EFFECTS OF *o*-PHENANTHROLINE, 2,2′-DIPYRIDYL AND NEOCUPROINE ON THE ACTIVITIES OF BLEOMYCIN TO INHIBIT DNA SYNTHESIS AND GROWTH OF CULTURED CELLS

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Effects of *o*-phenanthroline, 2,2′-dipyridyl and neocuproine, which form stable complexes preferentially with Fe(II), Fe(II) and specifically with Cu(I), respectively, on the inhibitory activity of bleomycin against DNA synthesis of rat ascites hepatoma AH66 cells were examined. The inhibitory activity of metal-free bleomycin was suppressed in the presence of *o*-phenanthroline or 2,2′-dipyridyl, but not by neocuproine, though these chelating agents also showed the inhibitory activity against the DNA synthesis of the cells by themselves alone. The activity of bleomycin-Cu(II) was also suppressed by *o*-phenanthroline, but bleomycin-Fe(II) and bleomycin-Fe(III) exhibited some activities in the presence of *o*-phenanthroline. The growth inhibitory activity of bleomycin against HeLa cells was also suppressed by *o*-phenanthroline. From these results, bleomycin-iron complexes were suggested to be responsible to the bleomycin action in cells.

Bleomycin (BLM) is a group of glycopeptide anticancer antibiotics discovered by UMEZAWA *et al.* in the culture filtrate of *Streptomyces verticillus*[1]. BLM forms an equimolar metal-complex with various kinds of metal ions such as Fe(II), Fe(III), Cu(II), Co(II), Co(III), Zn(II), *etc.*[2]. Among BLM-metal complexes, BLM-Fe(II) and BLM-Fe(III) with reducing agents cause DNA strand break *in vitro* in the presence of oxygen[3~6]. BLM-Fe(III) with hydrogen peroxide also causes DNA strand break in the absence of oxygen[5]. BLM-Cu(II) does not cause DNA strand break *in vitro*[7,8], but cleaves DNA *in vivo*[9] and shows cytotoxicity[1,10~13]. In the previous papers, we proposed that an active intermediate formed in the reaction of BLM-Fe(II) with oxygen should be BLM-Fe(III)-O_2^{2-} (or BLM-Fe(III)-O_2H^-)[5]. We also observed that the Cu(II) of BLM-Cu(II) is reductively removed in cells to yield metal-free BLM and the liberated Cu(I) is trapped by cellular thiol-proteins[9]. The metal-free BLM thus produced in cells appears to exhibit the cytotoxicity after binding with Fe(II). In recent years, BLM-Cu(I) plus oxygen[14], BLM-Mn(II) plus oxygen[15] or hydrogen peroxide[16,17], BLM-VO(IV) plus hydrogen peroxide[18] and BLM-Co(II) plus UV light[19] were reported to cause DNA strand break *in vitro*. Therefore, it is of importance to prove what kind of BLM-metal complex is responsible to the BLM action in cells. In this regard, LIN *et al.*[20] examined the effects of chelating agents on the cytotoxicity of BLM, and concluded that iron plays little role in the BLM cytotoxicity because deferoxamine, a specific chelating agent of Fe(III), caused no or slight suppression of the cytotoxicity. *o*-Phenanthroline and 2,2′-dipyridyl, which form complexes preferentially with Fe(II), have been known to permeate into cells in the study of iron metabolism in cells[21]. In the present study, effects of these chelating agents and neocuproine, a specific chelating agent of Cu(I), on the activities of BLM to inhibit DNA synthesis and growth of cultured cells were examined.

Fig. 1. Effect of *o*-phenanthroline on the inhibitory activity of metal-free BLM A_2 against DNA synthesis of AH66 cells.

Each point is a mean value of triplicate determinations throughout Figs. 1 to 5.

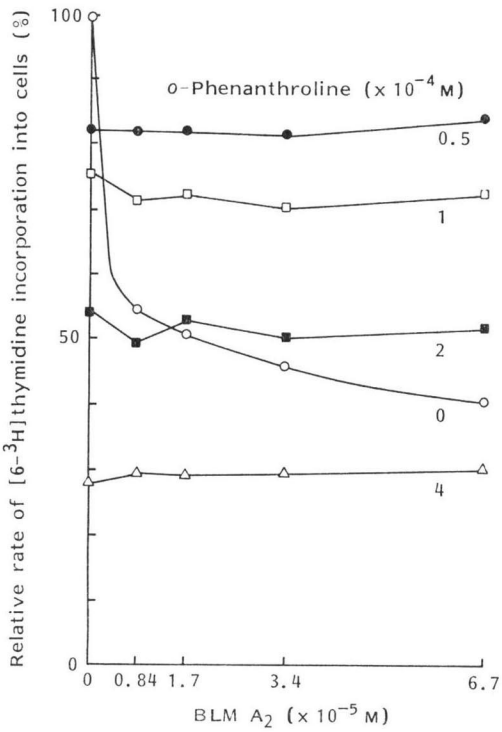
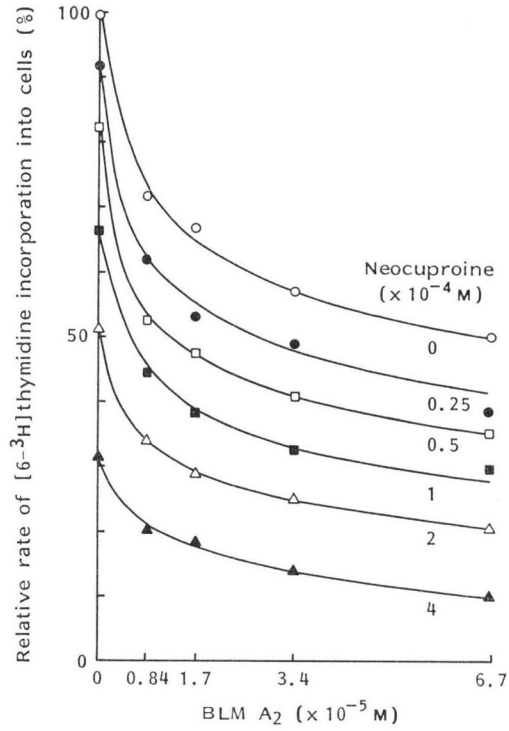


Fig. 2. Effect of neocuproine on the inhibitory activity of metal-free BLM A_2 agaisnt DNA synthesis of AH66 cells.



Materials and Methods

Chemicals

BLM A_2, which is a major component of the clinically used BLM, was used throughout the experiments. Metal-free BLM A_2 and BLM A_2-Cu(II) were prepared in Nippon Kayaku Co., Ltd., Tokyo, Japan. BLM-Fe(II) and BLM-Fe(III) were prepared by mixing equimolar aqueous solutions of metal-free BLM and ferrous sulfate or ferric chloride just prior to use. *o*-Phenanthroline, 2,2′-dipyridyl and neocuproine were purchased from Nakarai Chemicals Ltd., Kyoto, Japan. [6-^{3}H]Thymidine (5 Ci/mmol) was purchased from The Radio Chemical Centre, Amersham, U.K. These chemicals were dissolved or diluted with the culture medium described below unless otherwise noted.

Determination of Inhibition of DNA Synthesis

Rat ascites hepatoma AH66 cells were taken from the peritoneal cavity of the implanted rats with an injection syringe and washed 3 times by centrifugal separation with chilled saline. The 1×10^6 cells were suspended in 1.7 ml of EAGLE's MEM medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% calf serum (Res. Inst. Microb., Osaka Univ., Osaka, Japan) and pre-incubated at 37°C for 30 minutes. The pre-incubated cell suspension was mixed with 0.05 ml of BLM solution and 0.2 ml of chelator solution and incubated for 30 minutes at 37°C. After the incubation, 0.05 ml of [^{3}H]thymidine solution (1 μCi) was added to the mixture followed by further incubation for 30 minutes. The incorporation of [^{3}H]thymidine into DNA was terminated by addition of 2 ml of chilled 10% trichloroacetic acid solution. The precipitate obtained by centrifugation at 1,500 rpm for 5 minutes was washed twice with 5 ml of MeOH by centrifugal separation at 3,000 rpm for 5 minutes, solubilized with 0.7 ml of Hyamine-10X, and mixed with 9 ml of toluene scintillation fluid. The radio-

Fig. 3. Effect of 2,2′-dipyridyl on the inhibitory activity of metal-free BLM A_2 against DNA synthesis of AH66 cells.

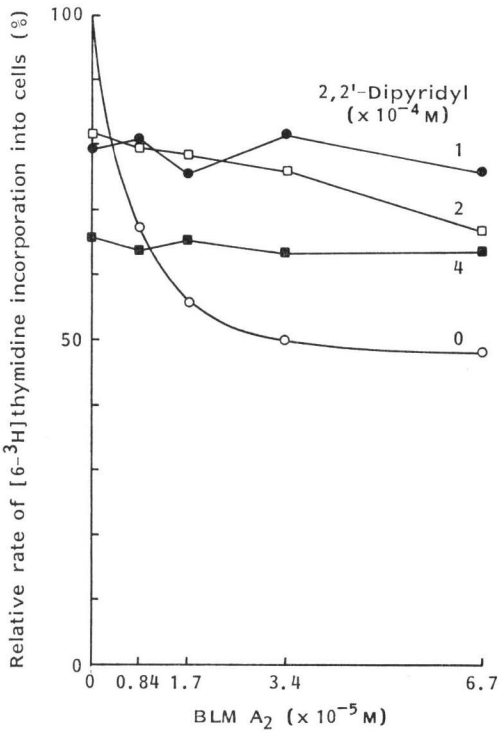
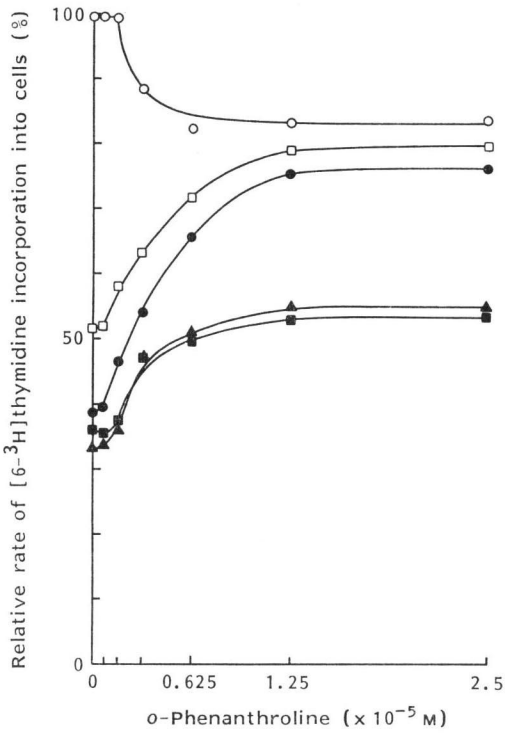


Fig. 4. Effect of *o*-phenanthroline on the inhibitory activities of metal-free BLM A_2, BLM A_2-Cu(II), BLM A_2-Fe(II) and BLM A_2-Fe(III) agaisnt DNA synthesis of AH66 cells.

BLM-Fe(II) and BLM-Fe(III) were prepared by mixing aqueous solutions of BLM and ferrous sulfate or ferric chloride as already described. BLM and BLM-Cu(II) were also dissolved in deionized water in this experiment. The aqueous solutions of the BLMs were added to the culture medium as described in Materials and Methods. ○; Control, ●; BLM A_2 (6.7×10^{-5} M), □; BLM A_2-Cu(II) (6.7×10^{-5} M), ■; BLM A_2-Fe(II) (6.7×10^{-5} M), ▲; BLM A_2-Fe(III) (6.7×10^{-5} M).



activity was determined with a Packard model Tri-Carb 3380 liquid scintillation counter.

Determination of Growth Inhibition of HeLa S_3 Cells

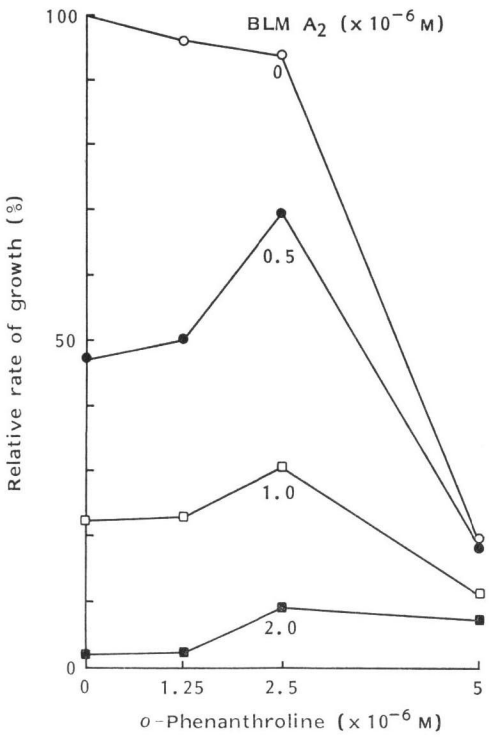
HeLa S_3 cells suspended in the above-described medium with the initial density of 2×10^4 cells/2 ml/plate were cultured at 37°C for 48 hours in a 5% CO_2 incubator. The medium was exchanged by 2 ml of the fresh medium containing various concentrations of BLM and *o*-phenanthroline and incubated further for 72 hours. The cell numbers before and after the drug treatment were counted with a Coulter Counter model ZB1.

Results

The inhibition of DNA synthesis of rat ascites hepatoma AH66 cells by metal-free BLM was examined in the presence or absence of *o*-phenanthroline. The result is shown in Fig. 1. Metal-free BLM markedly inhibited DNA synthesis of the cells in the absence of *o*-phenanthroline. *o*-Phenanthroline also inhibited the DNA synthesis in the absence of BLM. However, in the presence of *o*-phenanthroline at 0.5×10^{-4} M, the inhibitory activity of BLM was almost completely suppressed, while the inhibition caused by *o*-phenanthroline was not influenced by the presence of BLM at 6.7×10^{-5} M.

A similar experiment was performed using neocuproine, which is a dimethylated analog of

Fig. 5. Effect of *o*-phenanthroline on the inhibitory activity of metal-free BLM A_2 against growth of HeLa S_3 cells.



o-phenanthroline and a specific chelating agent of Cu(I). As shown in Fig. 2, neocuproine itself inhibited the DNA synthesis similar to *o*-phenanthroline, but did not suppressed the inhibitory activity of metal-free BLM. However, 2,2′-dipyridyl, which preferentially forms a stable complex with Fe(II) similar to *o*-phenanthroline, suppressed the inhibitory activity of metal-free BLM in a similar manner to *o*-phenanthroline (Fig. 3).

The inhibitory activities of metal-free BLM, BLM-Cu(II), BLM-Fe(II) and BLM-Fe(III) were examined in the presence of further lower concentration of *o*-phenanthroline. As shown in Fig. 4, the inhibitory activities of metal-free BLM and BLM-Cu(II) were suppressed with increase of *o*-phenanthroline concentration and almost completely suppressed at the concentration of 1.25×10^{-5} M. On the other hand, the inhibitory activities of BLM-Fe(II) and BLM-Fe(III) were not suppressed substantially at 2.5×10^{-5} M.

Metal-free BLM inhibited the growth of HeLa cells dose-dependently in the absence of *o*-phenanthroline (Fig. 5). *o*-Phenanthroline suppressed the growth inhibitory activity of BLM, though *o*-phenanthroline itself also inhibited the growth of the cells significantly at 5×10^{-6} M. The growth inhibitory activity of metal-free BLM was most significantly suppressed in the presence of *o*-phenanthroline at 2.5×10^{-6} M.

Discussion

In the present study, *o*-phenanthroline, 2,2′-dipyridyl and neocuproine showed dose-dependent inhibition of DNA synthesis of AH66 cells, and *o*-phenanthroline inhibited growth of HeLa cells. These chelating agents have been known to show antitumor[22] and bacteriostatic[23] effects, and *o*-phenanthroline was reported to inhibit DNA synthesis of mammalian cells[24]. Besides the effects shown by these chelating agents themselves, the suppressive effects on the BLM activities were shown by *o*-phenanthroline and 2,2′-dipyridyl at the low concentrations (for example; *o*-phenanthroline 0.5×10^{-4} M, 2,2′-dipyridyl 1×10^{-4} M), where the effects of the agents themselves were not so strong. *o*-Phenanthroline suppressed the activities of metal-free BLM to inhibit DNA synthesis of AH66 cells and growth of HeLa cells. Metal-free BLM has been known to cause inhibition of DNA synthesis[11] as well as DNA strand break in cells[25]. The inhibition of DNA synthesis is thought to be caused by the DNA strand break, because the activities of BLM analogs to inhibit DNA synthesis and growth of cultured cells are well correlated with those of the BLMs to cleave DNA *in vitro*[26]. Therefore, the above-described results suggest that metal-free BLM cleaves DNA, inhibits DNA synthesis and exhibits cytotoxicity after binding some metal ion present in the cells which is trapped by *o*-phenanthroline and 2,2′-dipyridyl. The activity of BLM-Cu(II) to inhibit DNA synthesis was also suppressed by *o*-phenanthroline. This result suggests that BLM-Cu(II) exhibits the cytotoxicity

after the following process: The Cu(II) of BLM-Cu(II) is reductively removed to yield metal-free BLM and the liberated Cu(I) is trapped by thiol-proteins in cells as reported previously[9].

As already described, BLM-Fe(II)[3~5], BLM-Fe(III)[5,6], BLM-Cu(I)[14], BLM-Mn(II)[15~17], BLM-VO(IV)[18] and BLM-Co(II)[19] have been reported to show DNA-cleaving activity *in vitro*. *o*-Phenanthroline and 2,2′-dipyridyl form complexes preferentially with Fe(II), but they also form the complexes with other metal ions[27]. Among the BLM-metal complexes, BLM-Co(II) requires UV light to cleave DNA, and shows no cytotoxicity and no antitumor activity[13]. In regard to the activity of BLM-Cu(I), SUZUKI *et al.*[28] re-examined the activity under the similar experimental conditions to the previous paper[14], and they concluded that BLM-Cu(I) had not the DNA-cleaving activity, because the observed DNA cleavage was inhibited by deferoxamine, a specific chelating agent of Fe(III), but not by vasocuproine, a specific chelating agent of Cu(I). In the present study, neocuproine, another specific chelating agent of Cu(I), did not show suppressive effect on the metal-free BLM activity to inhibit the DNA synthesis. This result suggests that BLM-Cu(I) is not an active metal complex of BLM in cells.

BLM-Fe(II) and BLM-Fe(III) were found to exhibit the inhibitory activities against DNA synthesis in the presence of *o*-phenanthroline, while metal-free BLM and BLM-Cu(II) did not. This result suggests that among BLM-metal complexes BLM-iron complexes seem to be responsible to the BLM action in cells. DNA-cleaving activities of BLM-Mn(II) plus oxygen[15], BLM-Mn(II) plus hydrogen peroxide[16] and BLM-VO(IV) plus hydrogen peroxide[18] were reported to be about 10-, 30- to 100- and 50-folds weaker than the iron complex, respectively. Tissue levels of manganese[29] and vanadium[30] are known to be much lower than that of iron. *o*-Phenanthroline and 2,2′-dipyridyl have much higher affinity to Fe(II) compared to Mn(II)[27]. These data support that BLM-iron complexes take part in the exhibition of the cytotoxicity.

The BLM-iron complexes showed the activities in the presence of *o*-phenanthroline, although the activities were slightly suppressed. This suppression is thought to be due to the partial transformation to metal-free BLM by *o*-phenanthroline.

From the results presented in this paper, BLM-iron complexes are supported to be the active complexes of BLM to exhibit cytotoxicity.

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