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Effect of Catechol on the Photo-Induced Activation of Bleomycin

HIROMASA MORI

*Laboratory of Theoretical and Molecular Biology, Faculty of Pharmaceutical Sciences,
Kumamoto University, Ohe-Honmachi 5-1, Kumamoto 862, Japan*

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Bleomycin decreases the melting temperature (T_m) of deoxyribonucleic acid (DNA) determined by the optical density and viscosity methods and breaks the DNA strand in the presence of 1,2-benzenediol (catechol). The effect of bleomycin in the presence of catechol is markedly in acidic solution. However, in contrast with the case of sulfhydryl compounds such as 2-hydroxy-1-ethanethiol (2-mercaptoethanol), catechol inhibits the interaction of ultraviolet (UV)-irradiated bleomycin with DNA. These results show that catechol has two functions, *i.e.*, increasing the activity of non-irradiated bleomycin and inhibiting that of UV-irradiated bleomycin. Moreover, it was demonstrated that irradiation is not effective for enhancing the antibacterial activity of bleomycin.

Keywords—bleomycin; catechol; sulfhydryl compound; UV irradiation; DNA melting temperature; DNA strand breakage; fluorescence; antibacterial activity

Introduction

Bleomycin, an antitumor antibiotic isolated from *Streptomyces verticillus*,¹⁾ can interact directly with double-helical deoxyribonucleic acid (DNA) *in vitro*. It is well known that bleomycin decreases the melting temperature (T_m) of DNA,²⁾ releases free bases from DNA³⁻⁷⁾ and breaks the DNA strand(s)⁸⁻¹⁰⁾ in the presence of a reducing agent or ferrous ions, and that these abilities of bleomycin are enhanced by ultraviolet (UV) irradiation.^{11,12)} Recently, it has been shown that the activities of bleomycin for decreasing T_m of DNA and breaking the DNA strand were also enhanced by UV pre-irradiation of bleomycin solution in the presence of sulfhydryl compounds.¹³⁾ When UV pre-irradiation was carried out in 48 μ M bleomycin solution at pH 7.4, the maximum activity was obtained at a short irradiation time, *e.g.*, about 10 min. Such an irradiation dose causing the greatest enhancement of the DNA T_m -decreasing ability and the strand breakage activity was in agreement with that causing the greatest quenching at 350 nm in the transition of the fluorescence maximum from 350 to 400 nm.¹³⁾ Although it has been reported that a photo-induced change occurs at the bithiazole moiety,¹¹⁻¹⁴⁾ the mechanism of this activation has not been made clear.

It would be of interest to verify whether the enhancement of the activities of bleomycin by irradiation is a common feature *in vitro* and *in vivo* or not. In this study, the interactions of UV-irradiated and non-irradiated bleomycin with DNA were investigated by measuring T_m and the strand breakage of DNA in the presence of 1,2-benzenediol (catechol) for comparison with the case of sulfhydryl compounds. The photo-induced change of the antibacterial activity of bleomycin was also examined.

Materials and Methods

Materials—Bleomycin was obtained from Nippon Kayaku Co. Highly polymerized salmon sperm DNA and 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide) were obtained from Sigma Co. and

catechol, 2-hydroxy-1-ethanethiol (2-mercaptoethanol), DL-*threo*-1,4-dimercapto-2,3-butanediol (dithiothreitol) and other reagents from Wako Pure Chemical Industries Co. So-called reversible DNA, salmon sperm DNA treated with HNO_2 , was prepared by the method of Geiduschek.¹⁵ Reversible DNA having 90% reversibility was used to test the strand breakage of DNA caused by bleomycin. The materials for assaying the antibacterial activity were obtained from Eiken Chemical Co.

Preparation of UV-Irradiated Bleomycin for Spectrophotometric Analyses and for Action on DNA—A solution (3 ml) of 48 or 480 μM bleomycin in 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer of pH 3.0, 7.4 or 10.6 in a cuvette was irradiated through a mercury lamp (15 W) at a distance of 23 cm. Under these conditions, the irradiation dose was estimated to be 35 erg/mm²/s. Bleomycin solutions (48 μM) irradiated under pH 3.0 and 10.6 were neutralized to pH 7.4 by adding 1 N NaOH or 1 N HCl. UV absorption and fluorescence spectra were measured with a Hitachi EPS-3T spectrophotometer and an MPF-3 fluorescence spectrometer, respectively.

Examination of Thermal Melting Curves of DNA Treated with Bleomycin by Using Optical Density and Viscosity Methods—For measurements of optical density, the following reaction mixture was prepared; 50 $\mu\text{g}/\text{ml}$ salmon sperm DNA ($A_{260}=0.6$), 40 μM UV-irradiated or non-irradiated bleomycin (in the preparation of the UV-irradiated solution, the bleomycin concentration fell from an initial value of 48 to 40 μM) and 1 mM catechol or 1 mM 2-mercaptoethanol in 50 mM Tris-HCl and 1 mM KCl buffer of pH 7.4. In the absence of a reducing agent, the reaction mixture contained 400 μM UV-irradiated or non-irradiated bleomycin (in the preparation of the UV-irradiated solution, the bleomycin concentration fell from an initial value of 480 to 400 μM) in place of 40 μM bleomycin in the above-mentioned reaction mixture.

These reaction mixtures were incubated at 37°C for 2 h, and the optical density of each reaction mixture in a cuvette (1 cm light path) with a stopper was measured at 260 nm to obtain the thermal melting curve in the temperature range from 37 to 95°C; the temperature was raised by one degree every 2 min.¹³ The parameter, T_m , characterizing the melting transition corresponds to the temperature at which the rise in A_{260} was half complete.

In the viscosity method, 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA ($A_{260}=1.2$) was added instead of 50 $\mu\text{g}/\text{ml}$ DNA used for measuring the optical density. A reaction mixture incubated at 37°C for 2 h (2 ml) was placed in an Ostwald-type capillary viscometer and the relative viscosity was measured to analyze the thermal melting curve in the temperature range from 37 to 95°C; the temperature was raised by one degree every 3 min.¹³ The relative viscosity was obtained by using the following equation:

$$\text{relative viscosity} = \frac{\text{flow time (s) of reaction mixture}}{\text{flow time (s) of blank solution}}$$

The parameter, T_m , characterizing the melting transition corresponds to the temperature at which the decrease in relative viscosity was half complete.

Examination of DNA Strand Breakage Caused by Bleomycin—The effect of pH on the DNA strand breakage activity of bleomycin in the presence of catechol or dithiothreitol was examined by using the method of Morgan and Pulleyblank.¹⁶ The reaction mixture was prepared in the same way as that for measurement of optical density. Reversible DNA at a concentration of $A_{260}=1.0$ was used instead of 50 $\mu\text{g}/\text{ml}$ salmon sperm DNA. The pH of the reaction mixtures was adjusted to various values and then the mixtures were incubated at 37°C for 3.5 h, denatured in boiling water for 3 min and quenched immediately in ice. Finally, 500 $\mu\text{g}/\text{ml}$ ethidium bromide, dissolved in a solution of 20 mM K_2HPO_4 and 200 μM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 11.5, was immediately added to each reaction mixture. The fluorescence intensity of the final solution of pH 11.3 was measured at 590 nm, with excitation at 525 nm.^{13,16} Reversibility (%) was estimated by using the following equation:

$$\text{reversibility (\%)} = \frac{\text{fluorescence intensity of the reaction mixture treated with bleomycin}}{\text{fluorescence intensity of the reaction mixture not treated with bleomycin}}$$

Assay of Antibacterial Activity of Bleomycin—The effect of UV irradiation on the antibacterial activity of bleomycin was tested by using the cylinder assay. Three milliliters of 480 μM bleomycin solution (pH 7.4) for assaying the antibacterial activity was irradiated with UV light for 60 min and diluted with 50 mM Tris-HCl buffer of pH 7.4 to give the concentrations shown in Fig. 7. A suspension (1 ml) of *Escherichia coli* (K-12) having the optical density of 0.24 was seeded in 50 ml of medium containing 1.5% agar and was divided into 2.5 ml test tubes. These test tubes were stored overnight at 5°C, then a bleomycin solution (0.4 ml) was gently laid on the agar layer in the test tube and it was incubated at 37°C for 20 h. The length of the inhibitory zone of each test tube was measured.

Results

Table I shows the DNA T_m -decreasing ability of bleomycin in the presence of catechol or 2-mercaptoethanol obtained by the optical density method. Bleomycin decreased T_m of DNA at pH 7.4 in the presence of 1 mM catechol more effectively than in the presence of 1 mM 2-mercaptoethanol. Moreover, bleomycin also decreased T_m in the presence of other poly-

TABLE I. Effects of Reducing Agents on T_m of DNA Treated with $40\ \mu\text{M}$ Bleomycin

Reducing agent	T_m of DNA ($^{\circ}\text{C}$)
Reducing agent-free	80
1 mM Catechol	65
1 mM 2-Mercaptoethanol	74

Each reaction mixture, containing $50\ \mu\text{g}/\text{ml}$ salmon sperm DNA, $40\ \mu\text{M}$ bleomycin with 1 mM catechol or 1 mM 2-mercaptoethanol in 50 mM Tris-HCl and 1 mM KCl buffer of pH 7.4, was incubated at 37°C for 2 h and T_m was measured in terms of optical density.

Fig. 2. Effect of UV Irradiation Time of Bleomycin on T_m of DNA in the Presence of Catechol or 2-Mercaptoethanol, Measured in Terms of Optical Density, and Decrease in UV Absorbance of UV-Irradiated Bleomycin

The reaction mixture contained $50\ \mu\text{g}/\text{ml}$ salmon sperm DNA, $40\ \mu\text{M}$ UV-irradiated or non-irradiated bleomycin and 1 mM catechol or 1 mM 2-mercaptoethanol in 50 mM Tris-HCl and 1 mM KCl buffer of pH 7.4.

Left and right scales represent T_m in the presence of catechol (—○—) or 2-mercaptoethanol (—●—) and the residual absorbance (%) of UV-irradiated bleomycin at 293 nm (---○---), respectively.

Fig. 1. Plot of DNA Strand Breakage Activity versus pH Value for Bleomycin in the Presence of Catechol or Dithiothreitol

The reaction mixture contained reversible DNA at a concentration of $A_{260}=1.0$, $40\ \mu\text{M}$ bleomycin and 1 mM catechol or 1 mM dithiothreitol in 50 mM Tris-HCl and 1 mM KCl buffer of various pH values.

—○—, 1 mM catechol; —●—, 1 mM dithiothreitol.

Fig. 3. Thermal Melting Curves of DNA Treated with UV-Irradiated Bleomycin in the Presence of Catechol or 2-Mercaptoethanol, Measured in Terms of Viscosity

The reaction mixture contained $100\ \mu\text{g}/\text{ml}$ salmon sperm DNA, $400\ \mu\text{M}$ UV-irradiated or non-irradiated bleomycin and $100\ \mu\text{M}$ catechol or $100\ \mu\text{M}$ 2-mercaptoethanol in 50 mM Tris-HCl and 1 mM KCl buffer of pH 7.4.

(1) DNA, (2) DNA + non-irradiated bleomycin + catechol, (3) DNA + UV-irradiated bleomycin for 60 min + catechol, (4) DNA + non-irradiated bleomycin + 2-mercaptoethanol and (5) DNA + UV-irradiated bleomycin for 60 min + 2-mercaptoethanol.

phenols such as 1,3-benzenediol (resorcinol), 1,3,5-benzenetriol (phloroglucinol) and 1,2,3-benzenetriol (pyrogallol). Figure 1 shows that bleomycin also broke the DNA strands in the presence of catechol and that this activity decreased linearly with increase in pH value. On the other hand, bleomycin in the presence of dithiothreitol showed different features of DNA strand breakage; the breakage was especially enhanced in the pH range higher than pH 7.5 as compared with that with catechol.

Figure 2 shows the change of the DNA T_m -decreasing ability of bleomycin, treated with UV irradiation, in the presence of catechol or 2-mercaptoethanol, obtained in terms of optical density. The T_m of DNA in the presence of catechol gradually increased with increasing irradiation time, whereas the DNA T_m -decreasing ability in the presence of 2-mercaptoethanol was enhanced by irradiation and became maximum after irradiation for about 10 min, when about 50% decrease of the residual absorbance at 293 nm and the greatest quenching of the fluorescence maximum at 350 nm were seen.¹³⁾ As shown in Fig. 3, the inhibition by catechol was also observed in terms of viscosity. Although non-irradiated bleomycin remarkably decreased the relative viscosity of DNA at low temperature in the presence of 100 μ M catechol (solid line 2), the activity of UV-irradiated bleomycin was hampered in the presence of catechol (solid line 3). In the case of 2-mercaptoethanol, the relative viscosity of DNA treated with bleomycin was enhanced by UV irradiation (dotted lines 4 and 5).

Figure 4 shows the effect of UV irradiation on the DNA T_m -decreasing ability of bleomycin in the absence of a reducing agent, obtained by using the optical density method, and the change of absorbance of bleomycin at 293 nm on UV irradiation. Although T_m of DNA treated with or without non-irradiated bleomycin was 80 °C, T_m of DNA treated with UV-irradiated bleomycin decreased at irradiation times of less than 60 min, then increased at 5 h. The absorbance of bleomycin at 293 nm also decreased with irradiation time. Decreases of 10 °C in T_m and of about 50% in absorbance at 293 nm were observed after irradiation for 60 min. These findings agree with those in the presence of 2-mercaptoethanol, presented in the previous report.¹³⁾ The thermal melting curves of DNA treated with 400 μ M UV-irradiated or non-irradiated bleomycin, obtained by using the viscosity method, are shown in Fig. 5. Although non-irradiated bleomycin did not affect the relative viscosity of DNA at these temperatures, the addition of UV-irradiated bleomycin markedly diminished the relative viscosity at 37 °C and resulted in loss of the typical melting pattern of DNA in the range of 37 to 95 °C.

Fig. 4. Effect of UV Irradiation Time of Bleomycin on T_m of DNA in the Absence of a Reducing Agent, Measured in Terms of Optical Density, and Decrease in UV Absorbance of UV-Irradiated Bleomycin

The reaction mixture contained 50 μ g/ml salmon sperm DNA and 400 μ M UV-irradiated or non-irradiated bleomycin in 50 mM Tris-HCl and 1 mM KCl buffer of pH 7.4.

Left and right scales represent T_m (—○—) and the residual absorbance (%) of UV-irradiated bleomycin at 293 nm (---○---), respectively.

Fig. 5. Thermal Melting Curves of DNA Treated with UV-Irradiated Bleomycin in the Absence of a Reducing Agent, Measured in Terms of Viscosity

The reaction mixture contained 100 μ g/ml salmon sperm DNA and 400 μ M UV-irradiated or non-irradiated bleomycin in 50 mM Tris-HCl and 1 mM KCl buffer of pH 7.4.

(1) DNA, (2) DNA + non-irradiated bleomycin, (3) DNA + UV-irradiated bleomycin for 5 min, (4) DNA + UV-irradiated bleomycin for 10 min, (5) DNA + UV-irradiated bleomycin for 20 min and (6) DNA + UV-irradiated bleomycin for 60 min.

Fig. 6. Changes of Fluorescence of Bleomycin with UV Irradiation at Various pH Values Before (A) and After (B) Neutralization to pH 7.4

Bleomycin solutions ($48 \mu\text{M}$) of each pH value (10.6, 7.4 and 3.0) were irradiated with UV light for 15 or 60 min.
Excitation, 304 nm.

The effect of bleomycin, irradiated with UV light for 15 min at pH 3.0, 7.4 and 10.6 and then neutralized to pH 7.4, on T_m of DNA obtained by using the optical density method is summarized in Table II. The DNA T_m -decreasing ability of $40 \mu\text{M}$ bleomycin in the presence of 2-mercaptoethanol was enhanced in order of pH 10.6, 7.4 and 3.0. The features of quenching of the fluorescence of bleomycin by UV irradiation differed depending on the pH values as shown in Fig. 6. However, as the solutions of pH 10.6 and 3.0 were neutralized to pH 7.4 under reducing agent-free conditions, these different fluorescence spectra became similar to that at pH 7.4.

Figure 7 shows the dose dependence of the antibacterial activity of UV-irradiated and non-irradiated bleomycin obtained by using the cylinder assay. The bleomycin solution ($480 \mu\text{M}$) was irradiated for 60 min, which caused the greatest enhancement of the *in vitro* activity. Inhibition of the growth of *Escherichia coli* was not linearly dependent on the concentration of UV-irradiated bleomycin (solid line), whereas it was in the case of non-irradiated bleomycin (dotted line). Moreover, the lengths of the inhibitory zone of UV-irradiated bleomycin were shorter than in the case of non-irradiated bleomycin.

TABLE II. Effects of Bleomycin Irradiated at Various pH Values on T_m of DNA in the Presence of 2-Mercaptoethanol

pH (during irradiation)	T_m of DNA ($^{\circ}$ C)
3.0	56
7.4 (non-irradiation)	74
7.4	65
10.6	71

Bleomycin solutions ($48\ \mu\text{M}$) of pH 3.0, 7.4 and 10.6 were pre-irradiated for 15 min and neutralized to pH 7.4 by adding 1 N NaOH or 1 N HCl. Each reaction mixture contained $50\ \mu\text{g}/\text{ml}$ salmon sperm DNA, $40\ \mu\text{M}$ bleomycin with or without UV irradiation and 1 mM 2-mercaptoethanol in 50 mM Tris-HCl and 1 mM KCl buffer of pH 7.4.

Fig. 7. Change of Antibacterial Activity of Bleomycin towards *Escherichia coli* during UV Irradiation, Determined by Cylinder Assay

Bleomycin solutions ($480\ \mu\text{M}$) with or without UV irradiation for 60 min were diluted to the indicated concentration and the antibacterial activity of bleomycin was assayed by means of the cylinder assay.

---●---, non-irradiated bleomycin; —○—, UV-irradiated bleomycin.

Discussion

It is well known that bleomycin decreases T_m of DNA and breaks the DNA strand(s) in the presence of 2-mercaptoethanol, dithiothreitol, hydrogen peroxide or ascorbic acid. In addition to these reagents, it was found out that similar effects occur in the presence of catechol (Table I and Fig. 1). The observation that the optimal pH range for strand breakage by bleomycin in the presence of catechol (below pH 6.0) differed from that in the presence of dithiothreitol (above pH 7.5) suggests that the activity of bleomycin to break the DNA strand is mediated by the reducing power of these reagents. That is, bleomycin is active in the pH range from pH 5 to 9 which was employed in this experiment. The present result is consistent with the previous report.¹⁷⁾

Catechol inhibited the DNA T_m -decreasing ability of bleomycin activated by UV irradiation, whereas the activity of bleomycin for decreasing T_m without a reducing agent or with 2-mercaptoethanol was remarkably enhanced by UV irradiation (Figs. 2—5). Therefore, it is presumed that catechol has two functions, *i.e.*, increasing the activity of non-irradiated bleomycin in the same manner as other reducing agents, and inhibiting that of photochemically activated bleomycin. The activity of bleomycin to break the DNA strand in the presence of catechol was lost with increase of pH (Fig. 1). Further, the enhancement of the activity of bleomycin by UV irradiation was weak in alkaline solution (Table II). These observations suggest that inhibition by catechol may be caused by the formation of a quinoid form due to the liberation of protons (oxidized form). It has been proposed that $\text{Fe(II)} \cdot \text{bleomycin}$ changes an active Fe complex.¹⁸⁾ Therefore, it is presumed that a quinoid form of catechol may specifically inhibit the formation of active Fe·bleomycin or the activated site formed by the photo-induced change. However, the mechanism of this inactivation remains unclear.

It has been reported that the activities of bleomycin to break the DNA strand, to release free bases from DNA and to decrease T_m of DNA were enhanced by UV irradiation.^{11–13)} The photo-induced activation is accompanied with change of the fluorescence maximum from

350 to 400 nm due to the alteration of the bithiazole rings in the bleomycin molecule.^{12,13)} Recently, it was shown that 2,4'-bithiazole in the bleomycin molecule and a model compound were photo-transformed to 4,4'-bithiazole by UV irradiation.¹⁴⁾ Hence, it is conceivable that the photo-induced change of the bithiazole moiety may cause the enhancement of interaction between bleomycin and DNA. On the other hand, it is well known that bleomycin can be classified into two functional parts. That is, the bithiazole rings of the C-terminal intercalate into the base stacking of DNA, and the other part, the active site of the N-terminal, is able to break the DNA strand.¹⁹⁻²²⁾ Therefore, it is reasonable to consider that the function of the bithiazole rings might be enhanced by UV irradiation. In addition, it was noticed that although the alteration of fluorescence due to UV irradiation differed at pH 10.6, 7.4 and 3.0 and all the spectra became similar on neutralization to pH 7.4, the DNA T_m -decreasing ability of bleomycin irradiated at each pH was enhanced in order of pH 10.6, 7.4 and 3.0 (Table II and Fig. 6). These findings seem to show that the photo-induced change for activating bleomycin might also be caused at the other site.

As bleomycin is photo-sensitive, a change of *in vivo* activity of bleomycin on UV irradiation would be expected. As shown in Fig. 7, the antibacterial activity of bleomycin towards *Escherichia coli* declined upon UV irradiation. As it has been reported that an enhancement of the permeability of the cellular membrane, induced by dibucaine, affected the activity of bleomycin on cell growth,²³⁾ the decline of the antibacterial activity of bleomycin caused by UV irradiation may be a result of decreased permeability through the cellular membrane or due to an inhibitor such as catechol in the cell. However, the mechanism of photo-induced inactivation remains unclear. The present results show that bleomycin should be used under 'dark' conditions for maintenance of the antibacterial activity.

In conclusion, although bleomycin decreases T_m of DNA and breaks the DNA strand in the presence of catechol, the activity of photochemically activated bleomycin is inhibited by catechol. Further, the antibacterial activity of bleomycin is lessened by UV irradiation. These results suggest that, as UV irradiation may reduce the activity of bleomycin, precautions against exposure to light may be necessary during bleomycin treatment.

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