STUDIES ON ANTITUMOR ACTIVITY OF PRUMYCIN

III. MODE OF ACTION OF PRUMYCIN ON HeLa S-3 CELLS*

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(Received for publication November 2, 1979)

The mode of action of prumycin was investigated using synchronized and asynchronous cultured HeLa S-3 cells. Prumycin inhibited significantly the growth of HeLa S-3 cells at the concentration over 5 mcg/ml. DNA synthesis as well as protein synthesis was strongly inhibited at the concentration of 10 mcg/ml of prumycin, but RNA synthesis was not inhibited by the same concentration. Prumycin did not block the transition of the cells from M phase to G_1 phase, however, G_2 phase cells were blocked clearly by this antibiotic.

Prumycin, an antifungal antibiotic with the structure of 4-N-D-alanyl-2,4-diamino-2,4-dideoxy-L-arabinose[1,2,3], has been reported to possess antitumor activity against several experimental tumors including mouse mammary adenocarcinoma, P-388 lymphocytic leukemia and AH-130 rat hepatoma[4]. SCHWARTZ *et al.*[5] has shown the mechanism of action of prumycin against microorganisms such as *Botrytis cinerea* and *Micrococcus lutea*.

However, the action of the antibiotic against mammalian cells was still unknown. Therefore, in order to elucidate this point the biosynthesis of macromolecules by HeLa S-3 cells under the presence of prumycin was studied. The effect of prumycin on HeLa S-3 cell cycle is also reported in this paper.

Materials and Methods

Chemical agents

Prumycin was obtained as previously reported[4]. Mitomycin C (Kyowa Hakko Kogyo), adriamycin (Kyowa Hakko Kogyo), actinomycin D (Makor Chemicals), chromomycin A_3 (Takeda Chem. Ind.), bleomycin (Nihon Kayaku), gentamicin (Shionogi Pharm. Co.), kanamycin (Meiji Seika), streptomycin (Kyowa Hakko Kogyo) were used as reference agents. Colcemid and thymidine were obtained from Nakarai Chemicals. Thymidine-6-^{3}H (50 Ci/mmol), uridine-5-^{3}H (30 Ci/mmol), L-leucine-4,5-^{3}H (52 Ci/mmol) were purchased from The Radiochemical Center, Amersham, England.

Tissue culture of HeLa S-3 cells

HeLa S-3 cells were cultured in monolayer in YLE basal medium (EARLE's balanced salt solution containing 0.1% yeast extract, 0.5% lactalbumin hydrolysate and 0.11% sodium bicarbonate) supplemented with 10% calf serum, penicillin (100 units/ml) and streptomycin (100 mcg/ml) as reported by KITAURA *et al.*[5] About 15×10^4 cells were inoculated into each culture tube with 1 ml of the medium and 24 hours after the incubation at 37°C, 0.2 ml of drug solution was added and further incubated for 3 days. Cell numbers were determined in a hemocytometer by counting cell nuclei stained with 0.05% crystal violet in 0.1 M citric acid solution.

Synchronous cell culture

Randomly growing HeLa cells in tissue culture flask (500 ml) were treated with 2 mM thymidine[7]

* Part II of this series appears in J. Antibiotics 33: 221~225, 1980

for 24 hours, then washed with medium twice, and cultured in fresh medium for 8 hours, followed by incubation in a medium containing 0.025 mcg/ml of colcemid for 5 hours. Mitotic cells were collected by the method of TERASHIMA and TOLMACH[8]. Prumycin (30 mcg/ml) was added immediately after the removal of colcemid and incubated at 37°C for 2 hours. And disappearance of mitotic cells was determined.

For observation of the effect of prumycin on the transition of HeLa cells from S to M phase *via* G_2 phase, cells in Lab-Tek chamber (Miles) were synchronized by the treatment with 2 mM thymidine, and prumycin was added at the various time after washing the cells with fresh medium (0 hour). Then colcemid was added in the medium at the concentration of 0.025 mcg/ml at 8 hour and mitotic cells were counted at 15 hour, according to the method of OKAMOTO *et al.*[16]

Cytological observation

Cells on Lab-Tek chamber or deposited directly on clean slide glass were fixed with 70% methanol for 1 minute and then with absolute methanol for 30 seconds. The number of mitotic cells were counted after GIEMSA staining.

Incorporation of labelled precursors into nucleic acid and protein

Logarithmically growing HeLa cells in short test tubes were incubated with 5 and 10 mcg/ml of prumycin at 37°C. And ^{3}H-thymidine (0.5 μCi/ml) or ^{3}H-uridine (0.5 μCi/ml) or ^{3}H-L-leucine (1.0 μCi/ml) was added into the medium at 1-hour or 4-hour and incubated at 37°C for 60 minutes.

Cells were washed with cold phosphate buffered saline (PBS) and treated with 4 ml of cold trichloroacetic acid, ethanol and ether. The dried cell residues were dissolved in 2.0 ml of 1 N NH_4OH and aliquots were measured for radioactivity by a liquid scintillation counter.

Results

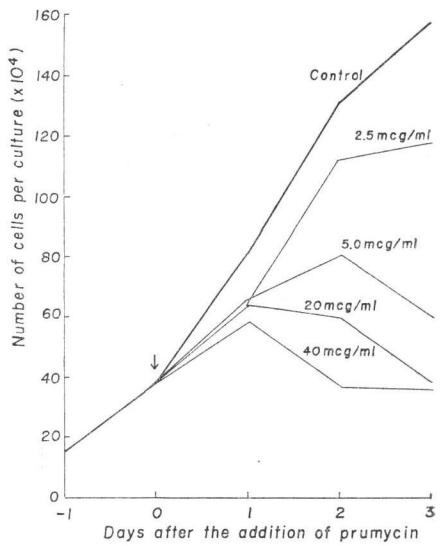
Effect of Prumycin on the Growth of HeLa S-3 Cells

The growth of HeLa S-3 cells was distinctly inhibited by prumycin at concentrations higher than 5 mcg/ml as shown in Fig. 1. The concentration required for 50% growth inhibition (IC_{50}) was about 3 mcg/ml at 3 days after the addition of prumycin, so that the cytotoxicity, and acute toxicity of prumycin is weaker than that of other antitumor antibiotics, and yet considerably strong when com-

Table 1. Acute toxicity and cytotoxic activity of some antitumor antibiotics and aminoglycoside antibiotics.

Antibiotics	Mouse LD_{50} (i.p.) (mg/kg)	Reference	IC_{50} (mcg/ml) to HeLa cell
Prumycin	155	(4)	3
Kanamycin	1,679	(15)	>100
Gentamicin	433	(15)	>100
Streptomycin	900 (s.c)	(15)	>100
Kasugamycin	2,000	(14)	
Mitomycin C	8.4		0.01
Adriamycin	13.7		0.03
Chromomycin A_3	2.1		0.004
Bleomycin	164		2.2
Actinomycin D	1.4		0.02

Fig. 1. Inhibitory effect of prumycin on the growth of HeLa S-3 cells.



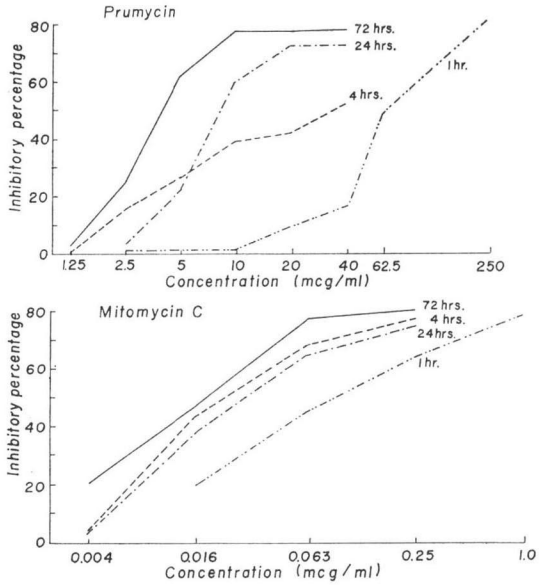
pared with other aminoglycoside antibiotics such as kanamycin, streptomycin, gentamicin and kasugamycin as seen in Table 1.

Inhibition of HeLa S-3 Cell Growth by Various Incubation Time with Prumycin and Mitomycin C

Logarithmically growing HeLa S-3 cells were incubated with prumycin and mitomycin C at 37°C for 1, 4, 24 and 72 hours. Cells were washed with PBS after exposure to prumycin and mitomycin C, and further incubated in fresh medium by 72 hours after the addition of agents. Inhibition percentage on the growth of HeLa S-3 cells was shown in Fig. 2. Comparing with mitomycin C, which has been proposed as concentration dependent drug by SHIMOYAMA *et al.*[9], the effect of prumycin on the growth of HeLa S-3 cells was more dependent on the incubation time.

Fig. 2. Inhibition of HeLa S-3 cell growth by various incubation time with prumycin and mitomycin C.

Logarithmically growing cells were incubated with prumycin and mitomycin C for indicated time, then washed and further cultivated in fresh medium by 72 hours after the addition of agents.



Effect of Prumycin on Biosynthesis of Macromolecules in HeLa S-3 Cells

Fig. 3 shows the effect of prumycin on the incorporation of ^{3}H-labelled precursors into DNA, RNA and protein. At the concentrations of 5 and 10 mcg/ml, RNA synthesis was not inhibited at all, however the incorporation of ^{3}H-L-leucine into protein and ^{3}H-thymidine into DNA was significantly inhibited by about 30% at the concentration of 10 mcg/ml.

Cell Cycle Phase-specific Effects of Prumycin on Transition of Synchronized HeLa Cells

Fig. 4 shows the effect of prumycin on transition from M phase to G_1 phase. The disappearance

Fig. 3. Effect of prumycin on incorporation of labelled precursors into DNA, RNA and protein.

The labelled precursors were added at indicated times after the addition of prumycin and the radioactivity was measured after 1 hour incorporation.

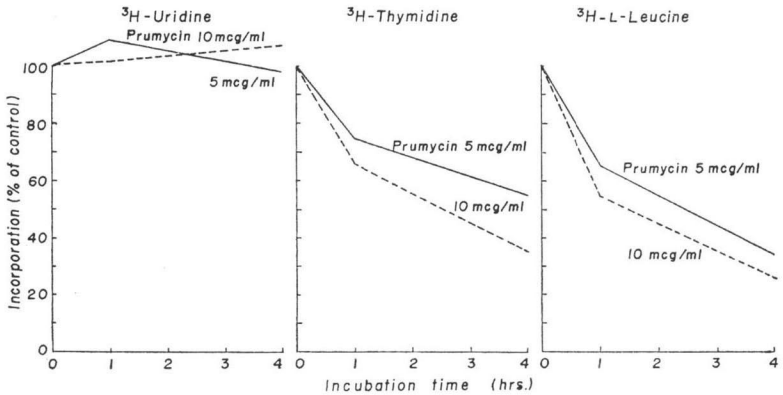


Fig. 4. Effect of prumycin on the transition of synchronized HeLa S-3 cells from M phase to G_1 phase.

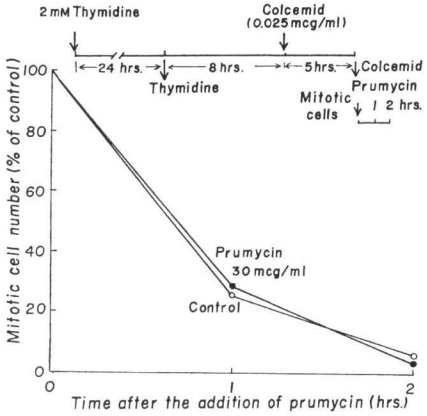
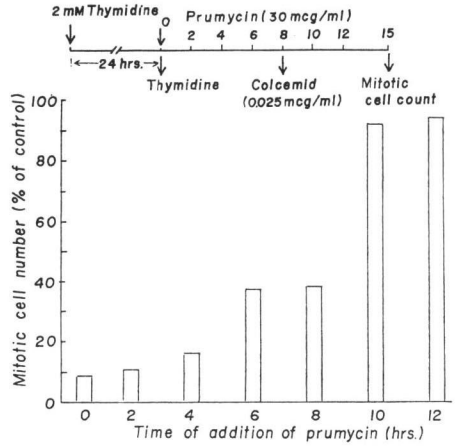


Fig. 5. Effect of prumycin on the transition of synchronized HeLa S-3 cells from S phase to M phase.



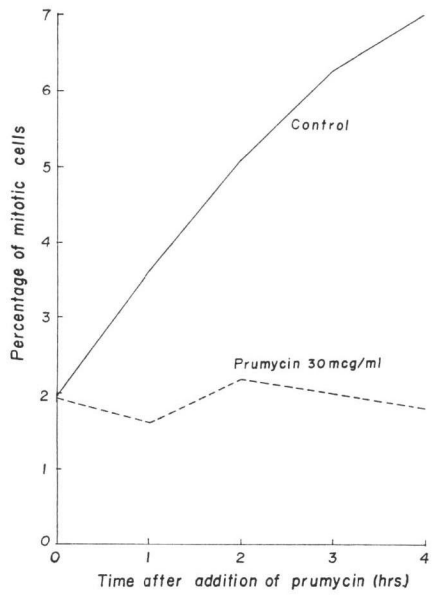
of mitotic cell population under the presence of prumycin after the removal of colcemid was determined. The addition of 30 mcg/ml of prumycin did not inhibit the division of mitotic cells. Thus, prumycin did not block the transition of cells from M to G_1 phase.

Inhibition of cell transition from S to M phase *via* G_2 phase by prumycin was examined and shown in Fig. 5. Prumycin significantly inhibited the appearance of mitotic cells when it was added by 8 hours after the removal of 2 mM thymidine. Therefore, it is obvious that prumycin blocks cells cycle from S phase to M phase *via* G_2 phase, although it was not clear either S phase or G_2 phase was sensitive to the antibiotic.

In order to elucidate the effect of prumycin on the cell transition from G_2 to M phase, the antibiotic (30 mcg/ml) was added to exponentially growing cells with colcemid of 0.025 mcg/ml, then 1 to 4 hours after, mitotic cell number was counted. As shown in Fig. 6, prumycin completely inhibited the accumulation of M phase cells with time which could be seen in control.

Fig. 6. Effect of prumycin on the transition of HeLa S-3 cells from G_2 phase to M phase.

Colcemid (0.025 mcg/ml) was added to logarithmically growing HeLa S-3 cells with or without prumycin (30 mcg/ml), thereafter mitotic cells were counted at indicated time.



Discussion

In general, aminoglycoside antibiotics are important agents in the management of severe bacterial infection. Prumycin, antifungal aminoglycoside antibiotic discovered and identified by HATA and ŌMURA *et al.*[1,2,3], has been shown to possess antitumor activity[4]. SCHWARTZ *et al.*[5] has re-

ported that the mechanism of action of prumycin on microorganisms such as *Botrytis cinerea* and *Micrococcus lutea* was inhibition of protein synthesis, and it appears to be similar to that of other aminoglycoside antibiotics[10~13]. However, the mechanism of action of the antibiotic on mammalian cells was not known. In the present study, it was shown that DNA synthesis as well as protein synthesis of cultured HeLa S-3 cells was strongly inhibited by prumycin, whereas RNA synthesis was not inhibited at all. And it is very interesting to note that prumycin has considerably strong cytotoxic activity against cultured HeLa S-3 cells compared with kanamycin, streptomycin and gentamicin, although prumycin has been shown to be inactive against most bacteria and yeasts[1,2]. Thus, contrary to other aminoglycoside antibiotics, prumycin appears to have preferential activity to mammalian cells rather than most microorganisms.

The data demonstrated that prumycin did not inhibit the HeLa S-3 cell transition from M to G_1 phase, but clearly inhibited the transition from S to M phase. Moreover, cell cycle from G_2 to M phase was blocked by prumycin. Therefore, although it is not clear the effect of prumycin on the cells at G_1 and S stages of the cycle, it can be mentioned that cells at least in G_2 phase are sensitive to prumycin. As stated above, prumycin inhibits both protein and DNA syntheses in HeLa S-3 cells, it will be of interest to investigate the combination treatment of tumor with other agents.

Acknowledgements

The authors wish to thank Mr. K. UCHIDA and Miss Y. WATANABE for technical assistance with the experiments and Miss E. NAKAGAWA for typing the manuscript.

References

1) HATA, T.; S. ŌMURA, M. KATAGIRI, K. ATSUMI, J. AWAYA, S. HIGASHIKAWA, K. YASUI, H. TERADA & S. KUYAMA: A new antifungal antibiotic, prumycin. J. Antibiotics 24: 900~901, 1971
2) ŌMURA, S.; M. KATAGIRI, J. AWAYA, K. ATSUMI, R. ŌIWA, T. HATA, S. HIGASHIKAWA, K. YASUI, H. TERADA & S. KUYAMA: Production and isolation of a new antifungal antibiotic, prumycin, and taxonomic studies of *Streptomyces* sp., strain No. F-1028. Agr. Biol. Chem. 37: 2805~2812, 1973
3) ŌMURA, S.; M. KATAGIRI, K. ATSUMI, T. HATA, A. A. JAKUBOWSKI, E. B. SPRING & M. TISHLER: Structure of prumycin. J. Chem. Soc., Perkin Trans I. 1974: 1627~1631, 1974
4) OKUBO, S.; N. NAKAMURA, K. ITO, H. MARUMO, M. TANAKA & S. ŌMURA: Antitumor activity of prumycin. J. Antibiotics 32: 347~354, 1979
5) SCHWARTZ, J. L.; M. KATAGIRI, S. ŌMURA & M. TISHLER: The mechanism of prumycin action. J. Antibiotics 27: 379~385, 1974
6) KITAURA, K.; R. IMAI, Y. ISHIHARA, H. YANAI & H. TAKAHIRA: Mode of action of adriamycin on HeLa S-3 cells *in vitro*. J. Antibiotics 25: 509~514, 1972
7) BOOTSMA, D.; L. BUDKE & O. VOS: Studies on synchronous division of tissue culture cells initiated by excess thymidine. Exp. Cell Res. 33: 301~309, 1964
8) TERASHIMA, T. & L. J. TOLNACH: Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. Exp. Cell Res. 30: 344~362, 1963
9) SHIMOYAMA, M. & K. KIMURA: Quantitative clonal growth of mammalian cells. Its application for quantitative study of cytocidal action of mitomycin C. Chemotherapy 20: 787~794, 1972
10) HAHN, F. E.; J. CIAK, A. D. WOLFE, R. E. HARTMAN, J. L. ALLION & R. S. HARTMAN: Studies on the mode of action of streptomycin. II. Effect of streptomycin on the synthesis of protein and nucleic acids in *Escherichia coli*. Biochem. Biophys. Acta 61: 714~749, 1962
11) TANAKA, N.; T. NISHIMURA, H. YAMAGUCHI, C. YAMAMOTO, Y. YOSHIDA, K. SASHIKATA & H. UMEZAWA: Mechanism of action of kasugamycin. J. Antibiotics 18: 139~144, 1965
12) NISHIMURA, T.; N. TANAKA & H. UMEZAWA: Inhibition of protein synthesis by kanamycin. J. Antibiotics, Ser. A 15: 210~215, 1962
13) HAHN, F. E. & S. G. SARRE: Mechanism of action of gentamicin. J. Infect. Dis. 119: 364~369, 1969
14) TAKEUCHI, T.; M. ISHIZUKA, H. TAKAYAMA, K. KUREHA, M. HAMADA & H. UMEZAWA: Pharmacology of kasugamycin and the effect on pseudomonas infection. J. Antibiotics 18: 107~110, 1965
15) ICHINO, K.; H. MUROYA, K .SUZUKI, S. NISHIO & N. TANAKA: "Handbook of Antibiotics". pp. 137~140, Sangyo Tosho Co., Tokyo, 1969
16) OKAMOTO, M.; H. TAKESHIMA, K. KOMIYAMA & I. UMEZAWA: Effects of acetylkidamycin on the cell cycle. Proc. Jap. Cancer Assoc. 35: p. 139, 1976