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EPR SPIN TRAPPING OF FREE RADICALS PRODUCED BY BLEOMYCIN AND ASCORBATE

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In the presence of ascorbate, bleomycin (BLM) is converted to a redox-inactive form that is incapable of inducing DNA strand scission. We have employed EPR spin trapping with 5,5-dimethylpyrroline-1-oxide (DMPO) to examine free radical production during this process. The introduction of ascorbate to an Fe(III)BLM-DMPO system results in the formation of three EPR observable free radicals. One of these radicals is the resonance-stablized ascorbate free radical (a^H = 1.8 G) that is not spin trapped by DMPO; the other two are the result of DMPO spin trapping. These radicals appear to be two carbon-centered radicals, DMPO/·CR_1, (a^N_1 = 15.75 G, a^H_1 = 22.30 G, a^N/a^H = 0.706) and DMPO/·CR_2 (a^N_2 = 15.20 G, a^H_2 = 19.20 *G*, a^N/a^H = 0.79). Although it is not possible to identify the exact structures of the carbon-centered radicals that are spin trapped, the hyperfine splittings, as well as the a^N/a^H values, are characteristic of the presence of electron-withdrawing groups, such as the oxygen atom when attached to the carbon atom. In fact, these parameters are characteristic of DMPO spin trapping results obtained when sugars are subjected to oxidative insult from HO·. Thus, these BLM-ascorbate produced radicals may well be derived from the sugar moiety of BLM.

KEY WORDS: ascorbate, bleomycin, electron paramagnetic resonance, free radical, spin trapping.
Abbreviations: Asc·$^-$: ascorbate free radical, AscH$^-$: ascorbate monoanion, BLM: bleomycin, DMPO: 5,5-dimethylpyrroline-1-oxide, ΔHpp: peak-to-peak EPR linewidth in gauss.

INTRODUCTION

Bleomycin (BLM) is a glycopeptide antibiotic commonly used in the treatment of several different carcinomas. It is thought that the antitumor activity of BLM is due to its ability to cause cellular DNA degradation.[1-3] *In vitro*, this degradation is thought to involve an iron-BLM complex that associates with DNA. The current view is that an Fe(II)BLM-DNA complex is formed; this complex reacts with O_2 and a reducing equivalent to form an "activated BLM", which then initiates the DNA strand scission.

The "activated BLM", HO_2^--Fe(III)BLM, can be formed from either Fe(III)BLM or Fe(II)BLM.[3] With Fe(III)BLM, H_2O_2 is required:

$$\text{Fe(III)BLM} + H_2O_2 \rightarrow HO_2^-\text{-Fe(III)BLM} + H^+ \quad (1)$$

With Fe(II)BLM and dioxygen the activated species can be formed, but another reducing equivalent is required:

$$2\text{Fe(II)BLM} + O_2 + H^+ \rightarrow HO_2^-\text{-Fe(III)BLM} + \text{Fe(III)BLM} \quad (2)$$

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This "activated BLM" is highly oxidizing; it can abstract H· from DNA,[1-3] generate HO· radicals,[4-7] homolytically cleave hydroperoxides,[8] produce thiyl radicals from thiols,[7,9] and oxidize HEPES to free radical products.[7]

In many studies, the activated BLM is commonly formed from Fe(III)BLM and reducing agents, such as thiols[7,9,10] or ascorbate.[11,12] Although ascorbate has been used as a tool in studying the chemistry of iron-BLM, no studies have focused on the actual ascorbate-iron-BLM chemistry. We report here our observations on this rich free radical chemistry, which appears to be involved in the production of redox-inactive BLM.

MATERIALS AND METHODS

Bleomycin, as Blenoxane®, was a gift of Mead Johnson, Evansville, IN; ascorbic acid, DMPO, and chelating resin were from Sigma Chem. Co., St. Louis, MO. Adventitious catalytic metals were removed from all buffers as outlined in Reference[13]; the absence of catalytic metals was verified using ascorbate.[13] DMPO was purified with charcoal and stored as a frozen 1.0 M aqueous solution before use;[14] stock solution concentrations were determined using $\varepsilon_{228} = 7.8 \times 10^3\,M^{-1}\,cm^{-1}$.[15] Metal-free EDTA was prepared by repeated recrystallizations from 18 Mohm-cm water of the tetraacid.[16] BLM stock solution concentrations were determined using $\varepsilon_{291} = 17{,}000\,M^{-1}\,cm^{-1}$.[17]

The spin trapping of FeBLM-ascorbate-derived radicals was accomplished with a solution of: 30 μM BLM; 10 μM Fe(III); 50 mM DMPO; and 500 μM ascorbate in pH 7.4 phosphate buffer. The sucrose and mannose radicals were generated with the aid of a Fenton system to generate HO·: 2 mM Fe(II) and 4 mM H_2O_2 in a spin trapping system that contained 200 mM sucrose or 200 mM mannose and 25 mM DMPO. EPR spectra were collected with a Bruker ES-300 EPR spectrometer using a TM_{110} cavity and aqueous flat cell.

RESULTS AND DISCUSSION

The formation of "activated BLM" from Fe(III)BLM and O_2 requires two reducing equivalents. Therefore, we reasoned that the introduction of Fe(III)BLM to an ascorbate solution should produce an increase from background levels[16] in the ascorbate radical EPR signal intensity,

$$\text{Fe(III)BLM} + \text{AscH}^- \rightarrow \text{Fe(II)BLM} + \text{Asc}\cdot^- + \text{H}^+ \qquad (3)$$

Indeed, when Fe(III)BLM is introduced into a solution of ascorbate, an increase in the concentration of $Asc\cdot^-$ is observed.[18] Because "activated BLM" brings about the oxidation and subsequent inactivation of DNA by free radical processes,[2] we also reasoned that the production of redox-inactive BLM from "activated BLM" may involve free radical oxidation processes within the FeBLM complex.

Direct EPR will detect only resonance stabilized radicals such as $Asc\cdot^-$ with the experimental conditions employed, i.e., room temperature aqueous solutions. Therefore, to search for the production of other free radicals during the reaction that yields redox-inactive BLM, we employed the EPR spin trapping technique.[19,20] When the spin trap DMPO was included in the Fe(III)BLM-ascorbate system, two additional free radicals were detected, Figure 1. The hyperfine splittings observed for

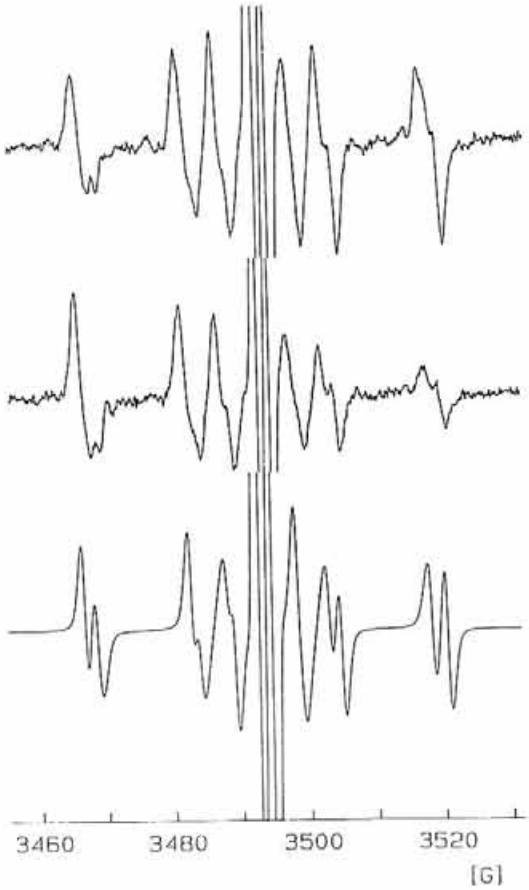

FIGURE 1 EPR Spin Trapping. Top and middle spectra are consecutive 80 gauss/11.2 min scans of an EPR spin trapping experiment in which 30 μM BLM that had been preincubated with 10 μM Fe(III) was introduced to a phosphate-buffered, pH 7.4, 500 μM ascorbate solution containing 50 mM DMPO. Receiver gain, 2×10^6; microwave power, 40 mW; modulation amplitude, 1.0 G. These spectra were simulated (bottom spectrum) assuming the presence of three distinct species; two DMPO spin adducts and the ascorbate free radical (the strong ascorbate radical signal is the off-scale center doublet). The parameters used for this simulation were: DMPO/adduct 1-a^N_1 = 15.75 G, a^H_1 = 22.30 G, ΔHpp = 1.60 G; for DMPO/adduct 2-a^N_2 = 15.20 G, a^H_2 = 19.20 G, ΔHpp = 2.0 G, Δg = −0.2 G; the ascorbate radical; a^H = 1.8 G, ΔHpp = 1.0 G, Δg = 0 G. Only the ascorbate radical is observed in the absence of BLM or DMPO. If BLM is omitted, only a weak ascorbate radical signal is observed. An oxygen-containing, near-neutral solution of ascorbate will have a very low level of the ascorbate radical present, even in the absence of significant adventitious metals.[16] This low level of $Asc^{\cdot -}$ is thought to arise from the autoxidation of the dianion of ascorbic acid.[13,16]

the DMPO spin adducts are: a^N_1 = 15.75 G, a^H_1 = 22.30 G, a^N/a^H = 0.706; a^N_2 = 15.20 G, a^H_2 = 19.20 G, a^N/a^H = 0.79. These parameters are characteristic of the spin trapping of carbon-centered radicals.[21,22,23] Although it is not possible to identify the exact radicals that were spin trapped, the hyperfine splittings as well as the a^N/a^H values[22,23] of 0.706 and 0.79 are characteristic of the spin trapping of carbon-centered radicals with electron withdrawing moieties, such as oxygen, on the carbon atom.[23]

TABLE 1

	DMPO Spin Trapping[a]		
Radical Trapped	$a^N/_G$	$a^H/_G$	a^N/a^H
BLM-1	15.75	22.30	0.706
BLM-2	15.20	19.20	0.79
Sucrose-1	16.2	22.9	0.707
Sucrose-2	15.5	19.4	0.79
Mannose	15.85	22.45	0.704
Adriamycin-1[b]	15.69	22.45	0.70
Adriamycin-2[b]	15.10	19.00	0.79

[a]Hyperfine splittings are for room temperature aqueous solutions of the spin adducts.
[b]Reference 24.

Sugars have numerous combinations of carbon-oxygen bonds. BLM contains a sugar moiety consisting of gulose and mannose, which could be a possible target for the self reaction of "activated BLM". We therefore undertook spin trapping experiments with typical sugars that were subjected to oxidation by ·OH generated with a Fenton system. In this spin trapping system, we detected DMPO spin adducts with hyperfine splitting parameters that are very much like those observed in the FeBLM-ascorbate-DMPO system (Table 1). This similarity suggests that the sugar moeity of BLM may be a target of the "self reaction" of activated BLM.

An interesting parallel can be seen between our observation with BLM and that of Li and Chignell with adriamycin.[24] They have observed two DMPO spin adducts having nearly identical hyperfine splitting parameters to those we have observed with FeBLM-ascorbate-DMPO when they subjected adriamycin to UV photolysis. They were not able to identify these radicals but suggested that one of them may be an acyl radical. The four-ring anthracycline structure of adriamycin has a sugar group as a substituent on the A-ring. Thus, their results are consistent with the homolytic cleavage of this sugar from the ring.

The formation of redox-inactive BLM is well understood with respect to the role of iron, O_2, and reducing agents. However, little is known about the actual changes in the BLM molecule that render it redox-inactive. Our results suggest that the highly oxidizing activated-BLM can initiate free radical processes on the BLM molecule. This may be the result of either self-reaction, i.e., reaction of the metal center with another part of the molecule, or it may be the result of an activated-BLM reacting with other BLM molecules in the solution. We have recently presented evidence that iron serves only as a catalyst in the ascorbate-driven formation of redox inactive-BLM.[18] Thus, we propose that activated-FeBLM produces redox-inactive BLM by self-oxidation or oxidizing other BLM molecules in the solution. Our data suggests that this is a free radical process and that a target of these oxidations may be the sugar moiety of BLM.

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References

1. S.M. Hecht (1986) The chemistry of activated bleomycin. *Accounts of Chemical Research*, **19**, 383–391.
2. J. Stubbe and J.W. Kozarich (1987) Mechanisms of bleomycin-induced DNA degradation. *Chemical Reviews*, **87**, 1107–1136.
3. D.H. Petering, R. Byrnes and W.E. Antholine (1990) The role of redoxactive metals in the mechanism of action of bleomycin. *Chemico-Biological Interactions*, **73**, 133–182.
4. L.W. Oberley and G.R. Buettner (1979) The production of hydroxyl radical by bleomycin and iron(II). *FEBS Letters*, **97**, 47–49.
5. D. Solaiman, E.R. Rao, D.H. Petering, R.C. Sealy and W.E. Antholine (1979) Chemical, biochemical, and cellular properties of copper and iron bleomycins. *International Journal Radiation Oncology & Biology Physiology*, **5**, 1519–1521.
6. Y. Sugiura and T. Kikuchi (1978) Formation of superoxide and hydroxy radicals in iron(II)-bleomycin-oxygen system. Electron spin resonance detection by spin trapping. *Journal of Antibiotics*, **31**, 1310–1312.
7. W.E. Antholine, B. Kalyanaraman, J.A. Templin, R.W. Byrnes and D.H. Petering (1991) Spin-trapping studies of the oxidation-reduction reactions of iron bleomycin in the presence of thiols and buffer. *Free Radical Biology & Medicine*, **10**, 119–123.
8. G. Padbury, S.G. Sligar, R. Labeque and L.J. Marnett (1988) Ferric bleomycin catalyzed reduction of 10-hydroperoxy-8, 12-octadecadienoic acid: evidence for homolytic O-O bond scission. *Biochemistry*, **27**, 7846–7852.
9. W.E. Antholine and D.H. Petering (1979) On the reaction of iron bleomycin with thiols and oxygen. *Biochemical Biophysical Research Communications*, **90**, 384–389.
10. P.L. Moseley and R. Chalkley (1987) Bleomycin-induced DNA clevage: Studies *in vitro* and in intact cells. *Journal Laboratory and Clinical Medicine*, **110**, 618–623.
11. E.A. Sausville, J. Peisach and S.B. Horwitz (1978) Effect of chelating agents and metal ions on the degradation of DNA by bleomycin. *Biochemistry*, **17**, 2740–2746.
12. A. Natrajan, S.M. Hecht, G.A. van der Marel and J.H. van Boom (1990) A study of O_2- versus H_2O_2-supported activation of Fe·Bleomycin. *Journal of the American Chemical Society*, **112**, 3997–4002.
13. G.R. Buettner (1988) In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *Journal of Biochemical & Biophysical Methods*, **16**, 27–40.
14. G.R. Buettner and L.W. Oberley (1978) Considerations in the spin trapping of superoxide and hydroxyl radical in aqueous systems using 5.5 dimethyl-1-pyrroline-N-oxide. *Biochemical Biophysical Research Communications*, **87**, 69–74.
15. G.R. Buettner (1990) On the reaction of superoxide with DMPO/·OOH. *Free Radical Research Communications*, **10**, 11–15.
16. G.R. Buettner (1990) Ascorbate oxidation: UV absorbance of ascorbate and EPR spectroscopy of the ascorbate radical as assays for iron. *Free Radical Research Communications*, **10**, 1–2:5–9.
17. R.M. Burger, S.B. Horwitz and J. Peisach (1985) Stimulation of iron(II) bleomycin activity by phosphate-containing compounds. *Biochemistry*, **24**, 3623–3629.
18. G.R. Buettner and P.L. Moseley (1991) Ascorbate both activates and inactivates bleomycin by free radical generation. *Biochemistry*, **31**, 9784–9788.
19. G.R. Buettner (1982) The spin trapping of superoxide and hydroxyl radicals. *Superoxide Dismutase*, **2**, 63–81, edited by L.W. Oberley, CRC Press, Inc., Boca Raton. FL.
20. G.R. Buettner and R.P. Mason (1990) Spin-trapping methods for detecting superoxide and hydroxyl free radicals *in Vitro* and *in Vivo*. *Methods in Enzymology*, **186**, 127–133.
21. G.R. Buettner (1987) Spin trapping: EPR parameters of spin adducts. *Free Radiation Biology Medicine*, **3**, 259–303.
22. A.S.W. Li, K.B. Cummings, H.P. Roethling, G.R. Buettner, Chignell and F. Colin (1988) A spin-trapping database implemented on the IBM PC/AT. *Journal of Magnetic Resonance*, **79**, 140–142.
23. A.S.W. Li and C.F. Chignell (1991) The NoH value in EPR spin trapping: a new parameter for the identification of 5,5-dimethyl-1-pyrroline-N-oxide spin adducts. *Journal Biochemical & Biophysical Methods*, **22**, 83–87.
24. A.S.W. Li and C.F. Chignell (1987) Spectroscopic studies of cutaneous photosensitizing agents-X. A spin-trapping and direct electron spin resonance study of the photochemical pathways of daunomycin and adriamycin. *Photochemistry Photobiology*, **45**, 565–570.