ENHANCEMENT OF BLEOMYCIN–IRON FREE RADICAL DAMAGE TO DNA BY ANTIOXIDANTS AND THEIR INHIBITION OF LIPID PEROXIDATION

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1. Introduction

The antitumour antibiotic bleomycin binds to and degrades DNA both in vivo and in vitro. This damaging activity has been shown to be dependent on the chelation of ferrous ions, which under aerobic conditions leads to the formation of the hydroxyl radical [1,2]. This damage can be measured by the release of malondialdehyde (MDA) which reacts with thiobarbituric acid (TBA) to give the chromogenic MDA–TBA adduct [3]. Iron-catalysed autoxidation of polyunsaturated fatty acids results in the formation of numerous lipid peroxides and long-chain aldehydes. When subjected to acid conditions and heating during the TBA test these decompose to give malondialdehyde and the same MDA–TBA adduct. Metal-ion catalysed peroxidation of phospholipid like that of bleomycin–iron damage to DNA has been shown to involve reduction intermediates of dioxygen [4]. Unlike bleomycin–iron-mediated damage to DNA, which takes only minutes, lipid peroxidation is a slower autocatalytic reaction characterised by a long induction period during which changes do not appear to take place.

Previous studies have shown that the antioxidant propyl gallate greatly enhances bleomycin–iron-dependent damage to DNA resulting in the increased formation of the MDA–TBA adduct [5]. This work has now been extended to examine the effect of a variety of water-soluble mono-, di- and trihydroxyphenols on the iron-catalysed free radical damage to lipids and DNA resulting in the formation of the MDA–TBA adduct. Phenols with reducing properties inhibited lipid peroxidation but greatly enhanced bleomycin–iron-dependent damage to DNA. One possible implication of this finding is that the clinical administration of phenolic compounds with reducing properties could potentiate the tumour damaging properties of the drug bleomycin while at the same time protecting normal lipid membranes from deleterious free radical damage.

2. Materials and methods

2.1. *Reagents*

Bathophenanthroline sulphonate, nitro blue tetrazolium (NBT) D,L-dihydroxyphenyl alanine (DOPA), L-adrenaline, catechol, phloroglucinol, L-tyrosine and DNA calf thymus type 1 were obtained from Sigma Chemical Co. Bleomycin sulphate complex was from Lundbeck Ltd. All other chemicals were of the highest grades available and obtained from BDH Ltd.

2.2. *Peroxidation of phospholipid*

The phospholipid and liposomes were prepared as in [6], the latter were used as a substrate for the lipid peroxidation studies. Phospholipid (5 mg/ml) was vortex-mixed with 0.15 M NaCl, buffered to pH 7.4 with sodium hydrogen carbonate, for 2 min and the preparation allowed to swell for 1 h at 4°C under nitrogen. Incubation mixtures consisted of 0.5 ml phospholipid liposomes, 0.1 ml phenolic compound, 0.3 ml phosphate saline buffer (pH 7.4) (0.024 M phosphate, 0.15 M NaCl) and 0.1 ml ferrous ions 0.5 mM freshly prepared. Samples were incubated for 2 h at 37°C followed by the addition of 1 ml 25% (v/v) HCl and 1 ml 1% TBA reagent then heated at 100°C for 15 min to develop the MDA–TBA chromogen which was read at 532 nm.

2.3. *Bleomycin–iron-mediated degradation of DNA*

DNA (0.5 ml) (1 mg/ml in 0.15 M NaCl, buffered

to pH 7.4 with sodium hydrogen carbonate) together with 0.1 ml 1 mg bleomycin/ml, 0.2 ml phosphate–saline buffer (pH 7.4) and 0.1 ml phenolic compound were mixed together and the reaction started by the addition of 0.1 ml 0.5 mM ferrous ions. The tubes were incubated at 37°C for 20 min followed by the addition of 1 ml 25% (v/v) HCl and 1 ml of 1% TBA reagent. The tubes were heated at 100°C for 15 min to develop the MDA–TBA chromogen which was read at 532 nm.

2.4. *Iron-reducing properties of phenols*

The method used for measuring the ferric ion reducing properties of the phenols was based on a vitamin E assay procedure [7]. Bathophenanthroline sulphonate (0.1 ml, 1 mg/ml), 0.1 ml ferric ions 0.5 mM and 0.1 ml phenolic compound were reacted together at room temperature for a few minutes in 0.6 ml phosphate–saline buffer (pH 7.4) before the addition of 0.1 ml 6% (v/v) orthophosphoric acid. Distilled water (2.0 ml) was added to each tube and the absorbance at 532 nm measured in a spectrophotometer.

2.5. *Reduction of NBT*

Phosphate–saline buffer (0.6 ml, pH 7.4), 0.1 ml EDTA 0.1 mM, 0.1 ml Triton X-100 16% (v/v) and 0.1 ml NBT 1 mM were mixed together before the addition of 0.1 ml test phenolic compound. The tubes were incubated at 37°C for 15 min followed by the addition of 2.0 ml phosphate–saline buffer (pH 7.4) and the absorbance read at 540 nm.

3. Results

Bovine brain phospholipids containing high levels of unsaturated fatty acids were used as a substrate in the form of multi-lamella liposomal membranes. Following iron-catalysed peroxidation, an MDA–TBA adduct was formed and measured as in [6]. Addition of a variety of mono-, di- and trihydroxyphenols either inhibited or had no effect on the iron-catalysed lipid peroxidation (table 1). Iron chelated by bleomycin enhanced lipid peroxidation by nearly 40% when the iron/bleomycin molar ratio approached 1. Similar enhancement of lipid peroxidation by chelated iron has been described for EDTA and diethylenetriaminepentaacetic acid (DETAPAC) and bathophenanthroline sulphonate [8]. Addition of phenols to bleomycin–iron-catalysed lipid peroxidation gave essentailly the same pattern of inhibition seen with iron alone, but with different degrees of inhibition (table 1).

Table 1
Inhibition of MDA formation during the iron-catalysed peroxidation of phospholipid by mono-, di- and trihydroxyphenols

Phenolic compound	Ferrous ions		Bleomycin–ferrous ions	
	A_{532}	% Inhibition	A_{532}	% Inhibition
Control	0.35		0.50	
Phenol	No change		No change	
Tyrosine	No change		No change	
Catechol	0.16	56%	0.13	74%
Adrenaline	0.27	25%	0.22	56%
Dihydroxyphenyl alanine (DOPA)	0.19	46%	0.35	30%
Resorcinol	No change		No change	
Quinol	0.22	38%	0.32	37%
Pyrogallol	0.22	38%	0.42	15%
Phloroglucinol	No change		No change	
Propyl gallate	0.17	54%	0.18	63%

Peroxidation of phospholipid catalysed by ferrous ions 0.05 mM and by 0.1 mg bleomycin/ml together with ferrous ions 0.05 mM. Inhibition by phenols 0.1 mM is expressed as a percentage of the control value, to which phenols were not added, based on the mean of 4 seperate assays

The TBA–MDA adduct formed from DNA after bleomycin–iron damage was measured by the same method used for the lipid MDA–TBA adduct. Those phenolic compounds which reacted as the most effective antioxidants against iron-catalysed lipid peroxidation were the most reactive pro-oxidants in the bleomycin–iron-catalysed damage to DNA (table 2). In the absence of added phenols but presence of bleomycin only ferrous ions were capable of mediating this DNA damage. Activity with ferric and enhancement with ferrous ions could be related to the reducing properties of the phenols (table 3). Measurement of the reduction of ferric ions as well as that of NBT indicated that the reducing properties of the phenols were essential to both its lipid antioxidant properties as well as to the enhancement of DNA damage by bleomycin–iron.

4. Discussion

Malondialdehyde can be formed by iron-catalysed damage to polyunsaturated fatty acids as well as to

Table 2
Enhancement of MDA formation during bleomycin-iron catalysed damage to DNA by mono-, di- and trihydroxyphenols

Phenolic compound	Bleomycin–ferrous ions A_{532}	% Enhancement	Bleomycin–ferric ions Increase in A_{532}
Control	0.42		0
Phenol	No change		No change
Tyrosine	No change		No change
Catechol	0.63	49%	0.063
Adrenaline	0.60	44%	0.355
Dihydroxyphenyl alanine (DOPA)	1.82	333%	0.480
Resorcinol	No change		No change
Quinol	1.78	326%	0.250
Pyrogallol	1.85	342%	1.600
Phloroglucinol	No change		No change
Propyl gallate	1.14	171%	0.393

Damage to DNA catalysed by 0.1 mg bleomycin/ml together with ferrous 0.05 mM or ferric ions 0.05 mM. Bleomycin–ferrous ion enhancement expressed as a percentage of the control value based on the mean of 4 separate assays. Bleomycin–ferric ion damage expressed as the change in absorbance at 532 nm

Table 3
Reducing properties of phenols

Phenolic compound	Reduction of ferric ions (measured as the BPS–ferrous complex) A_{532}	Reduction of NBT A_{540}
Phenol	No change	No change
Tyrosine	No change	No change
Catechol	0.190	0.022
Adrenaline	0.170	0.126
Dihydroxyphenyl alanine (DOPA)	0.180	0.148
Resorcinol	No change	No change
Quinol	0.200	0.115
Pyrogallol	0.215	0.640
Phloroglucinol	No change	No change
Propyl gallate	0.200	0.100

Reduction of 0.05 mM ferric ions and 0.1 mM NBT by 0.1 mM phenols

DNA. In both instances, evidence points to a dioxygen-dependent free radical mechanism. The peroxidation of unsaturated fatty acids has been studied in great detail during the last 40 years particularly with reference to the edible oils and fats of our foodstuff. Hydrogen abstraction by a free radical thereby leads to an autocatalytic sequence which eventually destroys the lipid molecule:

$$LH\,(\text{lipid}) + R^{\cdot}\,(\text{free radical}) \rightarrow L^{\cdot}\,(\text{lipid radical}) + RH$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}\,(\text{lipid peroxy radical})$$

$$LOO^{\cdot} + LH \rightarrow LOOH\,(\text{lipid hydroperoxide}) + L^{\cdot}$$

Transition metal ions, particularly iron and copper, can initiate radical formation as well as catalyse the branching sequence by decomposing lipid peroxides to further free radical intermediates. Most of the scavenging lipid antioxidants, such as the phenols, are reducing substances which act by hydrogen donation. Early studies [9], still valid today, suggest that the reducing antioxidants (AH) react primarily with the lipid peroxy radicals ($LOO^{\cdot}$) terminating the radical sequence by forming stable complexes (2A) or new radicals ($A^{\cdot}$) which do not continue the chain reaction:

$$AH + LOO^{\cdot} \rightarrow LOOH + A$$

$$A^{\cdot} + A^{\cdot} \rightarrow 2A$$

Application of the TBA test to peroxidised lipid results in the formation of an MDA–TBA adduct; most of the MDA being derived by the acid or thermal decomposition of peroxides during the test tube reaction. When the same test is applied to DNA damaged by bleomycin–iron it is possible that the MDA forming the TBA adduct is 'free MDA' derived from the deoxyribose sugar moiety of DNA. This iron–dioxygen-dependent damage to DNA is thought to occur by an iron-catalysed Haber-Weiss reaction which results in the formation of a hydroxyl radical opposite the deoxyribose sugar [10,11]. Attempts to inhibit this reaction, on linear duplex DNA, with specific and non-specific radical scavengers have been unsuccessful [5], with only metal chelators preventing such damage. This can be partly explained by the catalytic nature of the bleomycin–iron damage to DNA [12]. Bleomycin has been shown to act as a 'ferroxidase' catalysing the rapid oxidation of ferrous ions to the ferric state [13]. This 'enzymic' free radical producing reaction has here been shown to be enhanced by reducing phenols which re-cycle iron to the ferrous state

via a radical intermediate or the superoxide radical.

The ability of bleomycin to enhance iron-catalysed damage to lipid membranes could account for some of the deleterious effects of bleomycin observed during treatment with the drug. Activity of bleomycin towards the cancer cell is probably dependent on its free radical-mediated damage to DNA. Any enhancement of this activity by phenolic compounds, which at the same time protect normal lipid membranes from unwanted free radical damage, may suggest a rational basis for exploring their use as adjuvants to bleomycin therapy during cancer treatment.

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