PHAGE INACTIVATION AND DNA STRAND SCISSION ACTIVITIES OF 7-*N*-(*p*-HYDROXYPHENYL)MITOMYCIN C

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A new derivative of mitomycin C (MMC), 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83), had higher phage inactivation activity against phages ϕX174 and PM2 than MMC, and also higher DNA strand scission activity against their single- and double-stranded DNAs. M-83, at one third to one sixth concentration of MMC, showed the same level of phage inactivation and DNA strand scission activities. The mechanism of phage inactivation and DNA strand scission by M-83 were similar to those of MMC: (1) Reduction of M-83 was required for its action. (2) Oxygen radicals were involved in DNA strand scission, and metal ions possibly participated in the generation of oxygen radicals. (3) DNA strand scission was single strand scission, and dependent on temperature. The high DNA strand scission activity of M-83 is considered to reflect the rapid conversion to the active form.

A new derivative of mitomycin C (MMC), 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83)[1], has a higher antitumor activity than MMC against lymphocytic leukemia P388 and fibrosarcoma Meth 1[1,2], and a lower toxicity than MMC with myelosuppression and leukopenia[3]. M-83 is, therefore, expected to be more useful as a clinical antitumor agent. The mechanism of the increased antitumor activity of M-83 is unclear.

We have reported that MMC, when reduced with sodium hydrosulfite (sodium dithionite, $Na_2S_2O_4$) in the presence of cupric ion, causes a strand scission in ϕX174 single-stranded DNA (ϕX174 SS DNA) and subsequent phage inactivation[4], and a single strand scission in ϕX174 replicative from I DNA (RF I DNA)[5]. MMC reduced with sodium borohydride ($NaBH_4$) also causes the inactivation of phages ϕX174 and PM2 and strand scission in their single-stranded DNA[6] and double-stranded DNAs[6,7]. Oxygen radicals such as hydroxyl radical and singlet oxygen, and possibly MMC semiquinones radical are thought to be involved in the DNA strand scission[4~7]. Trace metal ions are believed to be involved in the generation of oxygen radicals[4~7].

To elucidate the mechanism of action of M-83, phage inactivation and DNA strand scission activities of M-83 have been investigated *in vitro*, and compared with those of MMC, using phages ϕX174 and PM2, and their DNAs as probes. M-83, at one third to one sixth concentration, showed the same degree of phage inactivation and DNA strand scission activities of MMC.

Materials and Methods

Chemicals and Enzymes

Mitomycin C and 7-*N*-(*p*-hydroxyphenyl)mitomycin C were kindly supplied by Kyowa Hakko Co. Ltd., Tokyo, Japan. Superoxide dismutase (EC 1.15.1.1, bovine blood, 2,900 U/mg protein) and catalase (EC 1.11.1.6, bovine liver, 2,500 U/mg protein) were purchased from Sigma Chemical Co.

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Sodium dextran sulfate 500 was purchased from Pharmacia Fine Chemicals. Other chemicals were obtained from Nakarai Chemicals Co.

Bacteria and Phages

Escherichia coli C_N and *Pseudomonas* BAL-31 were used as the indicator bacteria of phage ϕX174 and phage PM2, respectively.

Preparation of Phage ϕX174 and its DNAs

Phages ϕX174 and ϕX174 *am*3 were prepared as reported previously[8,9]. ϕX174 SS DNA was extracted from ϕX174 *am*3 particles by the hot phenol method[10]. ϕX174 RF I DNA was prepared as reported previously[5].

Preparation of Phage PM2

Pseudomonas BAL-31 was grown at 28°C to 4×10^8 cells/ml in 1 liter of AMS-broth[11], and infected with PM2 at a multiplicity of infection of 5 to 10. Incubation was continued for 2 to 3 hours after infection until complete lysis. After cooling in ice for 30 minutes, the lysate was concentrated according to the method described by SALDITT *et al.*[12]: Polyethylene glycol 6000, powdered, was slowly added to a final concentration of 43 g per liter and sodium dextran sulfate 500 was added to a final concentration of 2.35 g per liter. The mixture was vigorously shaken and then allowed to settle for 18 hours at 4°C. The bottom phase was collected and centrifuged at $10,000\times g$ for 20 minutes. The interphase was collected and suspended in 2-fold volume of 20 mM tris-HCl buffer (pH 7.1) containing 1 M NaCl and 10 mM $CaCl_2$ (NTC buffer). To the suspension was added 4 M KCl to a final concentration of 1.1 M. After allowing the mixture to stand for 2 hours at 4°C, the precipitate of dextran sulfate was removed by centrifugation at $8,000\times g$. For every gram of solution, 0.317 g of cesium chloride (CsCl) was added (average density 1.28 g/ml). After centrifugation (RP65TA rotor: Hitachi 55P ultracentrifuge) and $86,000\times g$ for 24 hours, the white virus band was collected by aspiration. The purified virus was dialysed against NTC buffer.

Isolation of PM2 DNA

PM2 covalently closed circular (ccc) duplex DNA was extracted from purified PM2 phage and further purified by CsCl equilibrium centrifugation essentially as described earlier[13,14].

Reaction of M-83 with ϕX174 or PM2 Virion

The reaction mixture (100 μl) contained 2×10^8 plaque-forming units (p.f.u.)/ml of ϕX174 or PM2 virion, 50 μM M-83 and 200 μM $NaBH_4$. The reaction with ϕX174 was carried out in 50 mM tris-HCl buffer (pH 7.1) at 37°C, and with PM2 in NTC buffer at 28°C. The reaction was started by adding freshly prepared $NaBH_4$ solution, continued for 1 hour with gentle shaking, and stopped by dilution with each ice-cold buffer. The survival of phage was assayed by the double agar layer technique[11,15].

Reaction of M-83 with DNA

The reaction mixture (20 μl) contained 0.17 μg (8.5 μg/ml) PM2 ccc DNA, ϕX174 SS or RF I DNA, 50 μM M-83 and 500 μM $NaBH_4$ in 50 mM tris-HCl buffer (pH 7.1), unless otherwise noted. Reactions were carried out for 1 hour at 37°C, and stopped by the addition of 5 μl of 0.1 M EDTA solution containing 50% (w/v) sucrose and 0.1% bromophenol blue. The sample in a final volume of 25 μl was analyzed by agarose gel electrophoresis.

Agarose Gel Electrophoresis

Electrophoretic analysis of PM2 DNA or ϕX174 RF I DNA was carried out in respectively 0.9% or 1.4% agarose slab gels at 3.5 volt/cm for 3 hours in 40 mM tris-acetate buffer (pH 8.1) containing 5 mM sodium acetate and 1 mM EDTA[16]. ϕX174 SS DNA was analyzed by electrophoresis in agarose slab gels (2.0%) at 10 volt/cm for 1 hour in 90 mM tris-borate buffer (pH 8.3) containing 2.5 mM EDTA[17]. The gel was stained by soaking in ethidium bromide solution (0.5 μg/ml) for 1 hour.

Determination of the Degree of DNA Strand Scission

The stained bands were visualyzed using an ultraviolet lamp (Funa UV light, model SL-800F, 254 nm) and photographed (Kodak Tri-X film, with red filter). The three topological forms of PM2 DNA or ϕX174 double-stranded DNA, which are ccc DNA (RF I DNA in the case of ϕX174), nicked,

open circular duplex (oc) DNA and full-length linear duplex (linear) DNA, and the two topological forms of ϕX174 single-stranded DNA, which are circular DNA (ϕX174 SS DNA), and full-length linear DNA, were detected as clearly separated bands in agarose gels[15]. PM2 ccc DNA or ϕX174 RF I DNA produces oc DNA following single strand scission, or linear DNA as the results of a double strand scission. A strand scission in circular ϕX174 SS DNA produces full-length linear DNA. Thus a single strand scission causes a decrease in the amount of PM2 ccc DNA, ϕX174 RF I DNA and ϕX174 SS DNA. The photographic negatives were scanned with a Shimadzu dual-wavelength TLC-scanner CS-900 to quantitate the amount of PM2 ccc DNA, ϕX174 RF I DNA and ϕX174 SS DNA, and to assay the degree of DNA strand scission. Amount of DNA was corrected by the calibration curve as shown previously[6].

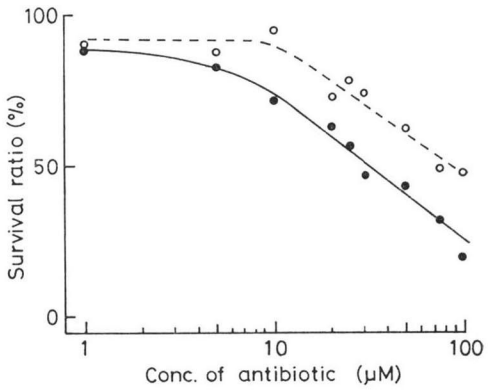
Results

Action of M-83 on ϕX174 Virion and Its Single-stranded DNA

M-83 was reduced *in situ* with $NaBH_4$ to cause the inactivation of phages ϕX174 (Fig. 1) and PM2 (Fig. 3) and strand scission in their DNAs (Fig. 2, 4) as was MMC[4~7]. M-83 had higher phage inactivation activity that MMC, *i.e.*, 30 μM of M-83 were required to inactivate phage ϕX174 by 50%, while 90 μM of MMC were required to show the same effect (Fig. 1). M-83 had also higher DNA strand scission activity than MMC, *i.e.*, 20 μM of M-83 were required to bring about 50% nicking of circular SS DNA, while 60 μM of MMC were required to show the same effect (Fig. 2). Both bands of circular and linear DNAs disappeared in Fig. 2N~Q, indicating that ϕX174 SS DNA was degraded to small fragments by reduced M-83.

Fig. 1. Inactivation of phage ϕX174 by MMC or M-83 reduced with $NaBH_4$.

Phage ϕX174 (2×10^8 p.f.u./ml) was incubated at 37°C for 1 hour with indicated concentrations of MMC (○) or M-83 (●) in 50 mM tris-HCl buffer (pH 7.1) containing 200 μM $NaBH_4$.



Action of M-83 on PM2 Virion and its Double-stranded DNA

M-83 had also higher phage inactivation activity against phage PM2 than MMC (Fig. 3), and higher DNA strand scission activity against PM2 ccc DNA (Fig. 4). Since the band of linear DNA did not appear (Fig. 4), reduced M-83, as well as reduced MMC, caused single strand scission, but not double strand scission of PM2 DNA.

Effect of Enzymes and Radical Scavengers

The effect of enzymes and radical scavengers on DNA strand scission by both antibiotics were similar (Fig. 5, Table 1). This indicates that oxygen radicals such as hydroxyl radical and singlet oxygen participate in DNA strand scission by both reduced M-83 and reduced MMC. Since the reactions were inhibited by EDTA (Fig. 5 I, Q), trace metal ions in the reaction mixture are presumed to be involved in the generation of oxygen radicals.

Other Reducing Agents in the Induction of DNA Strand Scission by M-83

M-83 reduced with dithiothreitol showed much higher DNA strand scission activity than MMC

Fig. 2. Induction of strand scission in ϕX174 single-stranded DNA by MMC (○) or M-83 (●) reduced with $NaBH_4$.

A: Drug-free control, B: 200 μM $NaBH_4$ alone, C~I: MMC, J~Q: M-83. Concentration of MMC or M83: C and J, 1 μM; D and K, 10 μM; E and L, 30 μM; F and M, 50 μM; G and N, 75 μM; H and P, 100 μM; I and Q, 500 μM.

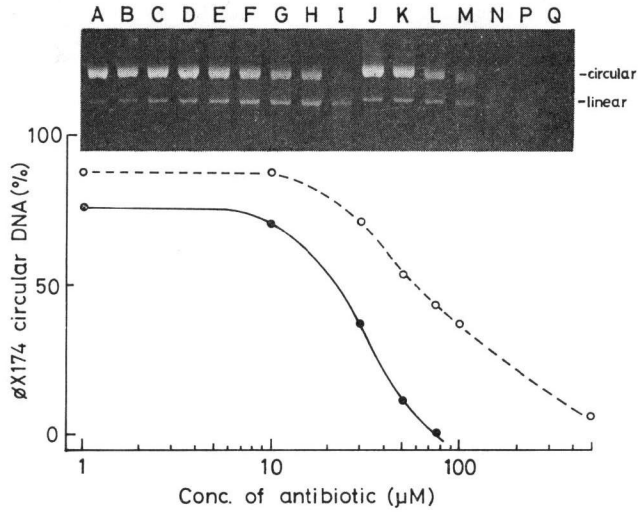


Fig. 3. Inactivation of phage PM2 by MMC or M-83 reduced with $NaBH_4$.

Phage PM2 (2×10^8 p.f.u./ml) was incubated at 28°C for 1 hour with indicated concentrations of MMC (○) or M-83 (●) in NTC buffer containing 200 μM $NaBH_4$.

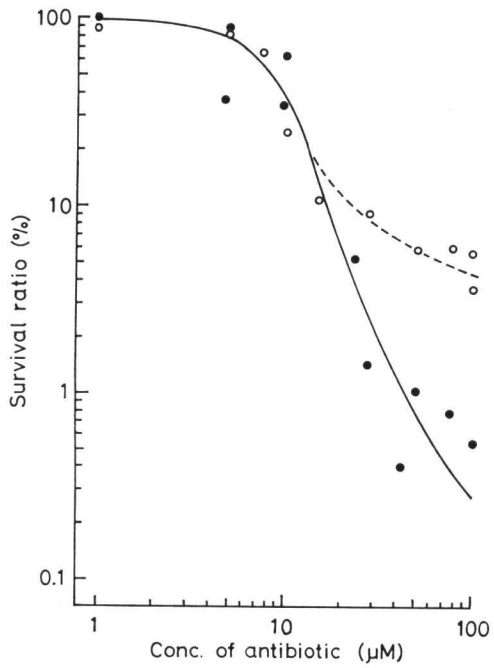


Table 1. Inhibition of DNA strand scission by catalase and superoxide dismutase*.

Antibiotic	Enzyme	Relative amount of ϕX174 RF I DNA (%)
MMC	—	43
	Catalase	90
	Superoxide dismutase	49
M-83	—	33
	Catalase	98
	Superoxide dismutase	21

* ϕX174 RF I DNA (0.2 μg) was reacted with 500 μM MMC or 50 μM M-83 in 50 mM tris-HCl buffer (pH 7.1) containing 100 μM $Na_2S_2O_4$ and 10 μM cupric ion in the absence of or in the presence of 10 μg/ml catalase or 20 μg/ml superoxide dismutase at 37°C for 1 hour.

reduced with dithiothreitol (Fig. 6B). M-83 reduced with 2-mercaptoethanol (Fig. 6C), NADH (Fig. 6D) or NADPH (Fig. 6E) caused DNA strand scission appreciably, but not MMC. These results suggest that M-83 is more readily reduced to an active form than MMC.

Fig. 4. Induction of single strand scission in PM2 ccc DNA by MMC (○) or M-83 (●) reduced with $NaBH_4$.
A: Drug-free control, B~I: MMC, J~Q: M-83. Concentration of MMC or M-83: B and J, 1 μM; C and K, 10 μM; D and L, 20 μM; E and M, 30 μM; F and N, 50 μM; G and P, 75 μM; H and Q, 100 μM; I, 500 μM.

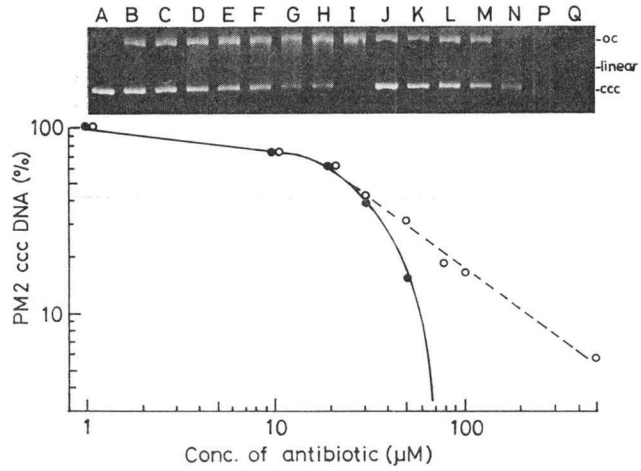


Fig. 5. Effect of enzymes, radical scavengers and EDTA on the induction of single strand scission in PM2 DNA.
A: Drug-free control. B: 0.5 mM $NaBH_4$ alone. C: 100 μM MMC, 0.5 mM $NaBH_4$. D: C+10 μg/ml catalase. E: C+25 μg/ml superoxide dismutase. F: C+4 mM Tiron. G: C+200 mM sodium benzoate. H: C+10 mM DABCO. I: C+10 mM EDTA. J: 50 μM M-83, 0.5 mM $NaBH_4$. K: J+10 μg/ml catalase. L: J+25 μg/ml superoxide dismutase. M: J+4 mM Tiron. N: J+200 mM sodium benzoate. P: J+10 mM DABCO. Q: J+10 mM EDTA.
DABCO: 2-Aminoethylisothiuroniumbromide·HBr.

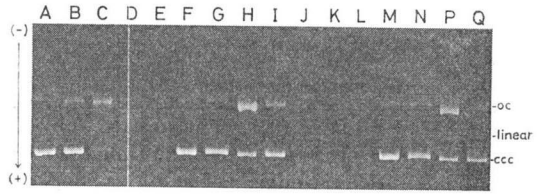
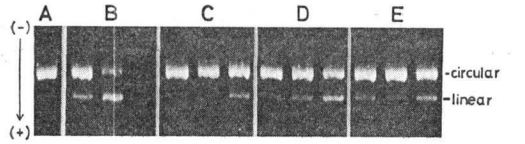


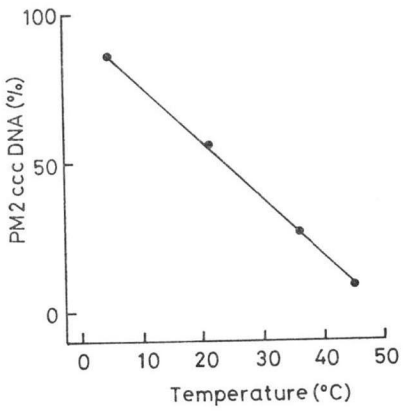
Fig. 6. Usefulness of reducing agent in the induction of DNA strand scission by M-83.
PM2 ccc DNA (0.17 μg) was reacted with 0.5 mM MMC or 0.5 mM M-83 reduced with 10 mM dithiothreitol (B), 0.1 mM 2-mercaptoethanol (C), 2 mM NADH (D) or 2 mM NADPH (E) in 50 mM tris-HCl buffer (pH 7.1) at 37°C for 1 hour. A: drug-free control. In B~E, left lanes: antibiotic-free control, middle lanes: MMC, right lanes: M-83.



Temperature Dependency of DNA Strand Scission

DNA strand scission by M-83 reduced with $NaBH_4$ was dependent on temperature, and was greatly depressed at 4°C (Fig. 7). Strand scission in φX174 SS DNA by reduced M-83 was also dependent on temperature (data not shown). Similar temperature dependency was also observed in DNA strand scission by reduced MMC (data not shown).

Fig. 7. Temperature dependency of DNA strand scission.
PM2 ccc DNA (0.17 μg) was reacted with 50 μM M-83 reduced with 500 μM $NaBH_4$ in 50 mM tris-HCl buffer (pH 7.1) at the indicated temperature for 1 hour.



Discussion

7-*N*-(*p*-Hydroxyphenyl)mitomycin C (M-83), a new derivative of MMC, possessed phage inactivation and DNA strand scission activities similar to those of MMC. M-83 was more active than MMC: M-83, at one third to one sixth concentration, showed the same degree of phage inactivation and DNA strand scission activities of MMC. The mechanism of phage inactivation and DNA strand scission by M-83 were similar to those by MMC: (1) Reduction of M-83 was requierd for its actions. (2) Oxygen radicals were involved, and metal ions possibly participated in the generation of these radicals. (3) The DNA strand scission was single strand and dependent on temperature. Oxygen radicals participate also in DNA strand scission by several antitumor antibiotics including bleomycin[18,19]. Metal ions are also associated with bleomycin-mediated DNA strand scission[19]. DNA strand scission by bleomycin in the absence of reducing agents is relatively insensitive to the change in reaction temperature from 4 to 60°C[20], however, in the presence of reducing agents DNA strand scission is dependent on temperature[21]. Reduction of M-83 or MMC to form an active intermediate may be dependent on temperature, and M-83 appears to be reduced to an active form more readily than MMC.

In the course of studies on phage inactivation and DNA strand scission activities of mitomycin derivatives, we have found that 7-aziridino and 7-anilino compounds show higher phage inactivation and DNA strand scission activities than MMC (unpublished results). M-83 belongs to the 7-anilino group. 7-Aziridino and 7-anilino compounds including M-83 may be readily reduced to an active form that binds to DNA, and generates oxygen radicals near the DNA molecule.

These results suggest that M-83 may be readily converted to an active form by reduction in tumor cells to induce DNA strand scission, and that the high antitumor activity of M-83 is due to high DNA strand scission activity.

Acknowledgement

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