUIREMENT FOR IRON, SEMIQUINONES AND HYDROGEN PEROXIDE

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(*Received* 14 *March* 1985; *accepted* 13 *May* 1985)

Abstract—Anthracycline, aureolic acid and aminoquinone antitumour antibiotics damage deoxyribose in cell-free systems when reduced in air by the enzyme ferredoxin reductase. Damage to deoxyribose is inhibited by the iron chelator desferrioxamine, the copper-containing protein caeruloplasmin and catalase but not by superoxide dismutase. Scavengers of the hydroxyl radical such as formate, butan-1-ol, ethanol and benzoate do not offer much protection, whereas mannitol and thiourea do. These findings point to a site-specific Fenton reaction in which the drug semiquinones reduce complexed iron and dioxygen leading to the formation of hydrogen peroxide and a ferrous complex.

Antitumour antibiotics isolated from microbial cultures, are an important group of drugs widely used in the treatment of human tumours. Like most forms of cancer chemotherapy they display a wide range of undesirable side effects. It is this undesirable toxicity which prevents the use at a dosage that would maximize cancer cell killing. A better understanding of the mechanisms by which antitumour antibiotics damage both target DNA and non-target sites is essential for their safe use and future development.

An important contribution to our understanding of the mechanisms by which antitumour antibiotics act, resulted from the work of Handa and Sato [1]. They showed that mitomycin c was reduced in the endoplasmic reticulum by an NADPH-dependent enzyme system to yield a semiquinone free radical. Activation of antitumour antibiotics to reactive radical intermediates has since provided a rational explanation for their damaging properties observed both *in vivo* and *in vitro* towards DNA, membranes and other sites [2–5].

Most, if not all, of the antitumour antibiotics bind to or intercalate with DNA. In addition, many form complexes with metal ions. It is the redox cycling of the bound metal ion [6, 7] or the quinone moiety of the drug [2, 8] or both [9, 10] that provide these drugs with their radical generating properties. The deoxyribose moiety of DNA is susceptible to oxidative damage when radicals are generated radiolytically [11, 12] or produced by redox-cycling drugs [13, 14]. The simple sugar deoxyribose provides, therefore, a sensitive yet inexpensive detector molecule for assessing iron and drug-dependent free radical damage in cell-free systems [15–19].

Many of the antitumour antibiotics display toxicities towards growing cancer cells, which depend on the tissue O_2 concentration [20]. Under certain circumstances hypoxic conditions enhance tumour cell killing [20], and deoxyribose degradation by promoting reduction of the drug to highly reactive intermediates [16–19]. In the present study the anthracycline drugs adriamycin, daunomycin and epirubicin and the related compound carminic acid, the aureolic drugs mithramycin, olivomycin and chromomycin A3 and the aminoquinone drugs mitomycin c and streptonigrin have been examined for their deoxyribose damaging properties under aerobic conditions in the presence of ferredoxin reductase and NADPH. The results obtained indicate that all the drugs tested within these groups readily form semiquinone radicals which, in the presence of molecular oxygen and traces of iron salts, lead to the formation of a highly reactive species which is probably the hydroxyl radical.

MATERIALS AND METHODS

2-Deoxy-D-ribose, ferredoxin NADP reductase (spinach leaves), catalase (bovine liver), superoxide dismutase (bovine erythrocyte), NADPH, caeruloplasmin (human type X), chromomycin A3, daunomycin, mithramycin complex, carminic acid, and mitomycin c were from the Sigma Chemical Co. Ltd (Poole, Dorset). Desferrioxamine "Desferal" was from Ciba Geigy Ltd. All other chemicals were of the highest grades available from BDH Ltd. Units of enzyme activity were as defined in the Sigma catalogue.

Deoxyribose degradation

The reaction conditions for adriamycin (doxorubicin), daunomycin and epirubicin were as follows: to new clean glass tubes were added the following reagents in the order stated 0.2 ml deoxyribose (10 mM), 0.4 ml NADPH (1.5 mM), 0.1 ml of antitumour antibiotic 1 mM, 0.1 ml of Chelex resin

treated distilled water and 0.1 ml ferredoxin reductase 0.6 units per ml. Where indicated, in relevant tables, 0.1 ml of the inhibitors were added instead of distilled water. The reaction conditions for the remaining drugs were as above except, 0.2 ml of NADPH and 0.02 ml of the drugs were added to the reaction.

Development of thiobarbituric acid-reactivity

After incubation, 0.5 ml of 1% (w/v) thiobarbituric acid in 50 mM NaOH was added to each tube followed by 0.5 ml of 2.8% (w/v) trichloroacetic. The tube contents were heated at 100° for 15 min. When cool absorbance values were read at 532 nm. Appropriate blanks were included to allow subtraction of the chromogenic properties of the drugs themselves.

All the results shown are the mean of three or more assays which differed by less than ± 6%.

RESULTS

Incubation of anthracycline, aureolic acid and aminoquinone antitumour antibiotics with ferredoxin reductase and NADPH under air resulted in the enhanced degradation of deoxyribose detected as release of thiobarbituric acid (TBA)-reactive material (Table 1). The anthracycline drugs were more reactive than the other drugs tested. They are also known to be effective metal chelators [9, 21, 22].

The ferric ion chelator desferrioxamine was a potent inhibitor of all drug stimulated deoxyribose degradations (Table 2) suggesting a key role for iron salts in the formation of the damaging species. Catalase, added as a specific indicator of the formation and participation of hydrogen peroxide was also strongly inhibitory. Its protective action could be abolished by heat-denaturation of the protein (Table 3), and the addition of albumin as a control

Table 1. Deoxyribose degradation stimulated by antitumour antibiotics in the presence of Ferredoxin reductase

	TBA-reactivity A532nm after 1 hr incubation at 37°		
	Control (Ferredoxin reductase omitted)	Test	% Stimulation of reaction by antibiotic
Deoxyribose 2.5 mM	0.008	0.008	
NADPH	0.017	0.017	
Ferredoxin reductase (FR)	—	0.011	
Deoxyribose + NADPH + FR (Blank)	—	0.077	
Mitomycin C 0.025 mM	0.027	0.158	70
Streptonigrin 0.025 mM	0.043	0.186	86
Mithramycin 0.025 mM	0.037	0.285	222
Olivomycin 0.025 mM	0.034	0.206	123
Chromomycin A_3 0.025 mM	0.055	0.182	64
Carminic acid 0.025 mM	0.046	0.325	266
Doxorubicin 0.10 mM	0.316	0.856	371
Daunomycin 0.10 mM	0.323	0.973	476
Epirubicin 0.10 mM	0.341	0.943	419

Results are shown after the subtraction of appropriate blanks and controls. High control values for doxorubicin, daunomycin and epirubicin are due to the chromogenic properties of the drugs at A532 nm. These drugs were incubated with NADPH at a conc of 0.6 mM; all others were incubated with 0.4 mM NADPH.

Table 2. The effect of desferrioxamine on deoxyribose degradation stimulated by antitumour antibiotics

	TBA-reactivity A532 nm after 1 hr incubation at 37°		
	Test	Test + desferrioxamine (0.2 mM)	% Inhibition by desferrioxamine
Deoxyribose + NADPH + FR (Blank)	0.077		
Mitomycin C	0.131	0.020	83%
Streptonigrin	0.143	0.021	85%
Mithramycin	0.248	0.022	91%
Olivomycin	0.172	0.015	91%
Chromomycin A_3	0.127	0.013	90%
Carminic acid	0.282	0.014	95%
Doxorubicin	0.546	0	100%
Daunomycin	0.668	0	100%
Epirubicin	0.602	0	100%

Control values (see Table 1) have been subtracted. Final reaction concentrations of reagents are as shown in Table 1.

FR = Ferredoxin reductase.

Table 3. The effect of Catalase on deoxyribose degradation stimulated by antitumour antibiotics

	TBA-reactivity A532 nm after 1 hr incubation at 37°			
	Test	Test + catalase 0.05 mM	% Inhibition by catalase	Test + heat denatured catalase
Dexoyribose + NADPH + FR (Blank)	0.077			
Mitomycin C	0.131	0	100%	0.182
Streptonigrin	0.143	0	100%	0.169
Mithramycin	0.248	0.008	97%	0.355
Olivomycin	0.172	0	100%	0.269
Chromomycin A_3	0.127	0	100%	0.235
Carminic acid	0.282	0	100%	0.299
Doxorubicin	0.546	0	100%	0.965
Daunomycin	0.668	0	100%	0.868
Epirubicin	0.602	0	100%	0.439

Control values (see Table 1) have been subtracted. Final reaction concentrations of reagents are shown in Table 1. Heat-denatured catalase did not inhibit these reactions. Its stimulatory properties can probably be ascribed to the release of iron.
FR = Ferredoxin reductase.

Table 4. The effect of superoxide dismutase on deoxyribose degradation stimulated by antitumour antibiotics

	TBA-reactivity A532 nm after 1 hr incubation at 37°		
	Test	Test + superoxide dismutase 0.05 mM	% Inhibition or stimulation by superoxide dismutase
Deoxyribose + NADPH + FR (Blank)	0.077		
Mitomycin C	0.131	0.120	8% Inhib.
Streptonigrin	0.143	0.150	4% Stim.
Mithramycin	0.248	0.208	16% Inhib.
Olivomycin	0.172	0.152	12% Inhib.
Chromomycin A_3	0.127	0.123	3% Inhib.
Carminic acid	0.282	0.242	14% Inhib.
Doxorubicin	0.546	0.576	5% Stim.
Daunomycin	0.668	0.662	1% Inhib.
Epirubicin	0.602	0.620	3% Stim.

Control values (see Table 1) have been subtracted. Final reaction concentrations of reagents are as shown in Table 1.
FR = Ferridoxin reductase.

Table 5. The inhibitory activity of caeruloplasmin on deoxyribose degradation stimulated by antitumour antibiotics

	TBA-reactivity A532 nm after 1 hr incubation at 37°		
	Test	Test + caeruloplasmin (3 units/ml)	% Inhibition by caeruloplasmin
Dexoyribose + NADPH + FR	0.077		
Mitomycin C	0.131	0.014	89%
Streptonigrin	0.143	0.005	97%
Mithramycin	0.248	0.078	69%
Olivomycin	0.172	0.080	53%
Chromomycin A_3	0.127	0.063	50%
Carminic acid	0.282	0.080	72%
Doxorubicin	0.546	0	100%
Daunomycin	0.668	0	100%
Epirubicin	0.602	0	100%

Control and blank values have been subtracted (see Table 1). Final concentrations of reagents are as shown in Table 1. Heat-denaturation of caeruloplasmin reduced its inhibitory values.
FR = Ferredoxin reductase.

Table 6. The effect of hydroxyl radical scavengers on deoxyribose degradation stimulated by epirubicin and mithramycin

	TBA-reactivity A532 nm after 1 hr incubation at 37°			
	Epirubicin (0.10 mM)	% Inhibition by $OH^{\cdot}$ scavengers	Mithramycin (0.025 mM)	% Inhibition by $OH^{\cdot}$ scavengers
Test (deoxyribose + NADPH + FR + Drug)	0.602		0.248	
+ Formate 13.9 mM	0.523	13.1%	0.222	11%
+ Benzoate 13.9 mM	0.508	15.6%	0.220	11%
+ Mannitol 13.9 mM	0.413	31.4%	0.088	65%
+ Urea 1.4 mM	0.639	0%	0.257	0%
+ Thiourea 1.4 mM	0.461	23%	0.186	15%
+ Ethanol 13.9 mM	0.569	6%	0.240	3%
+ Butan-1-ol 13.9 mM	0.599	1%	0.219	12%

Control and blank values (see Table 1) have been subtracted. Final reaction concentrations are shown.
FR = Ferredoxin reductase.

for non-specific radical effects showed no inhibitory properties (data not shown). Superoxide dismutase showed that $O_2^{\cdot}$ played no major role in the drug stimulated deoxyribose degradation except as a precursor of hydrogen peroxide. Its activity ranged from 5% stimulation to a maximum of 16% inhibition (Table 4). Addition of the protein caeruloplasmin produced a marked inhibition (Table 5); heat-denaturation of caeruloplasmin substantially reduced its inhibitory activity (data not shown). The hydroxyl radical scavengers formate, benzoate, ethanol and butan-1-ol offered little more protection than the control urea. However, mannitol and thiourea showed greater protection (Table 6).

DISCUSSION

Previous studies have shown that enzymically reduced anthracycline and aminoquinone antitumour antibiotics can degrade deoxyribose under very low O_2 concentrations [16–19]. In these studies superoxide dismutase was strongly inhibitory although there was no direct evidence for the participation of the superoxide radical in deoxyribose degradation and the lack of reactivity of $O_2^{\cdot}$ in aqueous solution makes its direct reaction with deoxyribose very unlikely. Its inhibitory properties were ascribed to a reaction between semiquinones and dioxygen in which the $O_2^{\cdot}$ radical is formed. SOD is believed to inhibit by pulling the equilibrium over to the right, comsuming the semiquinone [24]. Ferredoxin reductase dependent deoxyribose degradation stimulated by antitumour antibiotics in air is not inhibited by superoxide dismutase. This suggests that $O_2^{\cdot}$ itself is not an essential intermediate in the deoxyribose degradation, it serves as a precursor of hydrogen peroxide via the dismutation reaction, but superoxide dismutase merely accelerates this reaction.

The almost complete inhibition of drug reactivity by both catalase and desferrioxamine strongly suggests that a Fenton-type reaction (equation 5) is responsible for deoxyribose degradation. Caeruloplasmin is known to be able to catalyse oxidation of Fe^{2+} to Fe^{3+} (its ferroxidase activity). Inhibition by this protein adds further support to a Fenton reaction by indicating the essentiality of a ferrous ion species [23, 10]. "Hydroxyl radical scavengers" were generally poor as inhibitors of drug dependent damage with the exception of mannitol and thiourea, confirming results in previous studies with the scavengers [18, 19, 25]. It may well be that a site-specific formation of hydroxyl radicals is occuring which cannot readily be intercepted by added hydroxyl radical scavengers unless they themselves have a metal binding capacity such as mannitol and thiourea [25].

It therefore seems likely that a Fenton-type reaction is responsible for deoxyribose degradation (Fig. 1). The lack of inhibition by superoxide dismutase would suggest that either the semiquinone radical or hydrogen peroxide rather than $O_2^{\cdot}$ is responsible for the reduction of iron salts. The high reactivity of semiquinones with iron salts [26] makes it a likely candidate for this step (equation 4b) [18, 19].

All the drugs examined in this study were readily reduced by the enzyme ferredoxin reductase in the presence of NADPH, and such an enzyme system

(1) Antitumour antibiotic quinone moiety (Q) reduced by Ferredoxin reductase + NADPH to semiquinone ($SQ^{\cdot}$) radical
(2) $SQ^{\cdot} + O_2 \longrightarrow O_2^{\cdot} + Q$
(3) $2O_2^{\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$
(4) Reduction of iron complexes
  a) $Fe^{3+} + O_2^{\cdot} \longrightarrow Fe^{2+} + O_2$ (Inhibited by SOD and DEFOM)
  b) $Fe^{3+} + SQ^{\cdot} \longrightarrow Fe^{2+} + Q$ (Inhibited by DEFOM)
  c) $Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + 2H^+ + O_2^{\cdot}$ (Inhibited by CATALASE and DEFOM)
(5) Fenton reaction via semiquinone
  $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}$ (Inhibited by CATALASE, DEFOM and CAERULOPLASMIN)
  Deoxyribose + $OH^{\cdot} \longrightarrow$ TBA – reactive products (Inhibited by $OH^{\cdot}$ scavengers)

Net reaction $SQ^{\cdot} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{iron}} Q + OH^- + OH^{\cdot}$

Abbreviations: SOD = superoxide dismutase.
DEFOM = desferrioxamine

Fig. 1. Possible reactions leading to deoxyribose degradation

may provide a useful *in vitro* cell-free model for *in vivo* drug activation. However, the antitumour antibiotics used here are known to bind or intercalate DNA, and intercalated adriamycin can not be reduced by enzyme systems like ferredoxin reductase [27–29]. Whether an intercalated quinone-group drug can be activated will depend therefore on the ability of the reductant to reach the quinone moiety [29] or alternatively, the drug might first be activated before binding to DNA [30]. These limitations, however, do not prevent the drug participating in reactions leading to the toxic side-effects.

Carminic acid the food colouring additive E120 (cochineal) has been shown to possess antitumour properties, although it does not appear to bind to DNA [31]. Its structural and free radical activation similarities to the highly toxic anthracycline drugs cast doubt on the advisability of its use as a food additive.

Acknowledgements—We thank Farmitalia Carlo Erba for the gift of doxorubicin and epirubicin. The Natural Products Branch, Division of Cancer Treatment, NCI, Bethesda for streptonigrin and Dr Alexander Urumov for the sample of olivomycin.

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