Effect of FK973, a New Antitumor Antibiotic, on the Cell Cycle of L1210 Cells In Vitro

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Abstract—Our previous study showed that FK973 (11-acetyl-8-carbamoyloxy-methyl-4-formyl-14-oxa-1,11-diazatetracyclo[7.4.1.02'7010,12]tetradeca-2,4,6-trien-6,9-diyl diacetate), a novel substituted dihydrobenzoxazine, which is a derivative of the fermentation product of Streptomyces sandaensis No. 6897, had strong antitumor effects on experimental tumors in vitro and in vivo. In this report, we investigated its effect on the cell cycle of murine leukemia L1210 cells in vitro by means of DNA/5-bromo-2'-deoxyuridine double staining and compared these effects with those of other antitumor drugs. Both FK973 and mitomycin C arrested the cells in the G2 phase. Vinblastine arrested the cells in the M phase and cytosine arabinoside, in the G1 phase. Although FK973 and mitomycin C were shown to act on the cell cycle in a similar way, FK973 was slower in producing its effect. From the results, FK973 arrests the cells in the G2 phase, and it appears that FK973 must be converted into the activated form in the cells for the development of its antitumor effects.

It is well-known that an enhanced antitumor effect can be obtained by the combined use of various types of antitumor drugs which act on the different phases of the cell cycle in clinical application and experimental tumor models (7–9). Thus, in the present study, we studied the effect of FK973 on the cell cycle of L1210 cells by means of DNA/5-bromo-2'-deoxyuridine double staining (10) to learn how to use the drug most effectively.

Materials and Methods

Drugs: The drugs studied were FK973 (prepared in the Fujisawa Research Laboratories), mitomycin C (MMC, Kyowa Hakko Co., Ltd., Japan), vinblastine (VLB, Shionogi & Co., Ltd., Japan) and cytosine arabinoside (Ara-C, Nippon Shinyaku Co., Ltd., Japan). FK973, VLB and Ara-C were dissolved in and diluted with phosphate-buffered saline (PBS). MMC was dissolved in distilled water and diluted with PBS. Figure 1 shows the chemical structure of FK973.
Cell culture: L1210 cells were incubated in suspension in RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Japan) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, U.S.A.) and penicillin (50 units/ml)-streptomycin (50 μg/ml) (Flow Laboratories, Australia) at 37°C in a humidified atmosphere of 95% air / 5% CO₂. The doubling time of the cells was estimated to be about 12 hr. The cells (1 × 10⁵/ml) growing exponentially were exposed to a test drug (10 μl/ml) in plastic flasks (75 cm², Flask #25110, Corning Glass Works, U.S.A) for 8, 24 and 48 hr. The viable and dead cells were counted by the trypan blue dye exclusion method.

Flow cytometry: The experiment was performed according to the modified method of Dolbeare et al. (10). After exposure of L1210 cells to a drug for 8, 24 or 48 hr, 5-bromo-2'-deoxyuridine (BrdU, Sigma Co., U.S.A.) was added to the medium to give a final concentration of 5 μg/ml, and the culture was incubated at 37°C for 30 min. No drug was added in the control. The cells (2 × 10⁶) were harvested in tubes, washed twice with PBS/13 mM EDTA by centrifugation at 4°C, and fixed with 2 ml of 70% ethanol at 4°C for 30 min. The DNA of the ethanol-fixed cells was partially denatured by exposure to 4 N HCl (2 ml) at room temperature for 40 min. The cells were washed twice with PBS/0.5% Tween 20 and allowed to react with anti-BrdU antibody (fluorescein-isothiocyanate (FITC)-conjugated, #7583, Becton-Dickinson Co., Ltd., U.S.A.) to fluorescently label the incorporated BrdU in the dark at room temperature for 30 min. The cells were washed with PBS/13 mM EDTA by centrifugation, and 100 μl of propidium iodide (PI, Sigma Co., U.S.A.) solution (20 μg/ml) was added to the cells to stain the cellular double stranded DNA. Ten min later, the cells were sorted with a FACS IV (Becton-Dickinson, Co., Ltd., U.S.A.). DNA histograms were obtained for all phases of the cells (1 × 10⁴) as a three dimensional diagram expressing the fluorescent intensity of PI on the axis of the abscissae and that of FITC on the axis of the ordinates, and the number of cells was expressed by the contour lines. Excitation wavelength was 488 nm, and fluorescent wavelengths of PI and FITC were 525 and 610 nm, respectively. The fluorescent intensity of PI indicates DNA content in the cells, and that of FITC represents the amount of the BrdU incorporated in the cells, which shows the ability to synthesize DNA. The number of cells in the G₀+G₁, S and G₂+M phases were counted with a 9000 series 300 model computer (Hewlett-Packard Co., U.S.A.). The cells with low BrdU and DNA contents were defined as being in the G₀+G₁ phase; cells with low BrdU content and with DNA content twice that in the G₀+G₁ phase as being in the G₂+M phase; and those with high BrdU content, as being in the S phase. Cells other than those in the G₀+G₁, G₂+M and S phases and with greater DNA content than those in the G₂+M phase were defined as the multiploids.

Mitotic index: To distinguish the cells in the G₂ phase from those in the M phase, the number of cells with mitosis was counted. The cells were exposed to a test drug for an indicated period and then smeared on a glass slide. The preparations were dried in the air, fixed with methanol for 3 min and then stained with 3% Giemsa solution for 30 min. The number of cells with mitosis (cells in the M phase) in more than 1000 cells were determined under a microscope.

**Results**

**FK973**: FK973 concentration-dependently inhibited the proliferation of L1210 cells at 24 and 48 hr in concentrations up to 0.32 μM, although the drug did not kill the cells (Fig. 2). Table 1 and Fig. 3 show the effect of FK973 on the cell cycle of L1210 cells. Eight hr after addition of FK973, no marked changes
in the cell cycle were observed in DNA histograms. At 24 hr, the number of cells in the $G_2+M$ phase concentration-dependently increased, and those in the $G_1$ phase decreased. At 48 hr, $G_2+M$ phase cells markedly increased, with a clear decrease of the $G_1$ and $S$ phase cells, which suggests that FK973 inhibited DNA synthesis. The number of cells with mitosis ($M$ phase) did not differ from those of the controls in any case, suggesting that the increase in the $G_2+M$ phase cells was due to their being arrested in the $G_2$ phase. The multiploid cells increased concentration-dependently.

**MMC**: MMC concentration-dependently inhibited the proliferation of cells from 8 hr onwards in concentrations of 0.1 and 1.0 $\mu$M, and it did not kill the cells (Fig. 4). Table 2 and Fig. 5 show the effect of MMC on the L1210 cell cycle. The number of cells in the $G_2+M$ phase markedly increased, and those in the $G_1$ phase cells decreased from 8 hr after addition of MMC in concentrations of 0.1 and 1.0 $\mu$M. The $S$ phase cells decreased with time after addition of MMC in a concentration of 1.0 $\mu$M, but the number of $M$ phase cells did not change. The number of mul-

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### Table 1. Distribution of L1210 cells in each phase of the cell cycle at 8, 24 and 48 hr after exposure to FK973

| Concentration ($\mu$M) | Distribution (%) | 8 hr | 24 hr | 48 hr |
|------------------------|-----------------|------|------|------|
| 0                      |                 |      |      |      |
|                        | $G_1$           | 22.7 | 26.4 | 22.8 |
|                        | $S$             | 62.7 | 58.3 | 67.8 |
|                        | $G_2+M$         | 14.4 (1.8) | 15.3 (1.7) | 9.4 (2.6) |
|                        | $4n^a$          |      |      |      |
| 0.032                  |                 |      |      |      |
|                        | $G_1$           | 21.5 | 15.0 | 11.2 |
|                        | $S$             | 62.1 | 57.8 | 44.7 |
|                        | $G_2+M$         | 16.4 (2.6) | 26.3 (2.3) | 41.0 (3.0) |
|                        | $4n$            |      | 0.9  | 3.1  |
| 0.1                    |                 |      |      |      |
|                        | $G_1$           | 18.6 | 10.5 | 8.8  |
|                        | $S$             | 62.1 | 55.1 | 31.2 |
|                        | $G_2+M$         | 18.6 (2.2) | 31.3 (2.3) | 50.3 (2.7) |
|                        | $4n$            |      | 3.1  | 9.7  |
| 0.32                   |                 |      |      |      |
|                        | $G_1$           | 12.3 | 5.6  | 12.1 |
|                        | $S$             | 67.6 | 38.2 | 12.9 |
|                        | $G_2+M$         | 20.1 (2.2) | 52.6 (2.8) | 57.6 (2.7) |
|                        | $4n$            |      | 3.6  | 17.4 |

^a^: multiploidy. Values in parentheses indicate the number of $M$ phase cells as a percentage of the total cells tested.
tiploid cells was greater 48 hr after addition of MMC in a concentration of 1.0 μM.

**VLB:** VLB inhibited the proliferation of cells in a concentration of 0.0032 μM and was cytotoxic at a concentration of 0.01 μM, whereas the drug at a concentration of 0.0018 μM had no effect (data not shown). As shown in Table 3, the number of cells both in the G2+M and M phase was greater 8 hr after addition of VLB in concentrations of 0.0032 and 0.01 μM, suggesting that VLB arrests the cells in the M phase. Multiploid cells appeared. After exposure to VLB in a concentration of 0.0032 μM, the changes gradually returned to normal with longer periods of incubation. In a concentration of 0.01 μM, almost all the cells in the G1 phase disappeared and the number of cells in the S phase decreased. At 24 hr, multiploids occupied most of the cell population.

**Ara-C:** Ara-C inhibited the proliferation of cells in a concentration of 0.1 μM and showed strong cytotoxicity at a concentration of 0.32 μM (data not shown). The results with Ara-C at a concentration of 0.1 μM are given in Table 4. The accumulation of

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**Fig. 3.** The distributions of bivariate DNA/BrdU content of L1210 cells at 8, 24 and 48 hr after exposure to FK973. Fluorescent intensities on the X-axis and Y-axis indicate DNA content and BrdU incorporation, respectively. a–c: no drug; d–f: FK973, 0.032 μM; g–i: FK973, 0.32 μM.

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**DNA Content**
cells in the \( G_1 \) and \( G_2+M \) phase was observed at 8, 24 and 48 hr. The number of cells in the S phase decreased with time.

**Discussion**

The effect of FK973 on the cell cycle was studied and compared with those of MMC, VLB and Ara-C. MMC arrested cells in the \( G_2 \) phase at concentrations which inhibited cell proliferation. The effect of MMC appeared at 8 hr and persisted throughout the observation period (48 hr). FK973 behaved similarly, but its effect did not appear until 24 hr, and its effect at that time was stronger than that of MMC. The main mode of action of MMC is thought to be the formation of interstrand DNA-DNA and DNA-protein cross-links which block the traverse to the M phase of the cell cycle (11). In our previous study (6), FK973 also formed concentration- and time-dependent interstrand DNA-DNA and DNA-protein cross-links in cells. However, FK973 formed no detectable interstrand DNA-DNA cross-links when isolated nuclei of cells were exposed to the drug. Thus, FK973 may be converted into the activated form in the cytoplasm of cells (6). Both the carbamoyl moiety and aziridine ring of MMC were reported to bind to DNA after reduction of the quinone ring of MMC, the activated form, in

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**Table 2. Distribution of L1210 cells in each phase of the cell cycle at 8, 24 and 48 hr after exposure to MMC**

| Concentration (\( \mu M \)) | Distribution (%) |
|-------------------------------|------------------|
|                               | 8 hr             | 24 hr | 48 hr |
| 0                             |                  |       |       |
| \( G_1 \)                     | 22.9             | 26.4  | 22.8  |
| S                             | 62.7             | 58.3  | 67.8  |
| \( G_2+M \)                   | 14.4 (1.8)       | 15.3 (1.7) | 9.4 (2.6) |
| \( 4n^a \)                    |                  |       |       |
| 0.1                           |                  |       |       |
| \( G_1 \)                     | 16.4             | 14.7  | 15.4  |
| S                             | 60.7             | 55.4  | 53.7  |
| \( G_2+M \)                   | 22.7 (2.8)       | 27.9 (1.6) | 29.8 (1.7) |
| \( 4n \)                      |                  | 2.0   | 1.1   |
| 1.0                           |                  |       |       |
| \( G_1 \)                     | 10.3             | 4.3   | 16.8  |
| S                             | 55.1             | 32.1  | 17.2  |
| \( G_2+M \)                   | 32.4 (1.3)       | 57.6 (3.2) | 43.3 (1.5) |
| \( 4n \)                      | 2.2              | 6.0   | 22.7  |

\(^a\): multiploidy. Values in parentheses indicate the number of \( M \) phase cells as a percentage of the total cells tested.
cells (12, 13). FK973 also has a carbamoyl moiety and an aziridine ring in its molecular structure, but it has no quinone ring. Thus, we think that the activated form of FK973 may be produced in the cytoplasm, and the carbamoyl moiety and aziridine ring of FK973 may be binding sites to DNA. The induction of the activated form of MMC may be much easier than that of FK973 in cells. The mechanisms by which FK973 is activated in the cytoplasm have not been defined. FK973 gradually decreased the number of L1210 cells in the S phase, and this may have been the effect of arresting the cells in the G₂ phase. MMC had a similar effect, and this agrees with the findings of Takamoto et al. (7, 8).

VLB is known to inhibit the mitotic process by suppressing microtubule formation (14) and to arrest cells in the M phase (15). In the present study, VLB specifically arrested the cells in the M phase, and this probably caused the decrease in the number of cells in the G₁ and S phases. When used in high concentrations, FK973, MMC and VLB caused multiploids to develop; the reason for this is unknown.
Table 3. Distribution of L1210 cells in each phase of the cell cycle at 8, 24 and 48 hr after exposure to VLB

| Concentration (µM) | Distribution (%) | 8 hr | 24 hr | 48 hr |
|--------------------|------------------|------|-------|-------|
| 0                  | G₁ 19.7          | 17.3 | 26.2  |       |
|                    | S  66.3          | 68.3 | 49.6  |       |
|                    | G₂+M 14.0 (4.3)  | 14.4 (1.5) | 24.2 (0.7) |   |
| 4nᵃ                | —                | —    | —     |       |
| 0.0018             | G₁ 18.9          | 17.6 | 28.2  |       |
|                    | S  66.0          | 69.9 | 47.3  |       |
|                    | G₂+M 15.1 (5.3)  | 12.5 (3.2) | 24.5 (0.7) |   |
| 4nᵃ                | —                | —    | —     |       |
| 0.0032             | G₁ 18.8          | 15.8 | 20.6  |       |
|                    | S  59.6          | 70.1 | 66.2  |       |
|                    | G₂+M 17.6 (13.2) | 14.1 (11.1) | 13.2 (2.5) |   |
| 4nᵃ                | 4.0              | —    | —     |       |
| 0.01               | G₁ 1.6           | 5.9  | —     |       |
|                    | S  24.5          | 9.3  | —     |       |
|                    | G₂+M 55.3 (51.5) | 4.3 (29.4) | —     |       |
| 4nᵃ                | 18.6             | 80.5 | —     |       |

ᵃ: multiploidy. Values in parentheses indicate the number of M phase cells as a percentage of the total cells tested.

Table 4. Distribution of L1210 cells in each phase of the cell cycle at 8, 24 and 48 hr after exposure to Ara-C

| Concentration (µM) | Distribution (%) | 8 hr | 24 hr | 48 hr |
|--------------------|------------------|------|-------|-------|
| 0                  | G₁ 29.1          | 26.5 | 32.5  |       |
|                    | S  51.6          | 60.6 | 52.9  |       |
|                    | G₂+M 19.3 (2.1)  | 12.9 (2.3) | 14.6 (1.8) |   |
| 4nᵃ                | —                | —    | —     |       |
| 0.1                | G₁ 44.4          | 38.9 | 47.5  |       |
|                    | S  39.2          | 37.2 | 29.4  |       |
|                    | G₂+M 23.9 (1.6)  | 23.9 (1.6) | 23.1 (2.6) |   |
| 4nᵃ                | —                | —    | —     |       |

ᵃ: multiploidy. Values in parentheses indicate the number of M phase cells as a percentage of the total cells tested.

Ara-C is reported to inhibit DNA synthesis and RNA function (16). In the present study, Ara-C uniquely accumulated the number of cells in the G₁ phase with a slight increase of G₂+M phase, but decreased the number of cells in the S phase. This finding suggests that Ara-C arrests cells in the G₁ phase, and this leads to a change in G₂+M, and S phase cells.

The pharmacokinetic and toxicology profiles of a drug are important for its appropriate use in clinical combined therapy, and an understanding of its pharmacological profiles, especially the mode of action, is essential (7, 8). Our previous studies showed that FK973 was effective against a wide
variety of tumors including those resistant to MMC and adriamycin (5), and that FK973 seemed to act directly on DNA (6). The present results showed that FK973 acted on the cell cycle similarly to MMC. The facts that cells resistant to MMC are sensitive to FK973 might derive from the different modes of induction of the activated forms of FK973 and MMC in cells, although we have no explanation in detail for the mechanisms. Based on these results, we performed a preliminary experiment to study the effect of FK973 on L1210 cells in combination with other types of antitumor agents, and we found that the effect of treatment with the combination of FK973 and Ara-C was much greater than those of the individual treatments and treatment with the combination of MMC and Ara-C on the prolongation of life-span in mice bearing L1210 cells. All these results suggest that FK973 will be clinically beneficial in combined therapy.

In summary, the effect of FK973 on the cell cycle resembles that of MMC among the 3 reference drugs used. However, its onset of action was slower, as was the case in our previous studies (6).

References
1 Iwami, M., Kiyoto, S., Terano, H., Kohsaka, M., Aoki, H. and Imanaka, H.: A new antitumor antibiotic, FR900482 I. Taxonomic studies on the producing strain: A new species of the genus Streptomyces. J. Antibiot. (Tokyo) 40, 589-593 (1987)
2 Kiyoto S., Shibata, T., Yamashita, M., Komori, T., Okuhara, M., Terano, H., Kohsaka, M., Aoki, H. and Imanaka, H.: A new antitumor antibiotic, FR900482 II. Production, isolation, characterization and biological activity. J. Antibiot. (Tokyo) 40, 594-599 (1987)
3 Uchida, I., Takase, S., Kayakiri, H., Kiyoto, S., Hashimoto, M., Tada, T., Koda, S. and Morimoto, Y.: Structure of FR900482, a novel antitumor antibiotic from a Streptomyces. J. Am. Chem. Soc. 109, 4108-4109 (1987)
4 Shimomura, K., Hirai, O., Mizota, T., Matsumoto, S., Mori, J., Shibayama, F. and Kikuchi, H.: A new antitumor antibiotic, FR900482 III. Antitumor activity in transplantable experimental tumors. J. Antibiot. (Tokyo) 40, 600-606 (1987)
5 Shimomura, K., Manda, T., Mukumoto, S., Masuda, K., Nakamura, T., Mizota, T., Matsumoto, S., Nishigaki, F., Oku, T., Mori, J. and Shibayama, F.: Antitumor activity and hematotoxicity of a new, substituted dihydrobenoxazine, FK973, in mice. Cancer Res. 48, 1166-1172 (1988)
6 Masuda, K., Nakamura, T., Mizota, T., Mori, J. and Shimomura, K.: Interstrand DNA-DNA and DNA-protein cross-links by a new antitumor antibiotic, FK973, in L1210 cells. Cancer Res. 48, 5172-5177 (1988)
7 Takamoto, S., Kato, T. and Ota, K.: Study on the mechanism of action of anti-tumor agent by F.M.F. Report 1 —on MFC combination therapy—. Japan. J. Cancer Chemother. 5, 145-153 (1978) (Abs. in English)
8 Takamoto, S. and Ota, K.: Effect of antitumor antibiotics on the cell cycle traverse of cultured FL cells. Japan. J. Cancer Chemother. 6, 59-70 (1988) (Abs. in English)
9 Charcosset, J.Y.: Effects of antineoplastic agents on the cell cycle progression. Biol. Cell 58, 135-138 (1986)
10 Dolbeare, F., Gratzer, H., Pallavicini, M.G. and Gray, J.W.: Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc. Natl. Acad. Sci. U.S.A. 80, 5573-5577 (1983)
11 Dorr, R.T., Bowden, G.T., Alberts, D.S. and Liddil, J.D.: Interactions of mitomycin C with mammalian DNA detected by alkaline elution. Cancer Res. 45, 3510-3516 (1985)
12 Tomasz, M., Lipman, R., Gowdry, D., Pawlak, J., Verdine, G.L. and Nakashiki, K.: Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. Science 235, 1204-1208 (1987)
13 Kohn, H.: Studies concerning the mechanism of electrophilic substitution reaction of mitomycin C. J. Am. Chem. Soc. 105, 4105-4106 (1983)
14 Alberts, B., Bray, D., Lewis, J., Raff, R., Roberts, K. and Watson, J.D.: Cytoskeleton. In Molecular Biology of the Cell, p. 549-609, Garland Publishing, New York and London (1983)
15 Madoc-Jones, H. and Mauro, F.: Site of action of cytotoxic agents in the cell life cycle. In Antineoplastic and Immuno-Suppressive Agents, Edited by Sartorelli, A.C. and Johns, D.G., Tome I, p. 206-219, Springer-Verlag, Berlin (1974)
16 Kufe, D.W., Major, P.P., Egan, E.M. and Beardsley, G.P.: Correlation of cytotoxicity with incorporation of ara-C into DNA. J. Biol. Chem. 255, 8997-9000 (1980)