On the Formation of Oxygenated Radicals by Fredericamycin A and Implications to Its Anticancer Activity: An ESR Investigation

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ABSTRACT: It has been recently suggested that the exceptionally high antitumor and antibacterial activity of natural fredericamycin A (FMA) is related to its ability to spontaneously generate the superoxide anion (O_2^-) and hydroxyl ($^{\bullet}OH$) radicals in aerobic solutions [Hilton, B. D., Misra, R., & Zweier, J. L. (1986) *Biochemistry 25*, 5533]. With a view to understand the mechanistic details, attempts were made to reproduce earlier electron spin resonance (ESR) evidence for the oxygenated free radical formation in well-aerated solutions of natural FMA in dimethyl sulfoxide and dilute H_2O_2. Little or no evidence was obtained for the formation of the O_2^- and methoxy ($^{\bullet}OCH_3$) radicals, while the detected formation of the $^{\bullet}OH$ and methyl ($^{\bullet}CH_3$) radicals was attributable largely to mechanisms not involving FMA. These results thus reopen the question regarding the mechanism of its exceptionally high tumoricidal–bacteriocidal activity.

This paper reports on an electron spin resonance (ESR) investigation of the formation of oxygenated free radicals in solutions of natural fredericamycin A (abbreviated as FMA) which has recently been reported to be a highly potent antitumor antibiotic (Pandey et al., 1981; Warnick-Pickle et al., 1981). The impetus for this undertaking was provided by recent reports (Hilton et al., 1986; Stinson, 1986) suggesting that the strong tumoricidal activity with high therapeutic–toxic index of FMA was related to its ability for the spontaneous generation of superoxide anion (O_2^-) and hydroxyl ($^{\bullet}OH$) radicals. For example, the suggested mechanism for the formation of O_2^- by FMA was (Hilton et al., 1986)

$$\text{FMA–OH} + O_2 \rightleftharpoons \text{FMA–O}^{\bullet} + H^+ + O_2^-$$

Here FMA–OH is simply the FMA molecule emphasizing the –OH moiety which becomes FMA–O$^{\bullet}$, the free radical representing the oxidized form of the drug. While some other highly effective anticancer drugs in current clinical application, such as bleomycin, are also thought to exert their antitumor effects via the formation of reactive drug-based or oxygenated free radicals (Carmichael et al., 1985; Gianni et al., 1983), FMA was considered unique in that only in this case the free radical formation was thought to be spontaneous, rather than through a metal- or an enzyme-activated process (Hilton et al., 1986; Stinson, 1986). Thus it was suggested (Hilton et al., 1986; Stinson, 1986) that FMA could be the forerunner of a new class of anticancer drugs. However, we noted that while the hypothesis seemed plausible, the reported ESR spectral evidence appeared to be ambiguous. Because of the high significance of the earlier conclusions (Stinson, 1986), we undertook a systematic ESR study of the radical formation in FMA solutions, and as discussed below, our conclusions disagree with those reported earlier.

EXPERIMENTAL PROCEDURES

The FMA used in this work was natural fredericamycin from a stock similar to that employed earlier (Hilton et al., 1986; Kelly, personal communication) and was kindly supplied by Dr. T. R. Kelly of Boston College. The solvent was spectral-grade dimethyl sulfoxide (DMSO), used as received. All ESR measurements were made on a Bruker ER200D ESR spectrometer operating at X-band (~9.5 GHz). For accurate measurements of the g values and hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, Model ERO35M), and the microwave frequency was measured with a digital frequency counter (Hewlett-Packard, Model 5340A). The microwave power was about 20 mW, and the magnetic field modulation amplitude was adjusted (1–5 G) to obtain optimum signal intensity and resolution. The spin trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) was purchased from Aldrich and used without purification since little or no background ESR signals were observed when it was used by itself. An ASPECT 2000 computer was used for data acquisition and analysis and for ESR spectral simulations.

RESULTS AND DISCUSSION

Figure 1a shows a typical ESR spectrum of air-exposed FMA under normal room lighting. This spectrum was obtained from a 2 mM solution of FMA in DMSO, following closely the earlier reported procedure of exposing the solution to air for 1 week and then freezing in liquid nitrogen (77 K) just before ESR measurements (Hilton et al., 1986). However, this spectrum is quite different from that reported earlier [Figure 8 of Hilton et al. (1986)], in that the peaks assigned to O_2^-, corresponding to $g_{\parallel} = 2.524$ and $g_{\perp} = 2.073$ (field positions indicated by arrows), are not observed. To find if this difference could be due to photochemical effects, since FMA absorbs strongly in the UV–visible range (Hilton et al., 1986), the above experiments were repeated in the dark. The spectrum observed in the dark is shown in Figure 1b and is essentially identical with that in Figure 1a. This result shows that the ambient light is not related to the above-mentioned difference in ESR spectra. Moreover, an ESR spectrum of O_2^- in DMSO, produced by addition of KO_2 to DMSO, yielded (at 140 K) $g_{\parallel} = 2.100$ and $g_{\perp} = 2.004$ (Figure 1c), in agreement with an earlier study (Knowles et al., 1969) of O_2^- in DMSO. This result, together with the fact that the sharp, symmetric peak seen in Figure 1a,b, at $g = 2.004$, was essentially identical with the peak assigned earlier [Figure 6a of Hilton et al. (1986)] to FMA–O$^{\bullet}$, shows that the absence of O_2^--related peaks in Figure 1a,b was not due to any lack of sensitivity in our measurements.

Since the above-described direct ESR measurements indicated little or no formation of O_2^-, we reexamined the earlier spin trap ESR evidence for this radical. Figure 2a shows the

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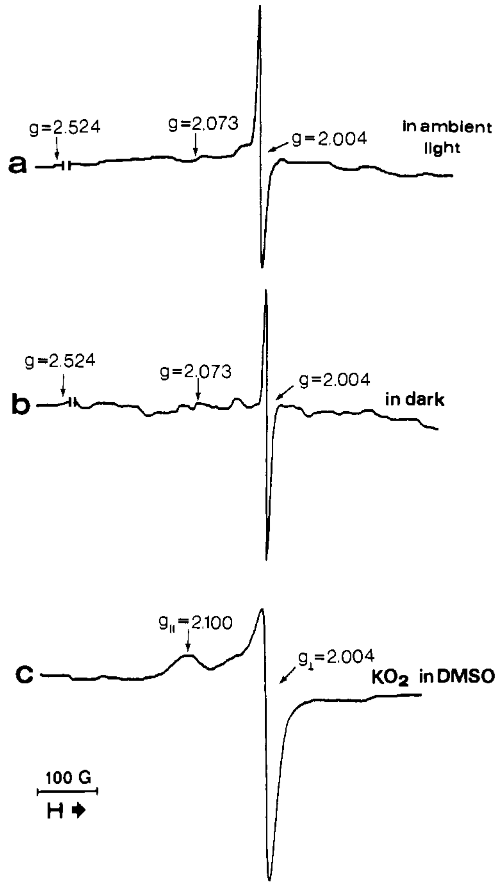

FIGURE 1: (a) ESR spectrum recorded at 77 K of 2.0 mM fredericamycin dissolved in DMSO and exposed to air for 1 week under ambient light; (b) same as (a) but in the dark; (c) ESR spectrum recorded (at 140 K) from KO_2 dissolved in DMSO.

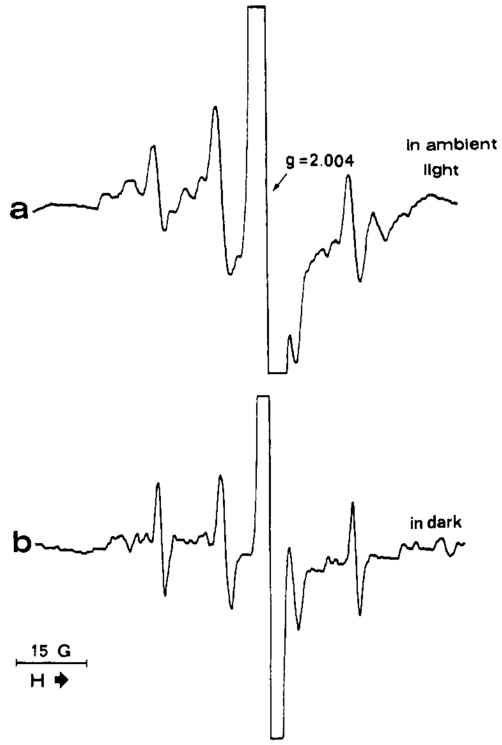

FIGURE 2: ESR spectra of 100 mM DMPO and 1.0 mM fredericamycin in DMSO exposed to air: (a) under ambient light and (b) in the dark.

ESR spectrum observed in the presence of the spin trap DMPO in the ambient light and Figure 2b that in the dark.

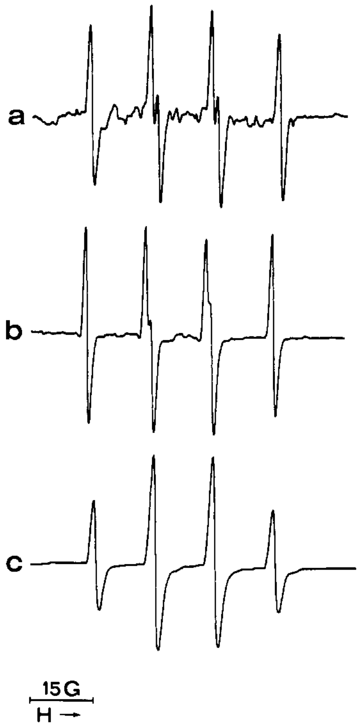

FIGURE 3: ESR spectra of 100 mM DMPO, 100 mM Na_2SO_3, and (a) DMSO:H_2O = 1:10, (b) DMSO:H_2O = 1:3, or (c) DMSO:H_2O = 1:1.

The similarity of these spectra again indicates that ambient light does not significantly affect the spin trap spectra.

The spin adduct spectra (Figure 2) are similar to that reported earlier [Figure 9 of Hilton et al. (1986)], except that we assign them to a DMPO–SO_3^- adduct rather than a DMPO–O_2^- adduct, since the observed splitting constants $a_N = 14.5$ G, $a_H = 15.3$ G, and $g = 2.0054$ are essentially equal to those known for DMPO–SO_3^-, $a_N = 14.7$ G, $a_H = 16.0$ G, and $g = 2.0056$ (Covello & Thompson, 1985; Mottley et al., 1982a). An essentially identical spectrum was obtained (using DMPO) when Na_2SO_3 was reacted with H_2O_2 in DMSO, as reported earlier (Mottley et al., 1982b; Norman & Storey, 1971). It has been reported that under alkaline conditions DMSO decomposes in air to form $^\bullet SO_3^-$ radical (Stolze & Mason, 1987). In fact, when Na_2SO_3, DMSO, and DMPO were mixed, the typical spectrum of the DMPO–SO_3^- adduct was observed as shown in Figure 3. Thus, the spectrum assigned earlier to a DMPO–O_2^- adduct (Hilton et al., 1986) should be reassigned to a DMPO–SO_3^- adduct.

The above result prompted us to study the spin trap ESR evidence for the formation of $^\bullet OH$ radicals by FMA in dilute H_2O_2 solution. A 1:2:2:1 quartet spectrum, characteristic of the DMPO–OH adduct, was indeed observed from a 1.0 mM aqueous solution of FMA containing 0.1% H_2O_2, in agreement with the earlier report [Figure 10a of Hilton et al. (1986)]. However, control experiments were needed to ascertain whether the $^\bullet OH$ radical generation involved FMA. In particular, since natural FMA was known (Hilton et al., 1986) to contain a significant amount of Fe^{2+} (<0.1% by weight of drug), chelating agents must be employed to minimize the production of the $^\bullet OH$ radicals via the well-known Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^\bullet OH + OH^-$$

Our measurements using diethylenetriaminepentaacetic acid (DETAPAC), the chelator used (Finkelstein et al., 1980) quite frequently for Fe^{2+}, showed that the $^\bullet OH$ radical production

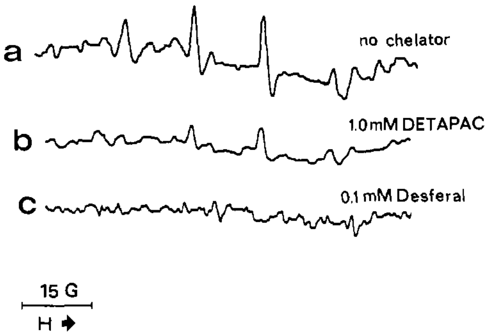

FIGURE 4: ESR spectra of 1.0 mM fredericamycin and 100 mM DMPO mixed with water in presence of 0.1% H_2O_2: (a) no DETAPAC added; (b) with 1.0 mM DETAPAC; (c) with 0.1 mM desferal.

decreased by more than 70% when 1.0 mM DETAPAC was employed. This may be noted from the spectra with and without DETAPAC, spectra a and b of Figure 4, respectively. Additional measurements with 0.1 mM desferal, a better chelator for Fe^{2+} (Morehouse & Mason, 1988), showed that essentially no $^{\bullet}OH$ radicals were trapped (Figure 4c). Thus, the $^{\bullet}OH$ formation can be largely attributed to the presence of Fe^{2+} in the sample.

We also find that the evidence provided earlier for the drug-related formation of the methoxy ($^{\bullet}OCH_3$) radical is in error. This evidence was the observation [Figure 10b of Hilton et al. (1986)] of a spectrum with $a_N = 16.0$ G and $a_H = 22.7$ G. However, the observed large β-hydrogen splitting (22.7 G) for a DMPO adduct is an indication that the trapped radical was carbon centered rather than oxygen centered (Janzen & Liu, 1973; Janzen et al., 1983). The known (Janzen & Liu, 1973; Janzen et al., 1973) a_N and a_H values for a DMPO–OCH_3 adduct are $a_N = 13.58$ G and $a_H = 7.61$ G. In fact, the reported parameters ($a_N = 16.0$ G and $a_H = 22.7$ G) match well with those for a DMPO–CH_3 adduct ($a_N = 16.1$ G and $a_H = 23.0$ G) (Mossoba et al., 1984). We thus propose that the spectrum assigned earlier to DMPO–OCH_3 should be reassigned to a DMPO–CH_3 adduct (Augusto & Montellano, 1981; Grover et al., 1987; Janzen & Liu, 1973; Janzen et al., 1973; Mossoba et al., 1984; Sinha, 1983), the $^{\bullet}CH_3$ fragment resulting from the reaction of DMSO with $^{\bullet}OH$ radicals (produced via the Fenton reaction).

In conclusion this study demonstrates, contrary to earlier reports (Hilton et al., 1986; Stinson, 1986), that natural FMA does not spontaneously reduce O_2 to O_2^- and H_2O_2 to $^{\bullet}OH$ radicals, nor does it form $^{\bullet}OCH_3$ radicals. It thus reopens the question as to what is the key factor in the exceptional activity of FMA. The answer might lead toward new clues in the strategies for the development of other highly potent anticancer antibiotics.

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Registry No. FMA, 80455-68-1; O_2^-, 11062-77-4.

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