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STREPTONIGRIN-INDUCED DEOXYRIBOSE DEGRADATION: INHIBITION BY SUPEROXIDE DISMUTASE, HYDROXYL RADICAL SCAVENGERS AND IRON CHELATORS

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Abstract—The aminoquinone antitumour antibiotic streptonigrin stimulates deoxyribose degradation in cell-free systems. This degradation is dependent both on reduction of the drug to a semiquinone and on traces of molecular oxygen in the reaction. Inhibition by a variety of hydroxyl radical scavengers and by catalase implicates a radical species with properties similar to the hydroxyl radical. Iron salts appear to play some part in radical formation as DETAPAC and desferrioxamine partly inhibit. Deoxyribose degradation under conditions of low oxygen concentration is strongly inhibited by superoxide dismutase.

Streptonigrin, a dark-brown pigmented aminoquinone antitumour antibiotic, isolated from cultures of *Streptomyces flocculus* [1], shows antitumour activity *in vivo* towards several transplantable animal tumours [2]. Its clinical use is limited because it induces severe and prolonged marrow depression.

The antitumour activity of streptonigrin has been attributed to several different cellular mechanisms which include interference with cell respiration and DNA synthesis and replication and also damage to DNA [3–6]. DNA strand scissions, both in bacteria and cell-free systems, appear to involve the formation of oxygen radicals [5, 6]. DNA damage by oxygen radicals has been proposed as a mechanism of action for other quinone-group containing antitumour antibiotics such as mitomycin C and the anthracyclines, adriamycin and daunomycin [7, 8].

The carbohydrate deoxyribose is readily attacked by hydroxyl radicals, or species with similar reactivity, to release material that reacts with 2-thiobarbituric acid (TBA) to give a coloured complex indistinguishable from that formed between TBA and malondialdehyde [9, 10]. Recent studies employing deoxyribose degradation in cell-free systems suggests that oxygen radical damage is increased at low oxygen concentrations [11–13] and similar effects have been observed in cells growing in culture under hypoxic conditions [14]. However, streptonigrin appears to be more toxic to well oxygenated tumour cells [14] than it is to hypoxic cells.

In the present study we have examined mechanisms by which streptonigrin can cause oxygen radical damage to deoxyribose at both normal and low oxygen concentrations in cell-free systems.

MATERIALS AND METHODS

2-Deoxy-D-ribose, hypoxanthine, diethylenetriaminepenta-acetic acid (DETAPAC), albumin (human fatty acid free), superoxide dismutase (bovine erythrocyte), xanthine oxidase grade I (28.1 units/ml), NADPH and ferredoxin reductase (1.5 units/mg) were from Sigma Chemical Co. Ltd. Desferrioxamine as "Desferal" was from Ciba-Geigy. All other chemicals were of the highest grades available from BDH Ltd. Units of enzyme activity were as defined in the Sigma catalogue.

Streptonigrin induced deoxyribose degradation. The following reagents, in the order stated, were added to new clean glass tubes (9 × 1.5 cm) with tight fitting screw caps: 0.2 ml deoxyribose 10 mM, 0.2 ml saturated aqueous hypoxanthine, and 0.1 ml streptonigrin 0.1 mM (dissolved in 1.25 mM NaOH). The tubes were gassed with N_2 for 1 min then tightly capped. 0.1 ml of xanthine oxidase (0.7 units/ml) was carefully added, allowing only a small amount of air to enter the tube, and then tightly capped. The reaction mixture was incubated in a shaking water-bath for 30 min at 37°. The pH of the final reaction mixture was 7.1. Where indicated, scavengers and inhibitors were added to the reaction before gassing with N_2. Appropriate controls and blanks were included with each batch of experiments. A change in the proportion of air to nitrogen within the tubes was made as previously described [11].

Thiobarbituric acid-reactivity. After incubation, 0.5 ml thiobarbituric acid (TBA) (1% w/v dissolved in 0.05 M NaOH) and 0.5 ml trichloroacetic acid 2.8% (w/v) were added to each tube followed by heating at 100° for 10 min. The absorbance of the pink colour formed was read at 532 nm. The results shown are a mean of three separate experiments which differed by less than 6%.

RESULTS

Streptonigrin dissolved in 1.25 mM NaOH to give a dark brown solution. At a concentration of 20 μM the absorbance of streptonigrin did not interfere with subsequent spectrophotometric measurements. The

rate of deoxyribose degradation was proportional to streptonigrin concentrations in the range 0.01–0.1 mM, at low concentrations of oxygen (data not shown).

In reaction mixtures exposed to air, streptonigrin (17 μM) was only slightly stimulatory towards deoxyribose degradation but it substantially enhanced the degradation of deoxyribose when most air was replaced by nitrogen (Table 1). Cytochrome c reduction was stimulated by 136% after the addition of streptonigrin under both normal and reduced oxygen concentrations (data not shown).

Addition of the hydroxyl radical scavengers formate, benzoate, ethanol, butan-1-ol and thiourea to the reaction systems shown in Table 1 revealed a similar pattern of inhibition under all conditions tested. The iron chelators DETAPAC and desferrioxamine, partly inhibited steptonigrin-stimulated deoxyribose degradation (Table 2), but EDTA did not. Deoxyribose degradation stimulated by streptonigrin at low oxygen concentrations was markedly inhibited by catalase (Table 2). It was also very sensitive to inhibition by superoxide dismutase, 3 μg/ml of superoxide dismutase giving over 70% protection under these conditions (Table 2). Albumin and heat-denatured proteins served as controls for non-specific protein effects. Altering the concentration of oxygen in the reaction tube changed not only the rate of deoxyribose breakdown but also the effect of SOD upon the breakdown. As the concentration of air was increased so a greater percentage of the reaction was inhibited by superoxide dismutase (Table 3).

Addition of hydrogen peroxide (1–100 μM) to the streptonigrin-dependent reaction under low O_2 concentrations stimulated deoxyribose degradation by less than 10% at a concentration of 10 μM (data not shown); at other concentrations it inhibited the reaction. Unlike xanthine oxidase, glutathione reductase and ferredoxin reductase did not significantly stimulate deoxyribose degradation under the conditions of low oxygen concentration, with control valves at A 532 nm of 0.073 and 0.086 respectively.

DISCUSSION

Micromolar concentrations of streptonigrin (SN) exposed to normal air in the presence of xanthine oxidase and its substrate hypoxanthine stimulated deoxyribose degradation only slightly. However, SN markedly stimulated deoxyribose degradation when the concentration of oxygen was decreased. Deoxyribose has been shown to be readily degraded to release TBA-reactive material by hydroxyl radicals or a species with similar reactivity [9, 10].

At low oxygen concentrations xanthine oxidase can transfer electrons to quinones to form semiquinone intermediates. These semiquinones appear to play an important role in the formation of hydroxyl radicals or a related species which causes the observed deoxyribose degradation [11–13]. The inhibitory effects of the different hydroxyl radical scavengers towards deoxyribose degradation were similar in the presence or absence of streptonigrin under conditions of both normal and low oxygen concentration. Partial inhibition of the streptonigrin dependent reaction by the metal chelators

Table 1. Effect of hydroxyl radical scavengers on deoxyribose degradation at different concentrations of air and in the presence of streptonigrin

	Deoxyribose degradation measured as TBA-reactivity/0.5 hr					
	Reaction 1 Normal air		Reaction 1 Low concentration of air		Reaction 2 Low concentration of air with streptonigrin	
	A 532 nm	% Inhibition	A 532 nm	% Inhibition	A 532 nm	% Inhibition
Blank 1 (DR + HXn)	0.028		0.028		0.028	
Blank 2 (DR + HXn + SN)	0.029		0.029		0.029	
Control for reaction 1 (DR + HXn + XOD + NaOH)	0.390		0.143		—	
Control for reaction 2 (DR + HXn + XOD + SN)	0.410		—		0.250	
Inhibitors added to reactions 1 and 2						
Formate 14.28 mM	0.280	28	0.106	26	0.178	29
Benzoate 14.28 mM	0.204	48	0.081	43	0.169	32
Ethanol 14.28 mM	0.285	27	0.119	17	0.201	20
Mannitol 14.28 mM	0.152	61	0.060	58	0.085	66
Butan-1-ol 14.28 mM	0.214	45	0.094	34	0.149	41
Thiourea 1.428 mM	0.177	55	0.064	55	0.136	46
Urea 1.428 mM	0.373	4	0.134	6	0.239	4

Blank values were subtracted from the readings shown.

DR, deoxyribose; HXn, hypoxanthine; SN, streptonigrin; XOD, xanthine oxidase. The reaction 1 control shows degradation of deoxyribose by a superoxide generating system under normal and low concentrations of air. The 'reaction 2 control' shows the same reaction in the presence of streptonigrin. Blanks were included to correct for small non-specific reagent effects (A 532 nm 0.028) together with chromogenic properties of streptonigrin (A 532 nm 0.029). These values were subtracted from the readings shown.

Table 2. Addition of metal chelators and proteins to streptonigrin-dependent deoxyribose degradation at low concentrations of air

	Deoxyribose degradation as TBA-reactivity 0.5/hr	
	A 532 nm	% Inhibition
Blank 1 (DR + HXn)	0.028	
Blank 2 (DR + HXn + SN)	0.029	
Control 1 (DR + HXn + XOD + NaOH)	0.143	
Control 2 (DR + HXn + XOD + SN)	0.250	
Control 2 + Inhibitors		
DETAPAC 0.285 mM	0.083	67
Desferrioxamine 0.285 mM	0.111	56
EDTA 0.285 mM	0.237	5
Superoxide dismutase 0.020 mg/ml	0.006	98
Superoxide dismutase 0.003 mg/ml	0.060	76
Superoxide dismutase 0.003 (heat-denatured)	0.163	35
Catalase 0.020 mg/ml	0.007	97
Catalase 0.003 mg/ml	0.147	41
Catalase 0.003 mg/ml (heat-denatured)	0.323	0
Albumin 0.020 mg/ml	0.240	4
Albumin 0.003 mg/ml	0.238	5
Albumin 0.003 mg/ml (heat-denatured)	0.238	5

Proteins were heat-denatured at 100° for 10 min.
Blank values were subtracted from the readings shown.

DETAPAC and desferrioxamine indicated some role for metal complexes in the degradation of deoxyribose. It is interesting to note that White and Yeowell found that iron was involved in the bacteriocidal action of streptonigrin [15].

Superoxide dismutase is a powerful inhibitor of streptonigrin-stimulated deoxyribose damage, suggesting the involvement of superoxide radicals. However, since xanthine oxidase would be expected to produce more superoxide under conditions of normal oxygen concentration than reduced oxygen concentration, it is likely that superoxide dismutase inhibits the reaction in some other way. Cytochrome c reduction at low oxygen concentrations suggests that SN is being reduced to its semiquinone. Inhibition by superoxide dismutase may be explained by a reversible reaction occurring between the semiquinone of streptonigrin ($SN^{\cdot}$) and oxygen (Equation 1) in which the resulting superoxide ($O_2^{\cdot}$) is removed by superoxide dismutase (SOD) thereby displacing the equilibrium to the right (equation 2) [16].

$$SN^{\cdot} + O_2 \leftrightarrows SN + O_2^{\cdot} \quad (1)$$

$$2O_2^{\cdot} \xrightarrow[2H^+]{SOD} H_2O_2 + O_2 \quad (2)$$

As the percentage of oxygen in the reaction mixture increases so superoxide dismutase inhibits a greater proportion of the observed deoxyribose degradation. When most of the oxygen had been removed from the reaction tube, by prolonged gassing with nitrogen, sufficient was apparently still present to allow a streptonigrin-dependent oxygen radical reaction to occur.

$$O_2^{\cdot} + Fe^{3+} \rightleftarrows Fe^{2+} + O_2 \quad (3)$$

$$SN^{\cdot} + Fe^{3+} \rightarrow Fe^{2+} + SN \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+} \quad (5)$$

Table 3. Inhibitory activity of superoxide dismutase with different concentrations of air in the reaction

	% of air in the reaction	Deoxyribose degradation as TBA-reactivity 0.5/hr	
		A 532 nm	% Inhibition
Blank 1 (DR + HXn)	0	0.028	
Blank 2 (DR + HXn + SN)	0	0.029	
Control 1 (DR + HXn + XOD + SN)	0	0.243	
Control + SOD 0.02 mg/ml	0	0.130	47
Control 1	5	0.232	
Control + SOD 0.02 mg/ml	5	0.114	51
Control 1	10	0.213	
Control 1 + SOD 0.02 mg/ml	10	0.071	67
Control 1	20	0.174	
Control + SOD 0.02 mg/ml	20	0.052	70

SOD, superoxide dismutase at final reaction concentrations shown.
Blank values were subtracted from the readings shown.

Net reactions:

$$(A)\quad O_2^{\cdot-} + H_2O_2 \xrightarrow[\text{catalyst}]{Fe} OH^{\cdot} + OH^- + O_2$$

$$(B)\quad SN^{\cdot-} + H_2O_2 \xrightarrow[\text{catalyst}]{Fe} OH^{\cdot} + OH^- + SN^+$$

The inhibition by catalase showed that hydrogen peroxide was required for the formation of a hydroxyl radical-like species. The concentration of hydrogen peroxide present in the reaction appeared to be important since addition of micromolar amounts of hydrogen peroxide either inhibited or slightly stimulated deoxyribose degradation. A requirement for both hydrogen peroxide and iron complexes suggest that formation of a radical species proceeds via a Fenton-type reaction (equations 3–5) in which reaction A predominates at normal oxygen concentrations and reaction B at low oxygen concentrations.

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