THE EFFECT OF KAZUSAMYCIN B ON THE CELL CYCLE AND MORPHOLOGY OF CULTURED L1210 CELLS

KEIKO TAKAMIYA, EISAKU YOSHIDA*, TAKEO TAKAHASHI†,
AKIRA OKURA and MASANORI OKANISHI

Exploratory Research Laboratories and †Central Research Laboratories,
Banyu Pharmaceutical Co., Ltd.,
2-9-3 Shimomeguro, Meguro-ku, Tokyo 153, Japan

KANKI KOMIYAMA and IWAO UMEZAWA

The Kitasato Institute,
5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

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The effect of a potent antitumor antibiotic, kazusamycin B, on the cell cycle of L1210 cells was examined. Kazusamycin B arrested synchronized L1210 cells at G1 phase. Retardation of metaphase initiation was also observed. Flow cytometric analysis of kazusamycin B-treated asynchronized cells also confirmed G1 arresting effect of kazusamycin B. In addition, an unidentified cell population with lower fluorescence intensity than G1 population was observed when the cells were exposed to the drug longer than 12 hours. Morphology of kazusamycin B-treated L1210 cells revealed that the intranuclear structure changed within 4 hours, and that abnormal condensation of nuclei coincided with the appearance of unidentified population. Kazusamycin B inhibited RNA synthesis moderately but specifically at 2 hours. However, this inhibition might be a secondary effect of the antibiotic-induced structural abnormality of the nuclei.

In 1987, a novel antitumor antibiotic named kazusamycin B was obtained from the fermentation broth of *Streptomyces* sp. No. 81-484[1] which was already known to produce kazusamycin A[2]. The fermentation, isolation, physico-chemical properties and biological activities of both antibiotics, and the taxonomy of the producing organism were reported previously[1~3]. Kazusamycins A and B are highly toxic to various tumor cells and showed potent antitumor activities on some experimental tumors in mice[4~6].

Although kazusamycin A was reported to inhibit macromolecular synthesis in HeLa S3 cells[4], their mode of cytotoxic action has not been characterized yet. Concerning leptomycin B, one of the congeners of kazusamycins A and B, HAMAMOTO *et al.* reported that leptomycin B arrested mammalian cell at G1 phase with inhibition of the DNA replication complex formation[7]. ROBERTS *et al.* mentioned that CI-940, which was considered to be identical to leptomycin B, caused cell cycle arrest at Gl-S interface[8]. They also noted that though CI-940 was a potent inhibitor of DNA synthesis, its mechanism of action was unknown, and appeared to novel[8].

In the course of studies on the cell cycle of kazusamycin B-treated cells, we found that it caused structural changes in the nuclei. The results are presented herein.

Materials and Methods

Reagents

Kazusamycin B was prepared according to the method described by FUNAISHI *et al.*[1]. The

antibiotic was used as a solution of dimethyl sulfoxide (Spectrosol, Dojin Chemical Co., Ltd., Tokyo, Japan), of which concentration was adjusted to 1% at each experiment. Control culture was given only the vehicle. Thymidine and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Radiolabeled macromolecular precursors, [^{3}H]thymidine (40 Ci/mmol), [^{3}H]-uridine (41 Ci/mmol) and [^{3}H]leucine (171 Ci/mmol), were obtained from Amersham (Amersham, UK).

Cells

L1210 cells purchased from Flow Labolatories (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum in a CO_2 incubator.

Determination of IC_{50} at Various Exposure Periods

L1210 cells (5×10^4 cells/ml) were treated with kazusamycin B for the periods indicated in the result section. The medium was replaced with kazusamycin-free medium and the culture was continued up to 72 hours from the addition of the antibiotic. The viability was examined using a Coulter counter (Coulter Electronics Inc., Hialeah, Fla., U.S.A.).

Effect on Synchronized Cells

L1210 cells were cultured in the medium containing 2 mM thymidine for 18 hours[9]. The synchronized cells were resuspended with the fresh medium (2×10^5 cells in 1 ml) and treated with 10 ng/ml kazusamycin B. DNA and RNA synthesis and cell number were monitored at every 1 hour. DNA and RNA synthesis was assessed by incorporation of ^{3}H-labeled precursors into acid-insoluble fraction. The labels (1 μCi/ml) were added 15 minutes before the determination. After the labeling, the cells collected onto glass filter were washed three times with ice-cold 10% trichloroacetic acid and then once each with 70% EtOH and 99.5% EtOH. The filters were dried and the radioactivity was counted by means of liquid scintillation methods. The tests were done in duplicate.

Flow Cytometry

L1210 cells (1×10^5 cells in 2 ml) co-cultured with 5 ng/ml kazusamycin B for various periods were treated with ice-cold 70% EtOH for 30 minutes. Fixed cells were stained with 100 μg/ml propidium iodide for 30 minutes in dark. The sample was filtered through stainless mesh (37~40 μm glid) to remove aggregated cells prior to the analysis. The fluorescence intensity was analyzed on 5,000 cells with a flow cytometer FCS-1 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Obtained DNA histograms of kazusamycin B-treated cells were compared with those of control cells.

Morphology

For optical microscopy, the cells smeared onto a slide glass were fixed with absolute MeOH and stained with hematoxilin and eosin.

For electron microscopy, the cells were treated with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and then with 1% osmium tetroxide in the same buffer. After dehydration with EtOH, the samples were treated with propylene oxide and embedded in Epon-812. The sections prepared using an ultramicrotome LKB-8800 were stained with uranyl acetate and lead citrate, and were subjected to the microscopy.

Effect on DNA, RNA and Protein Synthesis of Asynchronous L1210 Cells

The effect of 5 ng or 50 ng/ml kazusamycin B on DNA, RNA and protein syntheses of asynchronous L1210 cells were assessed by the same method described above, except that the precursors were added 1 hour before the determination. The test was done in triplicate, and the data were analyzed by Student's t-test.

Results

IC_{50} at Various Exposure Periods

The results are presented in Table 1. Cytotoxicity of kazusamycin B was time dependent, and

Table 1. Time dependent effect of kazusamycin B on the growth of L1210 cells.

Co-incubation period (hours)	IC_{50} (ng/ml)	
	Expt 1	Expt 2
2	>800	NT
4	>800	1,900
6	>400	NT
8	NT	400
10	200	NT
12	NT	6.3
16	NT	4.4
20	NT	4.0
24	4.5	3.9
48	3.4	NT

NT: Not tested.

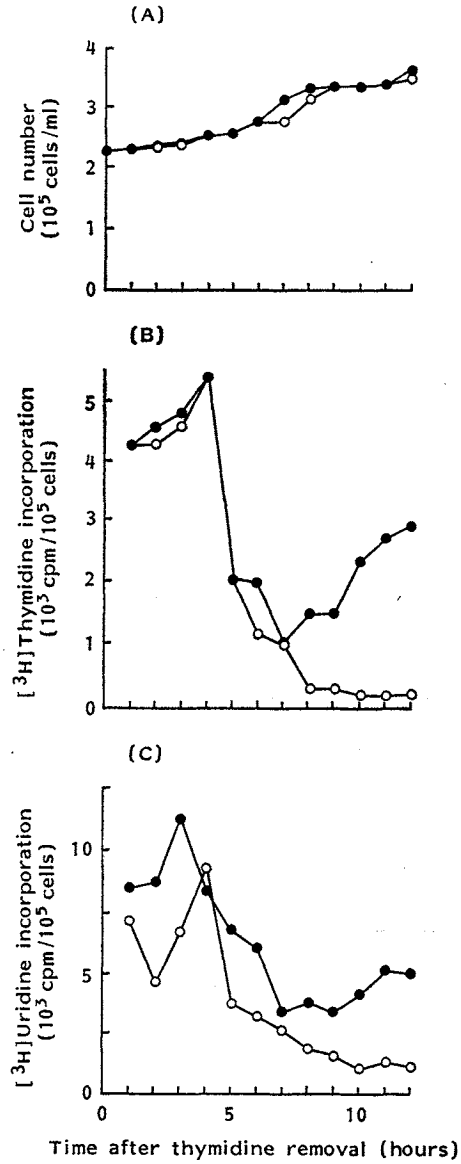
particularly there was threshold between 8 and 12 hours-exposure; IC_{50} at 12 hours-exposure was 6.3 ng/ml whereas IC_{50} at 8 hours-exposure was 400 ng/ml. These data were very similar to those on HeLa S3 cells which was reported in the previous paper[4].

Effect on Synchronously Cultured Cells

Fig. 1 shows the effect on the cell growth (A), and DNA (B) and RNA (C) syntheses of kazusamycin B. When 10 ng/ml kazusamycin B was added to the culture just after the thymidine removal (0 hour), partial inhibition of RNA synthesis in the first cycle, retardation of mitosis, and complete inhibition of both DNA and RNA syntheses after M-phase (5 to 8 hours), were observed. Inhibition of DNA synthesis was observed only after the M-phase. Since cell cycle synchronization using exess thymidine is known to cause artificial distortion in the cell cycle[10], the effect of kazusamycin B on cell cycle was investigated using flow cytometry as well.

Fig. 1. The effect of kazusamycin B on the growth (A), DNA (B) and RNA (C) syntheses of synchronized L1210 cells.

● Control, ○ +10 ng/ml kazusamycin B.



Flow Cytometry of Kazusamycin B-treated L1210 Cells

The results are shown in Fig. 2. The first column of the figure shows the analyses of control cells at every 4 hours from the start of the culture (A~G). The effect of 5 ng/ml kazusamycin B is shown in the second column (H~M). At 4 hours (H), the peak observed at channel 40 of abscissa corresponding to the G1-phase cells increased the height. And at 8 hours (I), the valley around channel 60 corresponding to S-phase cells deepened when compared to those of the control. G1 population of the control also increased after 12 hours (D~G) but it would be caused by the aging

Fig. 2. Flow cytometric analysis of kazusamycin B-treated L1210 cells.

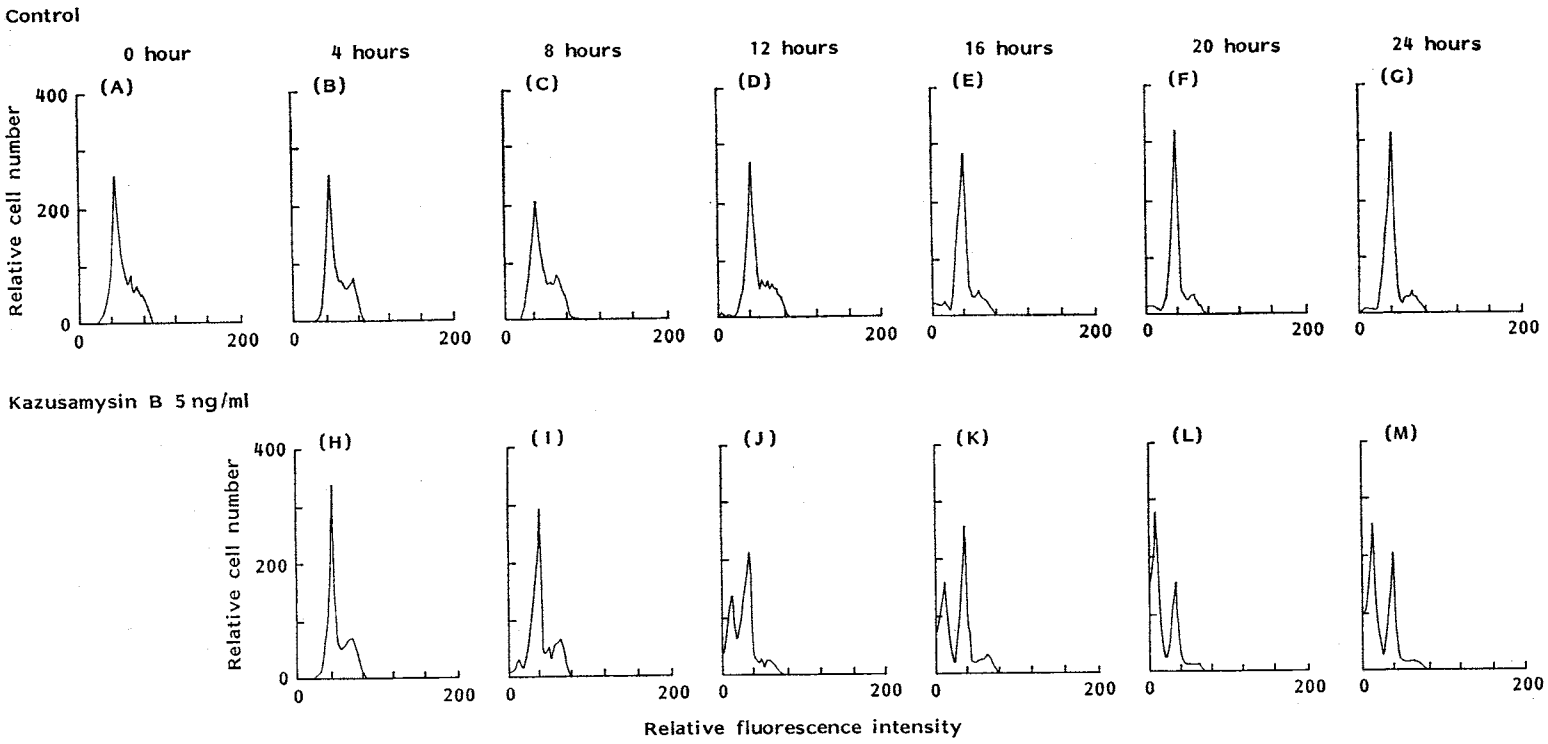


Fig. 3. Optical microscopy of kazusamycin B-treated L1210 cells.

(A) Control, (B~D) +5 ng/ml kazusamycin B, (B) at 4 hours, (C) at 8 hours, (D) at 12 hours. A bar in (D) represents 20 μm.

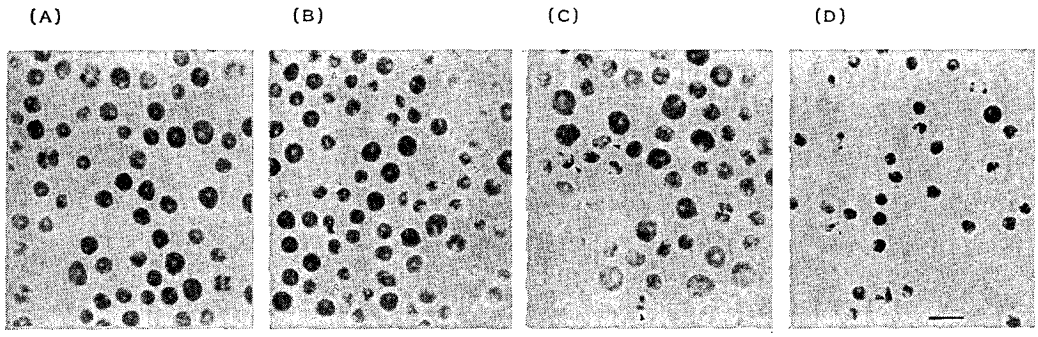
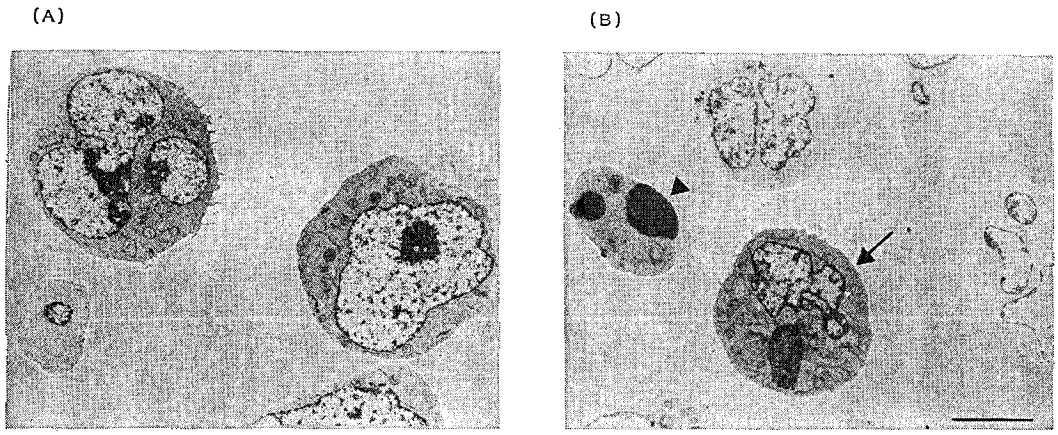


Fig. 4. Electron microscopy of kazusamycin B-treated L1210 cells.

(A) Control, (B) +5 ng/ml kazusamycin B, at 12 hours. A bar in (B) represents 5 μm.



of the culture. In addition, a peak of unknown cell population was observed at channels around 20 after 8 hours-exposure to the antibiotic (I). This peak grew time dependently and became obvious after 12 hours (J~M), at which cells in S and G2-phases (corresponded to cell population at channels 60~80) were scarcely observed.

Morphological Effect of Kazusamycin B on L1210 Cells

To examine the properties of the unidentified cell population on the flow cytometric analysis, kazusamycin B-treated L1210 cells were microscopically analyzed. The optical micrographs of L1210 cells exposed to 5 ng/ml kazusamycin B as well as the control cells are shown in Fig. 3. Control cells had large nuclei in which plexiform or granulated heterochromatine dispersed uniformly (Fig. 3A), and showed unchanged stained images throughout the examination period (0 to 24 hours, data not shown). On the other hand, the cells cultured with 5 ng/ml kazusamycin B for 4 hours showed pachychromatic nuclei (Fig. 3B, indicated by open arrowheads). At 8 hours, pyknotic cells, of which nuclei were highly condensed and sometimes splitted, appeared (Fig. 3C, indicated by closed arrowheads) and increased at 12 hours (Fig. 3D). These observations were further confirmed using electron

Table 2. Effect of kazusamycin B on DNA, RNA and protein syntheses of L1210 cells.

^{3}H-Labeled precursor	Addition		Mean±SD (% of control)[a]		
			Incubation period (hours)		
			2	4	6
Thymidine	Control		100±8.4	100±5.1	100±2.8
	Kazusamycin B	5 ng/ml	95.5±11.8	99.3±4.5	85.9±1.4[c]
		50 ng/ml	99.9±5.6	89.5±9.1	75.6±2.1[d]
Uridine	Control		100±1.8	100±6.4	100±1.8
	Kazusamycin B	5 ng/ml	66.5±2.3[d]	54.1±1.5[d]	38.1±0.9[d]
		50 ng/ml	64.2±0.8[d]	47.6±1.1[d]	35.7±2.4[d]
Leucine	Control		100±5.5	100±2.2	100±5.9
	Kazusamycin B	5 ng/ml	89.2±6.0	84.3±9.4[b]	58.1±3.9[d]
		50 ng/ml	89.6±17.1	70.0±5.0[d]	53.1±6.6[d]

[a] Mean incorporated dpm values of control determinations at 2, 4 and 6 hours are 188,222, 215,846 and 237,001 for [^{3}H]thymidine, 34,471, 38,594 and 47,442 for [^{3}H]uridine, and 2,840, 3,270 and 3,923 for [^{3}H]leucine, respectively.

[b] $P<0.05$, [c] $P<0.01$, [d] $P<0.001$ by Student's t-test.

microscopy (Fig. 4). Fig. 4B shows the electron micrograph of L1210 cells exposed to 5 ng/ml kazusamycin B for 12 hours. A cell, whose nucleus was characterized by the increase of heterochromatine-like structure near the nuclear membrane, is indicated by an arrow. And a cell with abnormally condensed nucleus is indicated by an arrowhead. The results of control cells are shown in Fig. 4A.

Effect on DNA, RNA, and Protein Synthesis

Since time dependent inhibition of DNA and RNA syntheses was observed on the experiment using synchronous culture, the effect of kazusamycin B on the macromolecular synthesis in asynchronized L1210 cells was examined. The results are shown in Table 2. Kazusamycin B (5 ng/ml) inhibited RNA synthesis of L1210 cells 33.5% ($P<0.001$) at 2 hours. The inhibition was time dependent; inhibition at 4 and 6 hours was 45.9 and 69.1%, respectively. Statistically significant inhibition of protein and DNA synthesis was not observed at 2 hours but observed at 4 and 6 hours, respectively. The inhibition of DNA synthesis at 6 hours was 14.1%, much less than that of RNA synthesis at 6 hours. The concentration dependency of the inhibitory effect of kazusamycin B on macromolecular synthesis seemed to be poor because 50 ng/ml of the antibiotic showed similar inhibition kinetics to that of 5 ng/ml antibiotic.

Discussion

Prior to examining the effect of kazusamycin B on cell cycle, we determined IC_{50} at various exposure periods. As in case of HeLa S3 cells[4], the toxicity of kazusamycin B towards L1210 cells was observed in a time dependent manner that IC_{50} at 1 hour exposure was 1,500-fold of that at 24 hours-exposure. It is considered important to assess the effect of kazusamycin B on cell cycle at a reasonable drug concentration. Therefore the effect of the antibiotic on synchronized cells was tested at 10 ng/ml corresponding to IC_{90} and other examinations were done at 5 ng/ml corresponding to IC_{50} at longer exposure periods.

The analysis on the effect of kazusamycin B on the cell cycle of synchronized L1210 cells clearly showed that, similar to leptomycin B[7], kazusamycin B had G1 arresting effect on the cells. G2 retarding effect was also observed. G1 arresting effect was further confirmed by the flow cytometry of kazusamycin B treated asynchronized L1210 cells. Since many other antitumor antibiotics are re-

ported to show G2 arrest on the treated cells[11], kazusamycin B will have a mode of action other than those of known antitumor antibiotics.

The flow cytometry also revealed the appearance of unknown cell population when the cells were exposed to the antibiotic for more than 8 hours. Following microscopy revealed that abnormal nuclear condensation of the treated cells coinsided with the appearance of unidentified cell population. Fluorescence microscopy of propidium iodide stained pyknotic cells revealed that the fluorescence intensity of the nuclei was weaker than that of other cells (data not shown). The appearance also coinsided with the time at which strong cytotoxicity of the antibiotic was fully observed. These data suggest that kazusamycin B has pyknotic effect on the nuclei of the treated cells and the effect strongly correlates to its cytotoxicity.

Leptomycin B was reported to inhibit the formation of DNA replication complex[7], however, present study suggests that it is not the case with kazusamycin B. Kazusamycin B inhibited RNA synthesis first within 2 hours, and then protein and DNA syntheses. It is conceivable that partial inhibition in RNA synthesis might be implicated in the delay of metaphase initiation and inhibition in protein synthesis, because a) the length of metaphase in kazusamycin B treated cells was not different from that in control cells, and it is known that RNA synthesis in G2 phase is necessary to the mitosis[12], and b) protein synthesis could be influenced by transcriptional rate. However, the inhibiton of RNA synthesis seemed to be concomitant action of kazusamycin B, because, a) inhibition in RNA synthesis was weaker when compared to other known RNA synthesis inhibitors such as actinomycin D (data not shown); b) the inhibition was concentration independent; and c) kazusamycin B did not inhibit RNA synthesis in isolated nuclei of L1210 cells (data not shown). It is likely that the inhibition of RNA synthesis by kazusamycin B is the result of structural abnormality in the nuclei caused by the antibiotic, since heterochromatin is known to be a transcription-inactive area on the chromosome and the antibiotic seemed to increase the heterochromatine-like structure in the nuclei of treated cells. This morphological effect of kazusamycin B is considered to be reversible until the nuclei are highly condensed. And it is still unclear whether this effect is the primary effect of the antibiotic. However, this finding may support that kazusamycin B has a unique mode of action. We think that further morphological studies on kazusamycin B treated cells would offer the key to clarify the primary action of the antibiotic.

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