A NEW ANTITUMOR ANTIBIOTIC, FR-900482: V. INTERSTRAND DNA-DNA CROSS-LINKS IN L1210 CELLS

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FR-900482 (Fig. 1) is a new antitumor antibiotic isolated from the culture broth of *Streptomyces sandaensis* No. 6897[1~3]. It has potent cytotoxic effects against murine and human tumor cells[2,4], and weak myelosuppressive effects in mice[5]. The mechanisms by which FR-900482 induces cytotoxic effects have not been defined. Some antitumor drugs induce interstrand DNA-DNA cross-links or DNA strand breaks in cells[6,7]. In this paper we describe the effects of FR-900482 on macromolecular syntheses in cultured murine L1210 leukemia cells and the interaction of the compound with DNA in the cells and plasmids.

First, cell growth and macromolecular syntheses were examined in L1210 cells exposed to FR-900482. The cells were grown in suspension in DULBECCO's modified EAGLE's medium supplemented with 10% fetal bovine serum in culture flasks in 5% CO_2 - 95% air atmosphere. In one experiment the cells (1×10^5) were incubated with FR-900482 for 48 hours at 37°C, and in another incubated for 1 hour, washed twice with cold phosphate-buffered saline (PBS) to remove the compound, and further incubated with the compound-free medium for 48 hours. The viable cells were counted by the trypan blue dye exclusion method, and the concentrations of FR-900482 required to inhibit cell growth by 50% were found to be approximately 2.5 and 7 μM, respectively, for the 48 and 1 hours treatments. In the experiment on macromolecular syntheses, the cells were incubated with the compound for 48 hours, and [^{14}C]thymidine, [^{14}C]-uridine or [^{14}C]leucine was added to the culture as indicators of synthesis of DNA, RNA or protein, respectively. The cells were further incubated for 2 hours, the TCA insoluble fraction was trapped on a Millipore filter (pore size: 0.4 μm), and the filter was immersed in toluene scintillator and measured for radioactivity with a liquid scintillation counter. FR-900482 inhibited incorporation of the radioactive compounds into the cells, with 50% inhibition of DNA, RNA and protein synthesis observed at FR-900482 concentrations of around 0.2, 4 and 3 μM, respectively (Fig. 2). The results suggest that FR-900482 preferentially inhibits DNA synthesis.

Next we studied the formation of interstrand DNA-DNA cross-links induced by FR-900482 in L1210 cells using the alkaline elution method[8]. Cellular DNA was radioactively labeled in the cells by incubation with [^{14}C]thymidine for 20 hours at 37°C. The cells were exposed to the compound at a concentration of 7 μM for 1 hour at 37°C, washed and resuspended in cold PBS,

Fig. 1. The chemical structure of FR-900482.

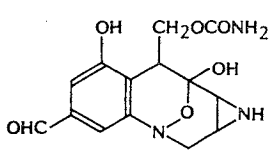
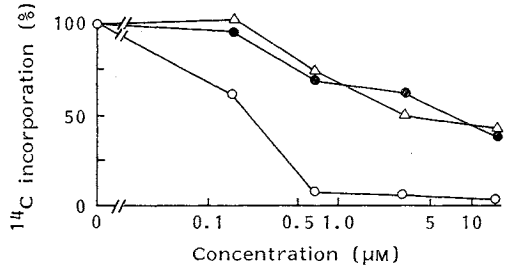


Fig. 2. Effect of FR-900482 on macromolecular syntheses in L1210 cells.

○ [^{14}C]Thymidine, ● [^{14}C]uridine, △ [^{14}C]leucine.

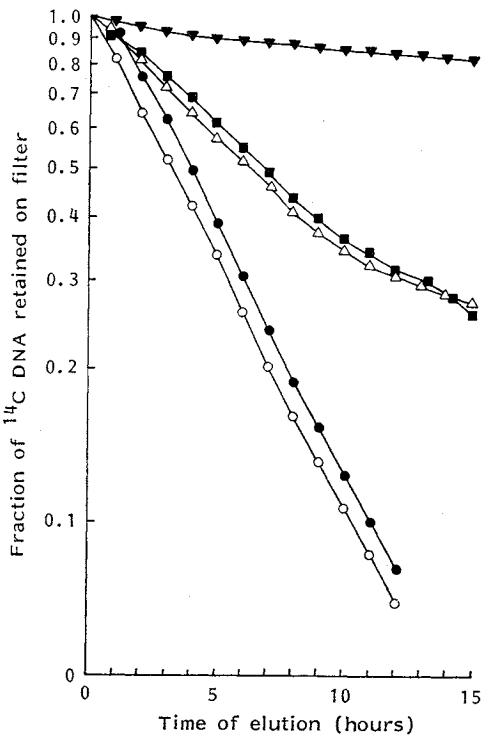


Cells were incubated with FR-900482 for 48 hours, and treated with [^{14}C]thymidine (0.2 μCi/ml), [^{14}C]uridine (0.4 μCi/ml) or [^{14}C]leucine (0.4 μCi/ml) for 2 hours. The radioactivity in the cells was measured.

and irradiated with 400 rads of X-ray on ice immediately before the alkaline elution was carried out. The cells (5×10^5) were collected on a polycarbonate filter (pore size: 2.0 μm), lysed with 2% sodium dodecyl sulfate (SDS)/0.025 M EDTA solution (pH 9.7), and treated with Proteinase K (0.5 mg/ml) at room temperature for 1 hour to digest all DNA-bound proteins, and eluted in the dark at a flow rate of 2.0 ml/hour with 0.1% SDS/0.02 M EDTA/tetrapropylammonium hydroxide solution (pH 12.1), and the fractions were collected at 1 hour intervals. The ^{14}C radioactivity (cpm) of each fraction was expressed as proportion of total radioactivity. As shown in Fig. 3, the treatment with FR-900482 for 1 hour did not increase the retention of ^{14}C-labeled DNA on the filter. Mitomycin C (MMC) at a concentration of 10 μM, which is known to produce interstrand DNA-DNA cross-links[9], increased the retention of the DNA. In the next experiment, the cells were incubated with FR-900482 for 1 hour, washed to remove the compound, and further incubated with the compound-free medium for 8 hours. In this case the retention of the DNA on the filter was increased, and this can be taken as evidence that FR-900482 is gradually activated after the compound is incorporated in the cells and, like MMC, forms interstrand DNA-DNA cross-links in the cells.

Fig. 3. Interstrand DNA-DNA cross-links formation in L1210 cells.

▼ No drug (no X-ray), ○ no drug (400 rads of X-ray), ● FR-900482 (7 μM), immediately after 1 hour exposure, ■ FR-900482 (7 μM), 8 hours after 1 hour exposure, △ mitomycin C (10 μM), immediately after 1 hour exposure.

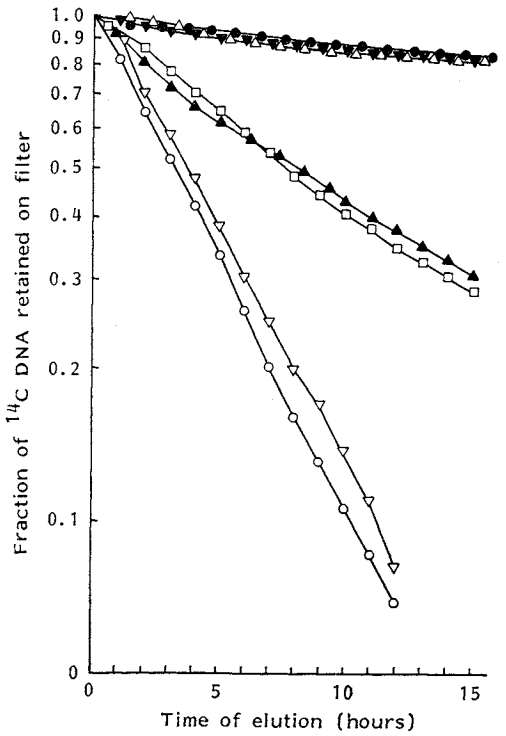


The cells were incubated with drug for 1 hour and further incubated with a compound-free medium for 8 hours at 37°C, irradiated with 400 rads of X-ray on ice. The DNA was eluted with a pH 12.1 solution.

The next experiment was performed to evaluate DNA single strand breaks in L1210 cells. DNA single strand breaks were assayed in the same way as for interstrand DNA-DNA cross-links[6],

Fig. 4. DNA single strand breaks in L1210 cells.

▼ No drug (no X-ray), ● FR-900482 10 μM, △ mitomycin C 10 μM, ▲ doxorubicin 1 μM, ▽ doxorubicin 3 μM, □ 200 rads of X-ray, ○ 400 rads of X-ray.



The cells were incubated with drug for 1 hour at 37°C. The DNA was eluted with a pH 12.1 solution.

but the cells were not irradiated with X-ray[8]. The cells were incubated with FR-900482 or MMC for 1 hour at 37°C, and the alkaline elution was performed. FR-900482 at 10 μM had no effect on the DNA elution (Fig. 4), and the results with MMC at 10 μM were similar. Furthermore, the cells were incubated with FR-900482 at a concentration of 10 μM for 1 hour, washed and further incubated with the compound-free medium for 8 hours. The DNA elution was not enhanced (data not shown). In doxorubicin and X-ray treated cells as control, the DNA elutions were enhanced dose-dependently (Fig. 4), confirming the formation of randomly distributed DNA single strand breaks[9,10]. We additionally evaluated DNA single strand breaks in plasmid pBR322 (10 μg) exposed to FR-900482 at 37°C for 30 minutes according to the method of MIRABELLI *et al.*[11]. The plasmid was electrophoresed through 1% agarose gels with a Tris-acetate buffer system (40 mM Tris-acetate, 2 mM EDTA, pH 7.8) at 5 V/cm, and stained with 1 μg/ml of ethidium bromide. FR-900482 up to 100 μM did not affect the migration of covalently closed circular DNA with or without 10 nM of $FeSO_4$ (data not shown). Bleomycin (0.7 μM), which is known to cause DNA break[11], with $FeSO_4$ (10 nM) showed open circular and linear DNAs (data not shown). Thus FR-900482 may cause no DNA single strand breaks in the cells and plasmids under these conditions.

The present results suggest that FR-900482 is activated in the cells and forms intrastrand DNA-DNA cross-links, which may play an important role for the induction of its cytotoxicity.

References

1) IWAMI, M.; S. KIYOTO, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: A new antitumor antibiotic, FR-900482. I. Taxonomic studies on the producing strain: A new species of the genus *Streptomyces*. J. Antibiotics 40: 589~593, 1987
2) KIYOTO, S.; T. SHIBATA, M. YAMASHITA, T. KOMORI, M. OKUHARA, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: A new antitumor antibiotic, FR-900482. II. Production, isolation, characterization and biological activity. J. Antibiotics 40: 594~599, 1987
3) UCHIDA, I.; S. TAKASE, H. KAYAKIRI, S. KIYOTO, M. HASHIMOTO, T. TADA, S. KODA & Y. MORIMOTO: Structure of FR-900482, a novel antitumor antibiotic from a *Streptomyces*. J. Am. Chem. Soc. 109: 4108~4109, 1987
4) SHIMOMURA, K.; O. HIRAI, T. MIZOTA, S. MATSUMOTO, J. MORI, F. SHIBAYAMA & H. KIKUCHI: A new antitumor antibiotic, FR-900482. III. Antitumor activity in transplantable experimental tumors. J. Antibiotics 40: 600~606, 1987
5) HIRAI, O.; K. SHIMOMURA, T. MIZOTA, S. MATSUMOTO, J. MORI & H. KIKUCHI: A new antitumor antibiotic, FR-900482. IV. Hematological toxicity in mice. J. Antibiotics 40: 607~611, 1987
6) LEE, Y. C. & J. E. BYFIELD: Induction of DNA degradation in vivo by adriamycin. J. Natl. Cancer Inst. 57: 221~224, 1976
7) DORR, R. T.; G. T. BOWDEN, D. S. ALBERTS & J. D. LIDDIL: Interactions of mitomycin C with mammalian DNA detected by alkaline elution. Cancer Res. 45: 3510~3516, 1985
8) KOHN, K. W.; R. A. G. EWIG, L. C. ERICKSON & L. A. ZWELLING: Measurement of strand breaks and cross-links by alkaline elution. *In* DNA Repair: A Laboratory Manual of Research Procedures. Vol. 1, Part B. *Eds.*, E. C. FRIEDBERG & P. C. HANWALT, pp. 379~401, Marcel Dekker, New York, 1981
9) KOHN, K. W.; L. C. ERICKSON, R. A. G. EWIG & C. A. FRIEDMAN: Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry 15: 4629~4637, 1976
10) ROSS, W. E.; D. GLAUBIGER & K. W. KOHN: Qualitative and quantitative aspects of intercalator-induced DNA strand breaks. Biochim. Biophys. Acta 562: 41~50, 1979
11) MIRABELLI, C.K.; C.-H. HUANG & S.T. CROOKE: Comparison of DNA damage and single- and double-strand breakage activities on PM-2 DNA by talisomycin and bleomycin analogs. Cancer Res. 40: 4173~4177, 1980