IDENTIFICATION OF MALONDIALDEHYDE AS THE TBA-REACTANT FORMED BY BLEOMYCIN-IRON FREE RADICAL DAMAGE TO DNA

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1. Introduction

Bleomycins are a group of metal-binding glycopeptides isolated from *Streptomycis verticillus.* These antibiotics possess antibacterial, antiviral and antitumour properties and are now in clinical use for the treatment of certain neoplasms. Various biochemical and pharmacological properties have been ascribed to the bleomycins to explain their cytotoxicity; these include inhibition of DNA and protein synthesis [1] inhibition of DNase I [2] and strand scission of DNA [3,4]. This latter property has been most widely studied and is likely to be the most important cytotoxic event. Two distinct properties of bleomycin (BLM) mediate this damage; its binding to DNA and transition metal chelation. Recent evidence suggests that autoxidation of chelated ferrous ions leads to the formation of oxygen free radicals (1) which are the reactive species responsible for damage to the DNA molecule [5,6]:

$$\text{DNA–BLM} + Fe^{2+} \rightarrow \text{DNA–BLM}Fe^{2+}$$

$$\text{DNA–BLM}Fe^{2+} + O_2 \rightleftharpoons \text{DNA–BLM}Fe^{3+} + O_2^{\cdot-}$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$\text{DNA–BLM}Fe^{2+} + H_2O_2 \rightarrow \qquad (1)$$

$$\text{DNA–BLM}Fe^{3+} + OH^{\cdot} + OH^-$$

Overall reaction for oxygen:

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2$$

Previous studies have shown that this damage leads to the release of oligonucleotides, free bases and a molecule with aldehyde function [7,8]. This aldehyde reacts with 2-thiobarbituric acid (TBA) to give colour reaction similar to that given by the three-carbon-atom molecule malondialdehyde, and has therefore been referred to as a malondialdehyde-like substance [8,9]. Using chromatographic techniques together with absorption and fluorescence spectrometry it has been possible to specifically identify this TBA-reactor as the molecule malondialdehyde.

2. Materials and methods

1,1,3,3-Tetraethoxypropane was obtained from K and K Chemicals, Eastman Kodak, Liverpool; calf thymus DNA grade 1, Sigma Ltd, Dorset; bleomycin injection (complex of mainly A2, B2) Lundbeck Ltd; ammonium ferrous sulphate 'AnalaR' BDH Ltd.

2.1. *Incubation of DNA with bleomycin*

Calf thymus DNA was dissolved in 0.15 M NaCl, 0.024 M phosphate buffer (pH 7.4) to give final conc. 0.25 mg/ml. Bleomycin was dissolved in Chelex treated metal-ion free water at 0.67 mM. Fe^{2+} were added as a solution of ammonium ferrous sulphate 1 mM, dissolved in nitrogen-purged water and used within 1 min of preparation. The incubation mixture consisted of 2 ml of the buffered DNA solution, 0.1 ml bleomycin solution mixed together before addition of 0.1 ml solution of Fe^{2+}. The tubes were then incubated at 37°C for 15 min in a shaking water-bath.

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2.2. *Thiobarbituric acid reactivity*

The chromogenic adduct was developed in the incubation mixture by adding 1 ml 25% (v/v) HCl, 1 ml 1% 2-thiobarbituric acid (TBA) and then heating at 100°C for 15 min. Malondialdehyde (MDA) standards were prepared from 1,1,3,3-tetraethoxypropane (TEP) by 3 different techniques: acid hydrolysis [10,11]; bis-bisulphite salt [12]; treatment with Dowex resin [13].

Fluorescent spectra presented were uncorrected and obtained on a Perkin-Elmer MPF-4. Absorption spectra were obtained with a Perkin-Elmer model 402.

2.3. *Ultrafiltration and distillation*

DNA together with bleomycin and iron were incubated in the ratios in section 2.1, to give final vol. 20 ml. An ultrafiltrate of this reaction mixture was obtained by passing the solution through a PM-10 membrane (Amicon) using a pressure filtration cell (C50, Chemlab). To the ultrafiltrate was added 1 ml 5 M HCl and the solution steam-distilled to collect the first 15 ml fraction at 100°C. Standard TEP solutions were similarly treated.

3. Results

A bleomycin dose–response curve was established for thiobarbituric acid reactivity and bleomycin concentration over 0.67–0.034 mM. Both iron, at final reaction conc. 0.045 mM, and bleomycin in the absence of added iron did not generate TBA-reactive compounds from DNA under the incubation conditions described. Evidence for the presence of malondialdehyde was sought by direct examination of the incubation mixtures before TBA chromogen formation and by characterisation of the TBA adduct itself. These were compared with the MDA standards.

3.1. *Direct techniques*

In aqueous solution MDA exists in two different forms according to the pH. At pH < 4.65 the undissociated cyclic chelate form is present with an A_{245} max. At pH > 7.0 the enolate anion becomes the major component with an A_{267} max [14]. Direct spectroscopy on the incubated mixtures was complicated by the strong absorption peaks within these regions given by DNA, bleomycin and free bases. Ultrafiltration, however, removed the DNA together with bound bleomycin, but still left considerable ultraviolet absorbing material which interfered with the characterisation of MDA (fig.1). The cyclic form of MDA is volatile and can be steam-distilled from acid solution [15]. Application of this technique to the DNA–BLMFe^{2+} ultrafiltrate yielded a solution giving a pH-dependant absorption spectra identical to that given by the MDA standards (fig.1).

Thin-layer chromatography (TLC) of the incubated mixtures (silica gel-60, Merck-BDH Ltd, in solvent: hexane:ether:*n*-butanol:ethanol, 60:40:1:1) and location in situ with acid–TBA reagent, confirmed the presence of a reactive zone isographic to one of those found in the MDA standards (table 1). Separation of the incubated DNA–BLMFe^{2+} mixture in a more polar solvent (*n*-butanol:ethanol:acetic:acid:water, 60:20:10:1) gave two TBA-reactive zones, one of which was isographic to a major zone found in both Dowex-treated and acid hydrolysed TEP with R_F 0.83.

Preparation from TEP of 'free monomeric' MDA is difficult due to polymerisation [11,16] giving rise to multiple zone formation when separated by TLC. Some of these zones reacted rapidly at room temperature with the TBA reagent, whilst others required

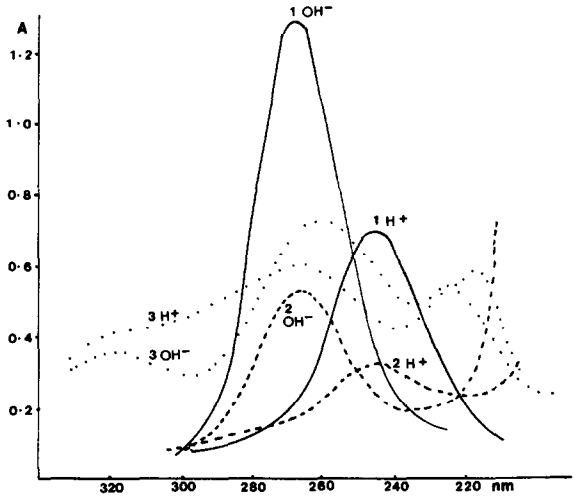

Fig.1. pH-dependant ultraviolet absorption spectrum. (1) (——) 10 μM MDA standard. (2) (- - -) DNA–BLMFe^{2+} incubate following ultrafiltration and distillation. (3) (· · · ·) DNA–BLMFe^{2+} ultrafiltrate against a blank similarly treated but not containing Fe^{2+}.

Table 1
TBA reactivity on thin-layer plates

	Colour development at 25°C		100°C (15 min)
	'Fast' (2 min) R_F	'Slow' (10 min) R_F	R_F
2 mM TEP 5 μl		0.56, 0.90	
2 mM TEP Acid hydrolysis 5 μl	0	0.11	0.44, 0.54
2 mM TEP Dowex treated 5 μl	0	0.11, 0.22, 0.56	
2 mM TEP Acid distilled 5 μl	0	0.11	0.44, 0.51
2 mM MDA bis-bisulphite acid distilled 5 μl	0	0.11, 0.37, 0.46	
DNA–BLMFe^{2+} ultrafiltrate 10 μl	0		
DNA–BLMFe^{2+} ultrafiltrate distilled 10 μl			0

heating. When the DNA–BLMFe^{2+} incubated mixture was distilled, the fast-reacting zone disappeared to give a TBA-reactor which required heating for colour development.

MDA can react with primary amino groups to give fluorescent amino-iminopropene structures R–N=C–C=N–R. Its reaction with DNA and free bases has been shown to give a distinct fluorophore with excitation maxima 390 nm and emission 460 nm [17]. The DNA–BLMFe^{2+} incubated mixture slowly formed such a Schiff's base with spectral characteristics similar to that given by an MDA–DNA standard (fig.2).

3.2. *Indirect techniques*

Indirect evidence for the formation of MDA was obtained by characterising the DNA–BLMFe^{2+} TBA adduct. This chromogen can be extracted into butan-1-ol and separated from other TBA chromogens, that are not MDA, using high performance TLC in two different solvent systems: (A) chloroform/methanol/acetic acid 60:20:0; (B) chloroform/methanol/acetic

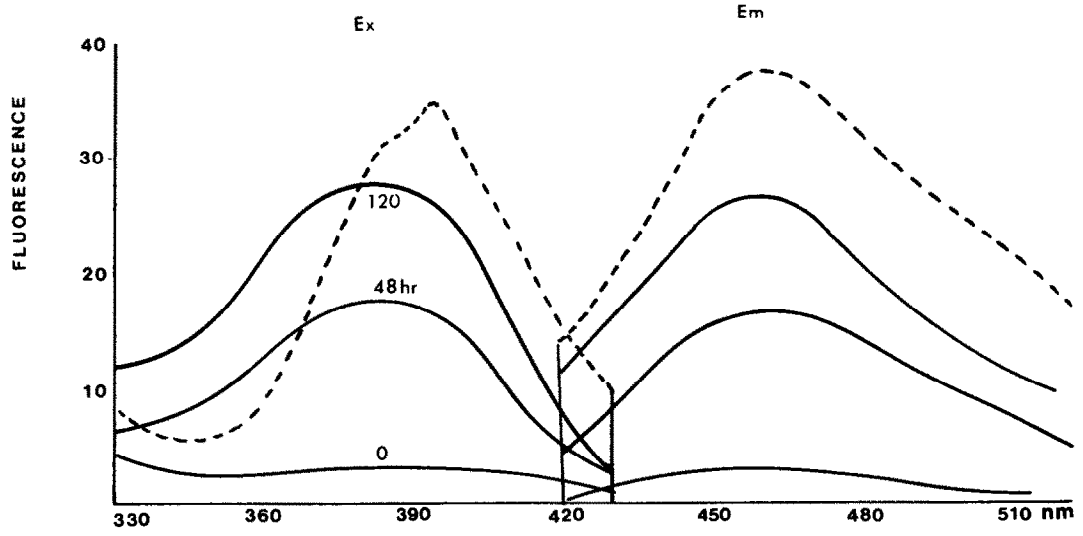

Fig.2. DNA–MDA fluorescence spectrum. (—) DNA–BLMFe^{2+} 0–120 h incubation 37°C. (- - -) DNA–MDA standard 2.5 mg DNA + 6 μM MDA (TEP-Dowex) reacted 18 h 37°C. Fluorimeter settings *Ex* 390, Em 460, slits 10 nm, filter 430 nm; sensitivity X 1.

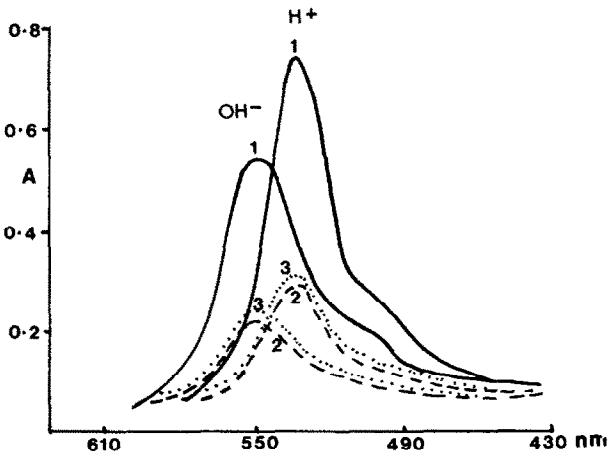

Fig.3. pH-dependant visible spectrum of TBA adduct. (1) (—) 1.0 mM MDA standard; (2) (- - -) DNA–BLMFe^{2+} ultrafiltrate distilled; (3) (· · ·) DNA–BLMFe^{2+} ultrafiltrate.

acid/butan-1-ol 60:5:10:10 [18,19]. The TBA adduct from the DNA–BLMFe^{2+} incubate was isographic with MDA standards showing an R_F in solvent (A) of 0.77 and in (B) 0.25.

The absorption maxima of the MDA–TBA adduct is pH dependant; showing a 532 nm peak in acid solution and 548 nm in alkaline solution. The pH characteristics of the chromogen formed from incubated DNA–BLMFe^{2+} were identical to those given by the MDA standards (fig.3).

The acid MDA–TBA adduct has a characteristic fluorescence spectra [18,20]. This shows an excitation peak at 532 nm and emission peak at 553 nm. Using the following instrument settings, both the ultrafiltrate and the distillate gave identical spectra to those given by the 0.05 μM MDA standards. Excitation 532 nm emission 553 nm, slits 10 nm, no filter, sensitivity X 0.3. Relative fluorescence intensity was measured with reference to rhodamine B, 3×10^{-6} M, excitation 480 nm emission 580 nm, slits 10 nm, no filter, sensitivity X 1.0, set to 100 units (% deflection).

4. Discussion

The detection of TBA-reactivity has long been associated with the measurement of free radical damage to polyunsaturated fatty acids. Recent work has confirmed that little free MDA is directly formed during lipid peroxidation and that the major reactants are peroxidic precursors which break down during the acid-heating stage of the TBA test to give MDA [21,22]. Like the much slower indirect formation from lipids, iron catalysed free radical damage to DNA gives rise to the direct formation of malondialdehyde. The bithiazole ring of bleomycin has been shown to intercalate between the base pairs of DNA containing a G–T or G–C sequence [23]. Within this specific orientation, chelation of ferrous ion, and its subsequent autoxidation leads to the formation of oxygen-free radicals. These free radicals generated in close proximity to the DNA molecule are involved in specific site damage leading to the release of pyrimidine bases, strand scission and oxidation of the deoxyribose moiety. It is likely that this occurs by the iron-chelate catalysed Haber-Weiss reaction (1) described by Halliwell [24]. Deoxyribose has a relatively high rate constant for the hydroxyl radical [25] and under these conditions is cleaved within a matter of seconds at the C3′–C4′ bond to liberate malondialdehyde into solution. This reactive bifunctional aldehyde can then crosslink amino group donors such as DNA, proteins and phospholipids, thereby giving rise to secondary damaging cellular events.

It appears that the measurement of malondialdehyde provides a sensitive and specific measure of free radical damage to DNA as well as to lipids.

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