EVIDENCE FOR INVOLVEMENT OF A FREE RADICAL IN DNA-CLEAVING REACTION BY MACROMOMYCIN AND AUROMOMYCIN

HIDEO SUZUKI, YUTAKA KIRINO* and NOBUO TANAKA

Institute of Applied Microbiology and *Faculty of Pharmaceutical Sciences,
University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

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The DNA-cleaving activity of auromomycin (AUR) was prevented by the free radical scavengers α-tocopherol and isopropanol. Participation of a free radical in DNA strand scission by AUR and macromomycin (MCR) was confirmed by electron spin resonance (ESR) spectroscopy, using spin trapping technique. In the mixture of AUR and the spin-trap, phenyl *N-tert*-butyl nitron (PBN), a definite signal was observed in the presence of ethanol or methanol, and addition of dithiothreitol increased the signal intensity about 5-fold, giving a hyperfine coupling constant of $a^N=15.9$ G and $a_\beta^H=3.6$ G. MCR exhibited no distinct signals in the same reaction mixture, but addition of dithiothreitol induced signals with ESR parameters similar to those of AUR. 2-Mercaptoethanol did not significantly affect the appearance and intensity of the ESR spectra generated by MCR or AUR. Chromophores, extracted from MCR and AUR, showed spectra similar to those of native MCR and AUR. The intensity of ESR signals parallels the degree of DNA strand scission by MCR, AUR and their free chromophores, suggesting that a free radical generated by these antibiotics is involved in the DNA breakage reaction. The ESR parameters of AUR or MCR radical adduct with spin-traps were compared to $\cdot OH$, $\cdot O_2^-$ or $CH_3\dot{C}HOH$ adduct, suggesting that the free radical formed in the presence of ethanol is $CH_3\dot{C}HOH$. A plausible intermediate radical species, generated by the antibiotics and transferred to ethanol, may be $\cdot OH$ (hydroxyl radical). The current experiments present evidence for involvement of free radical(s) in DNA breakage by macromomycin and auromomycin, although the precise radical species has not been identified.

We have studied the mechanism of action of the antitumor protein antibiotics macromomycin (MCR)[1] and auromomycin (AUR)[2]. We found that both antibiotics cause DNA strand scission *in vivo* and *in vitro*[3~6], and that the non-protein chromophore moiety is responsible for the cytotoxic and DNA-cleaving activities[7,8]. Although MCR requires a reducing agent, such as $NaBH_4$ or dithiothreitol (DTT), for DNA breakage, free MCR chromophore induces DNA strand scission without reducing agents as does AUR and its chromophore[9,10]. DTT enhances the DNA-cutting activity of AUR and the free chromophores of MCR and AUR approximately 4-fold[10]. Requirement for or enhancement by reducing agents for these antibiotics to cleave DNA is similar to DNA breakage by bleomycin and neocarzinostatin (NCS). The involvement of free radicals in DNA degradation by the latter two drugs has been shown by electron spin resonance (ESR) spectra[11,12].

In the current publication, we describe free radical generation by MCR, AUR and their free chromophores, detected by ESR using a spin trapping technique. Appearance and intensity of the signals generated by the drugs, and enhancement by DTT are in accord with the DNA-cutting activity, suggesting that a free radical plays an important role in DNA cleavage by the antibiotics.

Materials and Methods

MCR and AUR were generously supplied by Dr. K. WATANABE, Kanegafuchi Chemical Industry

Co., Takasago, Japan. Chromophores were extracted from the antibiotics in methanol by the method described previously[7]. The concentrations of free chromophores are expressed as equivalent concentrations of native MCR or AUR. The DNA-cleaving activity was quantitatively analyzed by agarose gel electrophoresis, using [^{3}H]thymidine-labelled pBR322 DNA, as reported in a previous paper[10].

ESR Spectra Measurement

Phenyl *N-tert*-butyl nitrone (PBN) and 5,5-dimethylpyrroline-*N*-oxide (DMPO) were synthesized by the procedure described previously[13]. ESR spectra were obtained at room temperature using a JEOL JES-PE-1X (X band) spectrometer with a cylindrical TE_{011} mode cavity and 100 kHz field modulation. The reaction mixture, in 50 μl, contained: 80 mM PBN or DMPO, antibiotic at the indicated concentration, 80 mM phosphate buffer, pH 7.0, 40% ethanol and a supplement, if mentioned. Hyperfine coupling constants were determined by comparison to the spectrum of an aqueous solution of Fremy's salt (a^N=13.0 G).

Results

The Effect of Radical Scavengers on DNA-cutting Activity of AUR

The effect of radical scavengers on DNA-cleaving activity of AUR was observed by electrophoretic analysis of [^{3}H]thymidine-labelled pBR322 DNA, and the degree of strand scission was calculated by decrease of radioactivity of form I DNA[10]. As presented in Table 1, α-tocopherol (antioxidant) markedly reversed the DNA-cutting activity of AUR. It agrees with the results reported by KAPPEN *et al.*[14]. Isopropanol (·OH radical scavenger) also blocked the AUR activity. The results suggest that free radical is involved in the reaction of DNA breakage induced by AUR.

ESR Signals Generated by AUR, MCR and Their Free Chromophores

First we tried but failed to detect ESR signals in a reaction mixture containing AUR and PBN in phosphate buffer, pH 7.0. Then we found that addition of ethanol or methanol is necessary for the appearance of ESR signals of the antibiotic. Fig. 1 shows the ESR spectrum of free radical formed by AUR (5 mg/ml) in the presence of 40% ethanol and 80 mM PBN. The spectrum gave parameters of a^N=15.9 G and a_β^H=3.6 G. When methanol was used instead of ethanol, the parameters were a^N=15.6 G and a_β^H=4.2 G.

Table 1. Effect of α-tocopherol and isopropanol on DNA-cleaving activity of AUR.

	Form I remaining	Degradation %
None	1,400 dpm	0
AUR (10 μg/ml)	751	46
+α-tocopherol (1 mM)	1,168	17
+ 〃 (0.2 mM)	1,058	24
+ isopropanol (1 M)	1,000	29
+ 〃 (0.2 M)	741	47

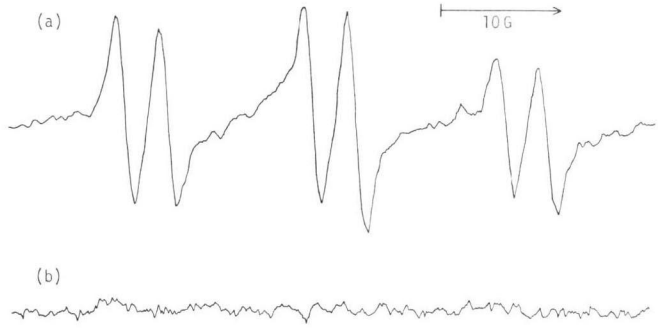
The reaction mixture of "None" contained [^{3}H]-thymidine-labelled pBR322 DNA (0.2 OD unit/ml, 1,400 dpm in form I), 1 mM DTT and 50 mM tris-HCl, pH 7.5. The incubation was carried out for 30 minutes at 37°C.

Table 2. Comparison of signal intensity of ESR spectrum generated by AUR, MCR and their chromophores.

	Relative signal intensity
AUR (500 μg/ml)	18
AUR (〃)+DTT (1 mM)	100
AUR (〃)+2-ME (10 mM)	18
AUR (〃)+DTT (1 mM)−EtOH*	0
MCR (5 mg/ml)	0
MCR (〃)+DTT (1 mM)	91
MCR (〃)+2-ME (10 mM)	0
AUR chromophore (200 μg/ml) +DTT (1 mM)	45
MCR chromophore (1 mg/ml) +DTT (1 mM)	18

PBN (80 mM) was used as a spin trap.
* EtOH: 40% Ethanol was omitted.

Fig. 1. ESR spectrum of the radical produced by AUR.
(a) Reaction mixture contained AUR (5 mg/ml), 80 mM PBN, 80 mM phosphate buffer, pH 7.0 and 40% ethanol.
(b) AUR was omitted from the reaction mixture of (a).



Similarly, radical formation by AUR, MCR and their free chromophores was determined in the presence or absence of DTT and 2-mercaptoethanol (2-ME). The results are summarized in Table 2. The reaction mixture contained 40% ethanol, unless otherwise noted, and signal intensity in each spectrum was measured by peak-to-peak height of the first derivative curve and shown as relative signal intensity. AUR gave small signal peaks at a concentration of 500 μg/ml, and addition of DTT at 1 mM stimulated radical formation about 5-fold. The ESR signals disappeared when ethanol was omitted from the incubation mixture. MCR at 5 mg/ml showed no significant peaks, but produced clear peaks upon addition of 1 mM DTT, indicating radical formation. 2-ME at 10 mM did not affect ESR spectra of AUR and MCR, showing no significant effects on radical production. Free chromophores of AUR and MCR also generated radicals, showing that the protein moiety of the antibiotics is not necessary for radical formation. The effects of DTT and 2-ME on radical production by AUR, MCR and their chromophores paralleled those on DNA strand breakage, suggesting that generation of free radical is involved in DNA cleavage by the drugs.

In order to identify the species of radicals generated by AUR and MCR, further ESR measurements were carried out, using DMPO as a spin trap instead of PBN, in the presence of ethanol. ESR parameters, obtained by MCR and AUR, were compared with those of some radical adducts of PBN and DMPO[15,16] (Table 3). The results suggest that the signals observed are due to the $CH_3\dot{C}HOH$ adduct of PBN and DMPO, but not by either the $\cdot OH$ or $\cdot O_2^-$ adduct of these spin traps. The possible identity of the free radical generated by AUR and MCR will be discussed below.

Table 3. Comparison of ESR parameters obtained by AUR and MCR to those of possible radicals.

	Spin trap				Reference
	PBN		DMPO		
	a^N	a_β^H	a^N	a_β^H	
AUR radical*	15.9 G	3.6 G	16.0 G	23.1 G	
MCR radical*	15.4	3.9	—	—	
$CH_3\dot{C}HOH$	16.2	3.34	15.8	22.8	15
$\cdot OH$	15.3	2.75	15.3	15.3	16
$\cdot O_2^-$	14.8	2.75	14.3	11.7	16

* Observed in 40% ethanol.

Discussion

In the current experiments, we have found that the intensity of ESR signals, generated by MCR and AUR, parallels the rate of DNA cleavage under various conditions, and suggested that a free radical is

involved in DNA-cutting reaction of MCR and AUR. Several antitumor antibiotics, such as bleomycin[17], adriamycin[18], aclacinomycin A[18], mitomycin C[19], and chartreusin[20], also cause DNA strand scission, which is enhanced by the presence of Fe^{2+} or Cu^{+}, and reducing agents. The radicals, produced by reduction and auto-oxidation of these antibiotics, may play an important role in DNA breakage. In contrast, DNA cleavage, induced by AUR and MCR, is not affected by addition of Fe^{2+}, Cu^{+} or EDTA (data not shown), as is the case with NCS[21]. Therefore, the mechanism of DNA strand scission by MCR, AUR and NCS may differ from the other antitumor antibiotics.

EDO *et al.*[12] reported that the radical formed by NCS is neither $\cdot O_2^-$ nor ·OH, but presumably a peroxy radical, by comparing ESR parameters of the NCS radical with those of $\cdot O_2^-$ and ·OH. We have found that ethanol or methanol is required for appearance of ESR signals generated by AUR and MCR, and that the ESR parameters, obtained with PBN and especially with DMPO, are similar to those of the $CH_3\dot{C}HOH$ adduct of PBN or DMPO. Therefore, the most plausible radical formed may be ·OH but not $\cdot O_2^-$, although it remains to be proven. The reason will be discussed below.

The ·OH adduct of PBN or DMPO is so labile that the concentration of the adduct radical rapidly decreases below the detection limit of ESR, unless ·OH is continuously produced[22]. This may be the reason why no radical signal is found without addition of ethanol or methanol. When ethanol is present in the reaction mixture at a high concentration, a radical $CH_3\dot{C}HOH$ is easily formed by α-hydrogen abstraction from ethanol by ·OH. The reaction is very fast, showing a second-order rate constant of *ca.* $10^9 M^{-1}$ second^{-1}[23]. The $CH_3\dot{C}HOH$ adduct of PBN or DMPO is more stable than that of ·OH, and is easily detected in ESR spectra. Another active oxygen species, $\cdot O_2^-$, cannot be detected in the current experiments, since it does not undergo hydrogen abstraction from CH bond. Furthermore, ·OH shows a high ability to cause DNA strand scission[24]. However, the possibility of generation of other unidentified radicals by AUR and MCR cannot be excluded by the present results.

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