Microbial Resistance to Mitomycins Involves a Redox Relay Mechanism

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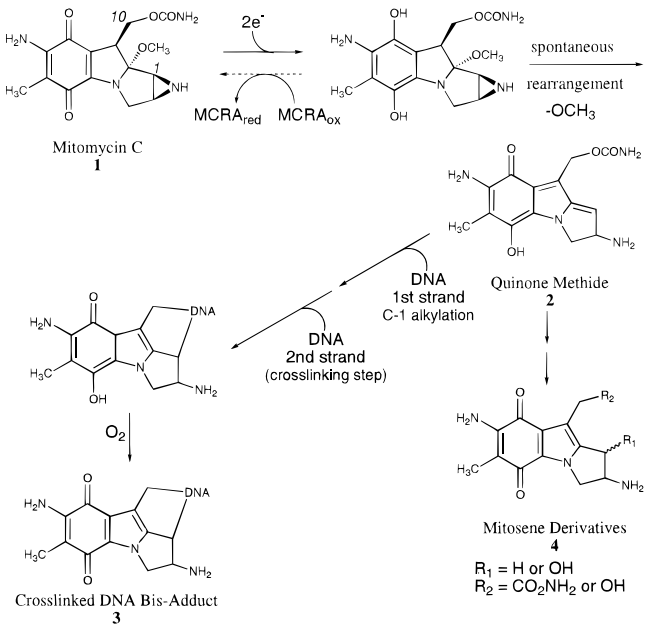
The antitumor antibiotic mitomycin C (MC, Scheme 1) produced by *Streptomyces lavendulae* was discovered 40 years ago,[4] and MC has since been recognized as the first in an expanding class of naturally occurring DNA-targeted agents that require reductive activation.[5] The drug cross-links DNA at 5′-CpG sequences,[6] and this property provides the molecular basis for using MC in the chemotherapeutic treatment of a variety of malignancies.[7] While the mode of action (Scheme 1) and target-site specificity of MC in DNA have been extensively studied,[8] little is known about the cellular resistance mechanism(s) the MC-producing bacteria employ to avoid self-destruction. Knowledge of this metabolic aspect of MC may enhance clinical applications of the drug.

Toward this end, a gene (*mcrA*) that confers resistance to MC had been isolated and cloned,[9] and it was found to be specifically induced by members of the mitosane class of natural products.[10] Sequence comparisons showed that the protein product (MCRA) is related to a special class of flavin adenine dinucleotide (FAD) dependent oxidoreductases.[9] Analysis of MCRA by electrospray mass spectrometry confirmed that it contains a covalently bound FAD cofactor,[11] thus suggesting that MCRA is also a redox enzyme. Here we show that MCRA effectively inactivates reduced MC through oxidation by the FAD cofactor. This is the first example of a redox-dependent resistance mechanism, which provides a unique and specific cellular foil against MC bioreductive activation.

Considering that the preliminary step for MC activation is single- or double-electron reduction, a mechanism in which MCRA oxidizes the reduced MC (to the prodrug form) before it can rearrange to the active quinone methide and alkylate DNA is the most appealing hypothesis (Scheme 1). To investigate this hypothesis, we purified MCRA[11] and designed an assay which exploited the ability of certain flavoenzymes to reductively activate MC.[12] CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase (E_3), a NADH-dependent flavoprotein from the ascarylose biosynthetic pathway in *Yersinia pseudotuberculosis*,[13] was found to catalyze the reductive activation of MC. When MC was added to a reaction mixture containing E_3, excess NADH, and MCRA, the absorbance of the FAD chromophore of MCRA centered at 455 nm decreased in a time-dependent manner (Figure 1). Control experiments showed that E_3 could not reduce MCRA even in the presence of excess NADH (data not shown). Thus, bleaching of the flavin coenzyme of MCRA apparently proceeds by an E_3-reduced MC intermediate via a redox relay process.

Scheme 1



Because mitosene products (**4**) absorb more strongly than MC in the 500−700 nm region, the fact that this region remained relatively unchanged during the FAD reduction phase (traces 1−5, Figure 1) indicated that reduced MC was oxidized by MCRA before the hydroquinone could spontaneously rearrange to the precursor quinone methide (**2**).[12,14] Only after the MCRA-bound FAD was completely quenched (trace 5, Figure 1) did the reduced MC hydroquinone rearrange to mitosene products (traces 6 and 7, Figure 1). With time and agitation, the flavin chromophore reappeared (data not shown), indicating that O_2 is an acceptor for reduced MCRA. These results are consistent with a mechanism that involves the regeneration of MC from the hydroquinone by a MCRA-catalyzed redox reaction (Scheme 2).

We next assessed whether MCRA can prevent DNA cross-linking by designing an *in vitro* assay in which various concentrations of MCRA were anaerobically incubated with constant concentrations of MC, NADH, E_3, and *Streptomyces* plasmid DNA (Figure 2). The absence of suitable electron acceptors for reduced MCRA and a stoichiometric ratio of NADH/MC ensured single-turnover conditions. The results show that the degree of DNA protection is directly proportional to the concentration of MCRA. A 1:1 ratio of MCRA/MC afforded greater than 90% of the plasmid DNA in denatured single-stranded form (compare lanes 1 and 2, Figure 2A). When the quantity of MCRA was gradually decreased (lanes 3−6,

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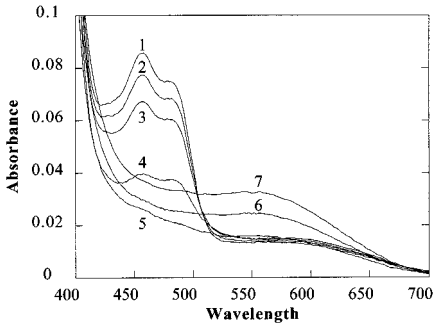

Figure 1. Quenching of the MCRA flavin in the presence of activated MC. The aerobic reaction of 61 μM MC, 560 μM NADH, 260 nM E_3, and 8.1 μM MCRA in 20 mM Tris-HCl, pH 7.5, was spectroscopically monitored at room temperature. After initiating the reaction by the addition of NADH, the solution was scanned at 30 s intervals. Trace 1 is the scan immediately after mixing, and traces 2−7 are selected scans at 4, 5.5, 6, 7, 8, and 10 min, respectively. Absorbance between 430 and 500 nm derives from the flavin chromophore of MCRA, while the broad band from 500 to 700 nm is primarily due to the mitosene derivatives, although MC does have a weak absorbance in this range.[14]

Scheme 2

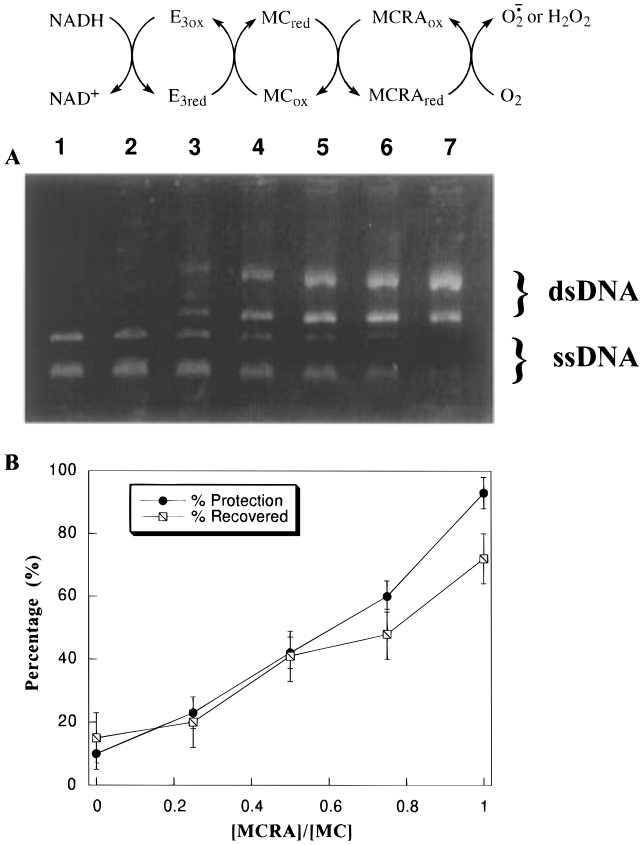

Figure 2. (A) Anaerobic MCRA-mediated DNA protection assay. This representative gel depicts a series of anaerobic DNA protection reactions: lane 1, denatured DNA control with no other reactants; lanes 2−7, 62, 50, 37, 25, 12, and 0 μM MCRA, respectively, plus 62 μM MC, 67 μM NADH, 0.4 μM E_3, and 11 μg of DNA in 20 mM Tris-HCl, pH 7.5 (59.5 μL total volume). The two bands labeled "ssDNA" are the linear (upper band of lane 1) and circular (lower band of lane 1) strands of the denatured plasmid DNA, while the "dsDNA" bands are the open circular (upper band of lane 7) and covalently closed circular (lower band of lane 7) forms of the native plasmid DNA.[17] (B) Percentage of DNA protection and MC recovery as a function of the [MCRA]/[MC] ratio.

Figure 2A), the DNA became increasingly cross-linked and thus resistant to denaturation. With no MCRA in the reaction mixture (lane 7, Figure 2A), the DNA was almost completely cross-linked. A control reaction with an equimolar amount of bovine serum albumin (BSA) substituted for MCRA showed complete cross-linking (data not shown); therefore, the DNA protection afforded by MCRA does not occur by nonspecific protein interference.

HPLC quantification of MC recovered from the reaction mixtures (Figure 2B) used in DNA protection experiments (Figure 2A) showed peaks corresponding only to the MC control. About 71% of the MC in the reaction mixture (lane 2, Figure 2A) was recovered at the same time the DNA was mostly protected against cross-linking ([MCRA]/[MC] = 1, Figure 2B). The high recovery of material indicates that MCRA does not sequester the drug. However, MC is expected to bind MCRA in order for electron transfer to occur, thus weak association of MC to MCRA could cause some loss of the drug in the assay. Also, it is likely that covalent binding of the drug to the DNA as a monofunctional (non-cross-linked) adduct[8] prevents 100% recovery of MC. Formation of mitosene derivatives (**4**), albeit at levels below the detection limit, before MCRA could inactivate the reduced MC might contribute to a lower recovery. Although unlikely, the possibility that MCRA can modify MC to a form that could not be detected by the analytical procedures employed can not be entirely ruled out.

Taken together, the spectrophotometric data, the DNA protection results, and the HPLC recovery of MC from the DNA protection reactions provide convincing evidence that MCRA confers cellular resistance by oxidizing reduced MC. Although many modes of cellular resistance have been described,[15] this is the first reported example of a redox-mediated resistance mechanism. The correlation between antimicrobial activity and a unique ability of MC to bioreductively alkylate DNA was established soon after its discovery, although conclusive evidence directly linking these processes as a natural biochemical phenomenon has been lacking. It is now apparent that *S. lavendulae* adapted its physiology to include a biochemically precise method for self-protection, against its unique ability to produce MC. Therefore, the discovery of a redox mechanism for MCRA confirms that bioreductive activation of MC is an authentic chemical process that evolved in a microbial milieu to confer optimal selective advantage.

As MC is a member of the growing class of bioreductively activated natural products,[16] other microorganisms that produce secondary metabolites in this class may employ a similar self-defense mechanism. The implications of this finding may be particularly significant in clinical cancer therapy in which individuals might be protected from higher doses of mitomycin C by transplantation with transgenic marrow expressing the MC-resistant oxidoreductase (*mcrA*) activity.

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Supporting Information Available: Experimental details for MCRA protein purification, electrospray mass spectrometry data and DNA protection assay and analysis (3 pages). See any current masthead page for ordering and Internet access instructions.

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