CELL KILLING MODE OF LIBLOMYCIN (NK313), A NOVEL DOSE-SURVIVAL RELATIONSHIP DIFFERENT FROM BLEOMYCINS

AKIKO KURAMOCHI-MOTEGI, HIROSHI KURAMOCHI, KATSUTOSHI TAKAHASHI and TOMIO TAKEUCHI†

Research Laboratories, Pharmaceuticals Group, Nippon Kayaku Co., Ltd.,
3-31-12 Shimo, Kita-ku, Tokyo 115, Japan
†Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication September 13, 1990)

Liblomycin (NK313) is a novel derivative of bleomycin (BLM) and peplomycin (PEP). The cell kill kinetics of NK313 on rat ascites hepatoma AH66 were compared with those of PEP. NK313 induced intracellular DNA cleavage and arrested cell cycle progression at the G_2 phase similarly to PEP. The cytocidal effect of NK313, however, was found to be different from that of PEP as described below: 1) The dose-survival curve for cells exposed to PEP for 1 hour was upward concave, whereas in case of NK313, the survival curve was linear. PEP was more effective to AH66 than NK313 at lower concentration, but at higher concentration, NK313 was much more effective. 2) The time-survival curve for cells treated with either NK313 or PEP was biphasic. NK313, however, did not induce temporary resistance of AH66 cells to NK313, while PEP induced resistance to PEP. 3) NK313 was effective against the cells which became temporarily resistant to PEP by the treatment of PEP. These differences suggest that NK313 might be of value to treat PEP-insensitive tumor cells.

Since the discovery of bleomycin (BLM) from *Streptomyces verticillus* by UMEZAWA in 1966[1], BLM has been used widely in cancer chemotherapy. However, its use is limited by pulmonary toxicity. Many analogs were screened for stronger antitumor activity and less pulmonary toxicity and peplomycin (PEP) has been developed as a second generation of BLM[2,3]. PEP has been also used clinically since 1981, but pulmonary toxicity still remains the dose-limiting factor. Liblomycin (NK313) is a novel analog of BLM selected in a new screening program[4,5]. NK313 showed a different antitumor spectrum from that of PEP and BLM, and exhibited activity against PEP-unresponsive tumors[6]. Pulmonary toxicity of NK313 was much lower than that of BLM and PEP in mice and dogs[5,7]. In the present study, using BLM sensitive rat ascites hepatoma AH66 cells, we examined *in vitro* actions of NK313 and PEP, including as the cell-killing modes, the effect on cell cycle progression, and the intracellular DNA breakage. We found that NK313 has a quite different cell-killing mode from PEP, although it cleaves intracellular DNA strands similarly to PEP.

Materials and Methods

Chemicals

NK313, [^{3}H]NK313 and PEP were prepared by Nippon Kayaku Co., Ltd., Tokyo, Japan. EAGLE's minimal essential medium for suspension culture (MEM) and RPMI1640 medium were purchased from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan and Gibco, Santa Clara, U.S.A., respectively. Fetal calf serum (FCS) was obtained from Hyclone Laboratories (a division of Sterile Systems, Inc.) Utah, U.S.A., and agar noble was purchased from Difco Laboratories, Detroit, U.S.A. Sodium *N*-lauroylsarcosinate and tetra-*n*-propylammonium hydroxide were purchased from Wako Pure Chemical Industries Ltd., Tokyo, Japan. Nitrocellulose filter and [^{14}C]thymidine for alkaline elution was from Millipore Co., and Amersham Japan Co., Tokyo, Japan, respectively. Propidium iodide was purchased from Sigma Chemical Co., Saint Louis, U.S.A.

Cell Preparation

Rat ascites hepatoma AH66 was maintained by intraperitoneal passage of ascites in Donryu rats. For experiments, the cells were obtained from the peritoneal cavity 5 or 6 days after tumor inoculation. The cells were suspended in EAGLE's MEM and incubated for 2 hours in a 5% CO_2 incubator at 37°C before each experiment.

Colony Formation

AH66 cells were suspended at a density of 2×10^5 cells/ml in the MEM supplemented with 10% FCS, cultured with NK313 or PEP at 37°C for the indicated time in a 5% CO_2 incubator, and centrifuged for 5 minutes at $170 \times g$ at room temperature. The cell pellets were resuspended in RPMI1640 medium containing 10% FCS, 50 μM 2-mercaptoethanol and 60 μg/ml kanamycin, and washed with the medium and then centrifuged twice. The cells were diluted with the RPMI1640 medium successively and incubated in soft agar for colony formation as described previously[8]. Briefly, 1 ml of the cell suspension was mixed with 3 ml of RPMI1640 medium containing 0.133% agar in a Falcon 2054 tube, and the cells were incubated for 12~14 days in a 5% CO_2 incubator at 37°C. The colonies formed in soft agar were counted and the survival fraction was calculated.

Alkaline Elution

AH66 cells were suspended at a density of 10^5 cells/ml in MEM as described above, and exposed to 0.1 μCi of [^{14}C]thymidine at 37°C. The labeled cells were washed twice with the medium and incubated at 37°C in the presence of NK313 or PEP for 1 hour. After the incubation, the cells were washed twice with PBS (−), and subjected to alkaline elution to examine DNA cleavage. The elution was performed according to KOHN[9].

Cell Cycle Progression

To 6.9 ml of cell suspension containing 5×10^5 cells in MEM, 0.1 ml of NK313 or PEP solution was added. After incubation for 24 hours at 37°C in a CO_2 incubator, 0.5 ml of 2% EDTA solution was added to the suspension and the cells collected by centrifugation were fixed with cold 70% ethanol. The cellular DNA was stained with 0.05 mg propidium iodide dissolved in 1 ml of 1.12% sodium citrate[10], and DNA distribution in cell cycle phases was analyzed by EPICS V.

Results

Dose-dependency of Cell Killing Effect

The survival curves of AH66 cells as functions of PEP and NK313 concentrations are shown in Fig. 1. In case of PEP, the survival curves for cells were biphasic and upward concave similar to BLM. The curves for AH66 cells bent around 2.5 μM PEP, where 90% of the cells were killed. On the other hand, the survival curve for cells treated with NK313 were linear. NK313 killed 90% of AH66 cell at 3 μM.

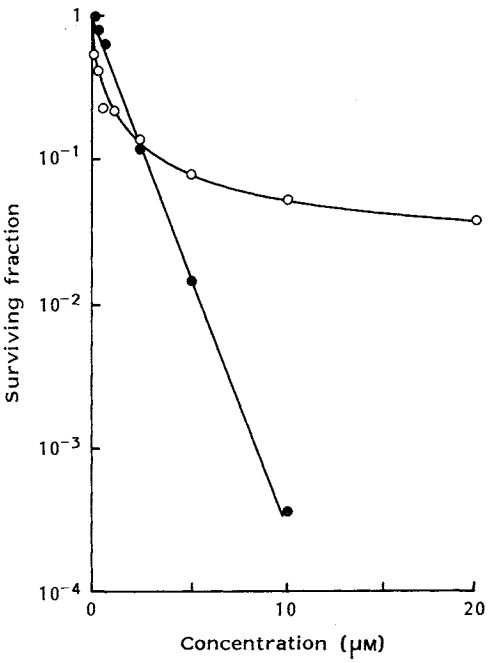
Time-dependency of Cell Killing Effect

Fig. 2 shows the survival curves of AH66 cells as functions of drug exposure time. The drug concentration was fixed at 2.5 μM, because NK313 and PEP gave similar surviving fractions after treatment at this concentration for 1 hour. In contrast to dose-response curves, PEP and NK313 exhibited similar time-survival patterns with upward concavity. Surviving fractions decreased greatly for the first 1 hour treatment, but thereafter they became almost flattened.

TERASHIMA *et al.*[11] showed that cells exposed to BLM acquired temporary resistance to the drug. They suggested that induction of resistance was the cause of the observed biphasic time-response curve. AH66 cells were incubated with NK313 or PEP for 2 hours, washed once to remove the drug, and again incubated with the drug at the same concentration. For comparison, the cells were incubated without

Fig. 1. Dose-response curve for AH66 cells treated with NK313 and PEP.

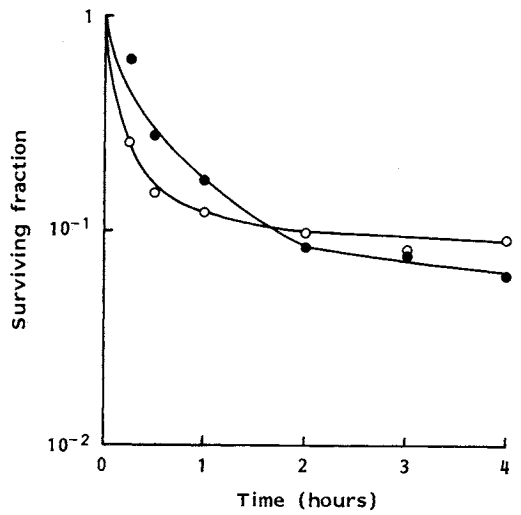
AH66 cells were treated with NK313 (●) and PEP (○) for 1 hour.



The condition for drug treatment and colony formation were performed as described in Materials and Methods. After incubation for 12~14 days, the numbers of colonies in soft agar were counted. Surviving fraction was calculated by dividing the plating efficiency for cells treated with drug by that of control cells.

Fig. 2. Exposure time-survival relationships of NK313 and PEP.

AH66 cells were treated with 2.5 μM NK313 (●) or PEP (○).



Materials and Methods and calculation of surviving fraction were described in legend of Fig. 1.

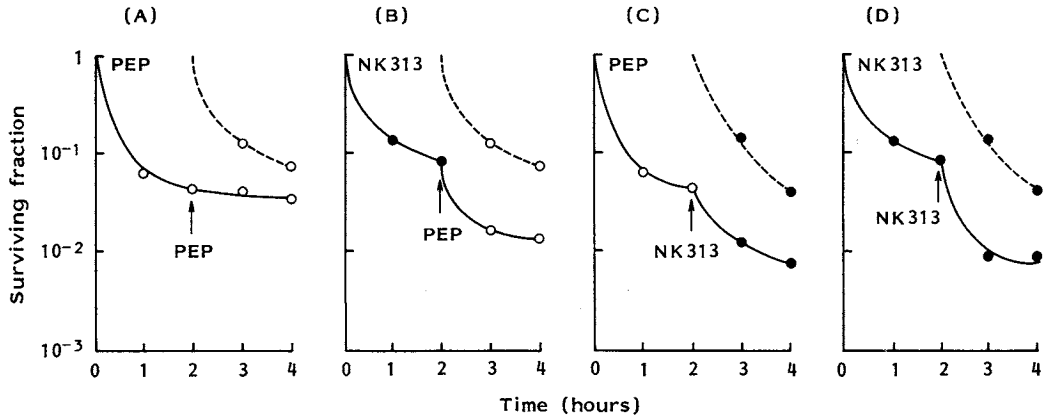
drugs for 2 hours and thereafter treated as described above. In the case of PEP, the surviving fraction did not decrease by the second drug treatment, while the cells preincubated for 2 hours in the absence of PEP responded to the drug treatment (Fig. 3A). In contrast, in the case of NK313, the surviving fraction decreased by the second drug treatment (Fig. 3D). Furthermore, when the cells pretreated with PEP were treated with NK313, cell survival decreased (Fig. 3C). Also in the reverse order, PEP treatment to the cells pretreated with NK313 exhibited cytotoxicity (Fig. 3B). These results demonstrated that unlike PEP, NK313 did not induce temporary resistance in AH66 cells and the temporary resistance induced by PEP did not exhibit cross-resistance to NK313. Consequently, the biphasic time-survival curve of NK313 can not be explained by induction of temporary resistance. Furthermore, the biphasic curve was not explained by stability of the drug in medium and/or release of some factors which inactivate the drug (data not shown).

Intracellular DNA Cleavage

BLM cleaves intracellular DNA strands to cause cytocidal effect[12,13]. The ability of NK313 to cleave intracellular DNA of AH66 cells was compared with that of PEP using alkaline elution method. As shown in Fig. 4, both NK313 and PEP cleaved intracellular DNA strands dose-dependently. At the lowest concentration of the drugs, the activity of PEP was slightly higher than that of NK313, while at higher concentrations, the activity of NK313 was much greater than that of PEP. This dose-response relationship is consistent with the above dose-survival relationship. These results suggest that NK313 exhibits its cytocidal effect by cleavage of intracellular DNA strands similarly to PEP.

Fig. 3. Change of sensitivity of AH66 to NK313 and PEP after treatment with NK313 and PEP.

A and C: After cells were treated with 2.5 μM PEP for 2 hours (○) in EAGLE's MEM, cells were washed once with medium for colony formation and were treated with equal concentration of PEP (○) or NK313 (●). Broken line presents the survival curve for cells those were incubated in medium without drug and then treated with PEP (○) or NK313 (●). B and D: Cells were incubated with NK313 (●) for 2 hours and then treated as described above. The procedure after washing was identical as described in A and C.

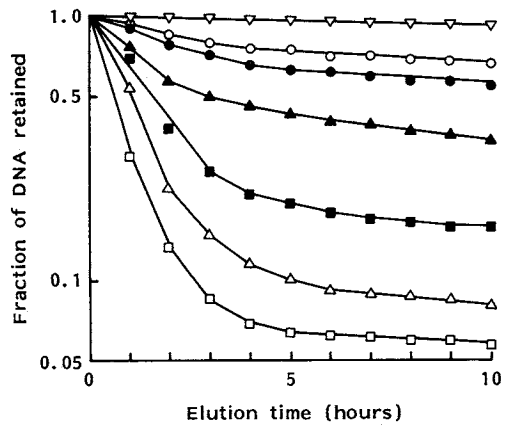


Effect on Cell Cycle Progression

AH66 cells were treated with NK313 or PEP for 24 hours, and DNA distribution in cell cycle phase was examined with flow cytometry. As shown in Fig. 5, G_1 peak decreased and G_2+M increased with the concentration of NK313 and PEP. At this time, increase of the mitotic index was not observed (data not shown). This result shows that NK313 arrests cell cycle progression at the G_2 phase similarly to PEP.

Fig. 4. Cleavage of intracellular DNA strands of AH66 by NK313 and PEP.

▽ Control. AH66 cells were treated with NK313 (open symbols) or PEP (closed symbols) at the concentration of 2.5 (○, ●), 10 (△, ▲) and 40 (□, ■) μM.



Discussion

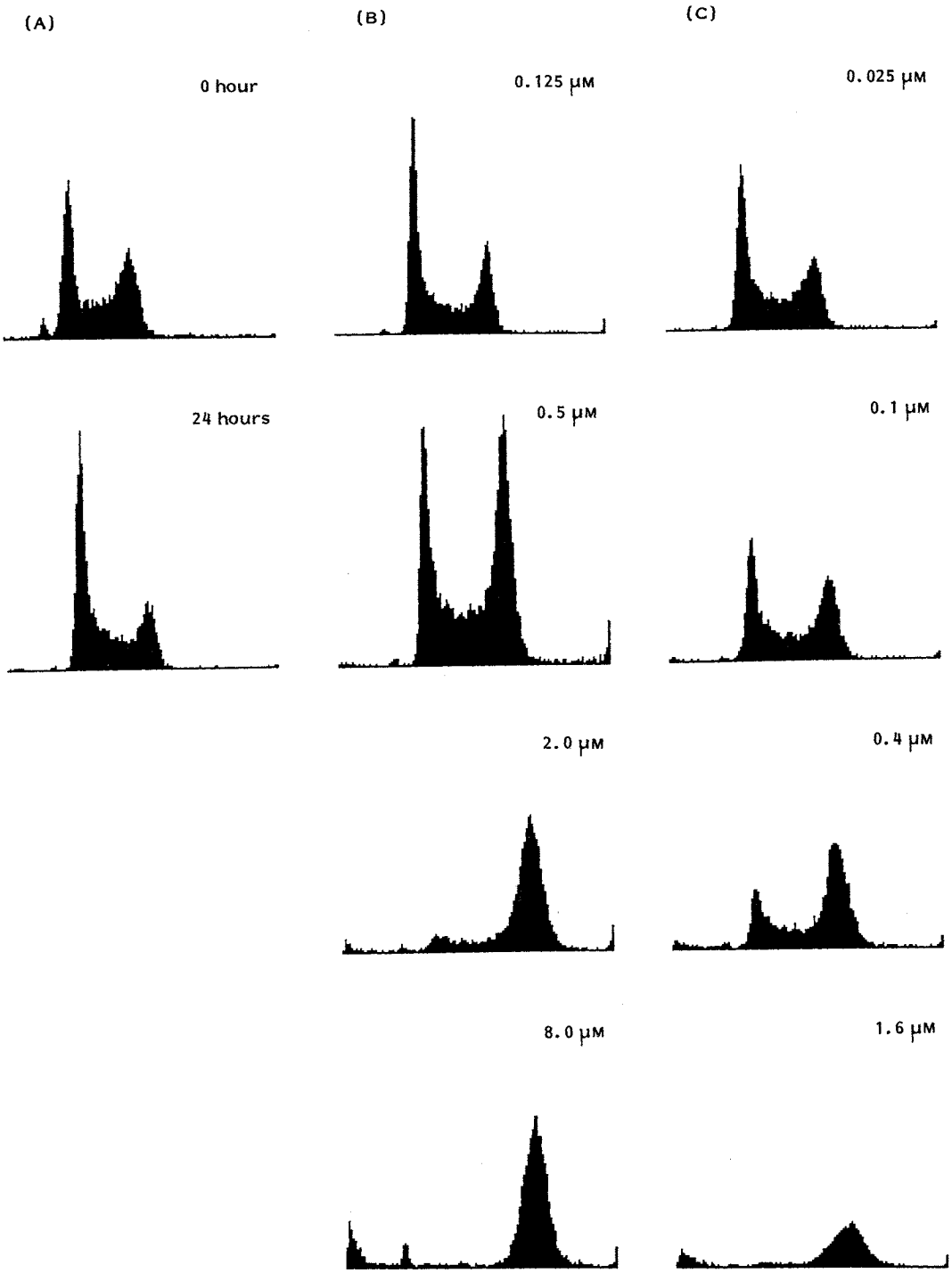
In the present study, it was shown that NK313 cleaved intracellular DNA strands, arrested cell cycle progression at G_2 phase, and killed tumor cells. These results show that NK313 exhibits activity by similar mechanisms to PEP and BLM. However, NK313 was found to be distinguishable from PEP in activity and cell-killing modes.

A time-survival curve of NK313 for AH66 cells was biphasic similar to that of PEP (Fig. 2). MIYAMOTO *et al.*[14] and SAITO *et al.*[15] observed that survival of the cells treated with PEP or BLM decreased steeply within the first 10 minutes, recovered quickly within the next 10 minutes and again decreased slowly. They concluded that a repair system was newly induced due to the DNA damage by the drugs, and suggested that the newly induced repair activity was the cause of biphasic cell-killing pattern and temporary resistance of tumor cells.

As reported by TERASHIMA *et al.*[11], the cells pretreated with PEP for 2 hours became temporarily resistant to the second treatment by freshly prepared medium containing PEP, but NK313 did not induce temporary resistance to NK313. This indicates that the biphasic time-survival curve of NK313 is not due

Fig. 5. Effects of NK313 and PEP on cell cycle progression.

(A) Control, (B) NK313, (C) PEP. DNA distribution pattern is shown in A and B.



AH66 cells were treated with no drug for 0 or 24 hours. DNA distribution patterns were obtained by treatment with 0.125, 0.5, 2.0 and 8.0 μM NK313 for 24 hours. And DNA distribution patterns in right column are obtained after 24 hours incubation with 0.025, 0.1, 0.4 and 1.6 μM PEP.

to the induction of resistance, therefore its mechanism is different from that of PEP. Furthermore, this was not the result of inactivation of NK313 in culture medium (data not shown) or the decrease of its uptake, because cellular uptake of NK313 was shown to increase time-dependently as well as that of PEP[16] (data not shown).

Different from the biphasic dose-survival curve of PEP, the curve of NK313 for AH66 cells was linear. Since all the curves of PEP and BLM for various tumor cell lines reported to be biphasic, this linearity of dose-response is specific character to NK313. This character of NK313 may be related to the disappearance of the induction of temporary resistance. Further, it is conceivable that lack of induction of temporary resistance by NK313 is the cause of effectiveness of the drug for BLM-unresponsive tumor cell lines. Therefore, the aquisition of linearity of dose-response may be considered to have led NK313 to the extension of the antitumor spectrum.

In the present study, it was clarified that cell killing modes of NK313 were distinct from those of BLM and PEP.

As we reported previously[17], NK313-Fe(II) complex cleaved isolated DNA as PEP did. Therefore it is interesting to know why NK313 exhibited different cell-killing mode from PEP and BLM. On the other hand DNA cleavage activity of NK313 to isolated DNA was lower than that of PEP, while the activity of NK313 to intracellular DNA was higher than that of PEP. NK313 has a bulky lipophilic group at the terminal amine moiety, but other structure including ligands for Fe(II) chelation is common to PEP. The role of the lipophilic group in the different characteristics of NK313 from PEP is now under further study.

References

1) UMEZAWA, H.; K. MAEDA, T. TAKEUCHI & Y. OKAMI: New antibiotics, bleomycin A and B. J. Antibiotics, Ser. A 19: 200~209, 1966
2) TAKAHASHI, K.; H. EKIMOTO, S. AOYAGI, A. KOYU, H. KURAMOCHI, O. YOSHIOKA, A. MATSUDA, A. FUJII & H. UMEZAWA: Biological studies of the degradation products of 3-[(S)-1'-phenylethylamino]propylaminobleomycin: A novel analog (pepleomycin). J. Antibiotics 32: 36~42, 1979
3) EBIHARA, K.; H. EKIMOTO, Y. ITCHODA, F. ABE, H. INOUE, S. AOYAGI, T. YAMASHITA, A. KOYU, K. TAKAHASHI, O. YOSHIOKA & A. MATSUDA: Studies on antitumor activities and pulmonary toxicity of pepleomycin sulfate (NK631). Jpn. J. Antibiotics 31: 872~885, 1978
4) UMEZAWA, H.; T. TAKITA, S. SAITO, Y. MURAOKA, K. TAKAHASHI, H. EKIMOTO, S. MINAMIDE, K. NISHIKAWA, T. FUKUOKA, T. NAKATANI, A. FUJII & A. MATSUDA: New analogs and derivatives of bleomycin. *In* Bleomycin Chemotherapy. *Ed.*, B. I. SIKIC *et al.*, pp. 289~301, Academic Press, 1985
5) TAKITA, T. & T. OGINO: Peplomycin and liblomycin, a new analogues of bleomycin. Biomed. Pharmacother. 41: 219~226, 1987
6) TAKAHASHI, K.; H. EKIMOTO, S. MINAMIDE, K. NISHIKAWA, H. KURAMOCHI, A. MOTEGI, T. NAKATANI, T. TAKITA, T. TAKEUCHI & H. UMEZAWA: Liblomycin, a new analogue of bleomycin. Cancer Treat. Rev. 14: 169~177, 1987
7) TAKAHASHI, K. & T. NAKATANI: Liblomycin. Drugs Future. 13: 519~521, 1988
8) CHU, M. & G. A. FISCHER: The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukemia cells (L5178Y). Biochem. Pharmacol. 17: 753~767, 1968
9) KOHN, K. W.; R. A. EWIG, L. C. ERIKSON & L. A. ZWELLING: Measurement of strand breaks and cross-links by alkaline elution. *In* DNA Repair. *Ed.*, E. C. FRIEDBERG *et al.*, pp. 379~401, Academic Press, 1979
10) KRISHAN, H. A.: Rapid flow cytometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol. 66: 188~195, 1975
11) TERASHIMA, T.; Y. TAKABE, T. KATSUMATA, M. WATANABE & H. UMEZAWA: Effect of bleomycin on mammalian cell survival. J. Natl. Cancer Inst. 49: 1093~1100, 1972
12) SUZUKI, H.; K. NAGAI, H. YAMAKI, N. TANAKA & H. UMEZAWA: On the mechanism of action of bleomycin: Scission of DNA strands *in vitro* and *in vivo*. J. Antibiotics 22: 446~448, 1969
13) HAIDLE, C. W.: Fragmentation of deoxyribonucleic acid by bleomycin. Mol. Pharmacol. 7: 645~652, 1971
14) MIYAMOTO, T.; M. WATANABE & T. TERASHIMA: New type of recovery in Hela cells exposed to bleomycin. Br. J. Cancer 49: 247~249, 1984
15) SAITO, T.; R. KAMATA & S. OKADA: Two types of lethal damage induced by pepleomycin in cultured chinese hamster V79 cells. Gann 75: 143~150, 1984
16) KURAMOCHI, H.; A. MOTEGI, K. TAKAHASHI & T. TAKEUCHI: DNA cleavage activity of liblomycin (NK313), a novel analog of bleomycin. J. Antibiotics 41: 1846~1853, 1988
17) KURAMOCHI, H.; K. TAKAHASHI & T. TAKEUCHI: Tumor cell permeability to peplomycin. J. Antibiotics. 42: 1163~1170, 1989