THE NATURE OF *IN VIVO* CELL-KILLING OF DEOXYSPERGUALIN AND ITS IMPLICATION IN COMBINATION WITH OTHER ANTITUMOR AGENTS

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The mode of *in vivo* cell-killing by 15-deoxyspergualin (NKT-01) was assessed by measuring change of whole body radioactivity of mice inoculated with ^{125}I-iododeoxyuridine-labeled P388 leukemia cells. Although NKT-01 showed strong life prolonging effect on P388 leukemia-bearing mice, significant excretion of ^{125}I was not observed within 4 days after the start of treatment with NKT-01. Thereafter, the remaining ^{125}I was reduced gradually and reached about half of control level on day 7. Colony forming ability in soft agar media of peritoneal tumor cells taken after completion of 5-day treatment with NKT-01 was markedly reduced to less than 3%. These results suggested that life prolongation of NKT-01 was produced both by a cytostatic effect, which lasts for an extraordinarily long period, and by a subsequent cytotoxic effect. Cell cycle distribution analysis using flow cytometry showed the cytostatic action of NKT-01 caused G_0/G_1 arrest of the tumor cells. Therefore, the drug-sensitive cycling population of the tumor cells was reduced, and combination with other antitumor agents was antagonistic, if they were administered simultaneously or consecutively with NKT-01. In contrast, if the other drugs such as cyclophosphamide, cisplatin and cytosine arabinoside, were administered prior to NKT-01, a synergistic combination effect was obtained. This synergism might be due to prolongation of the period of cell cycle perturbation caused by other drugs (such as G_2 arrest by cisplatin) by the cytostatic effect of NKT-01. Although the precise mechanisms of the cytostatic action of NKT-01 remain unclear, it might play an important role in the combination with other antitumor drugs.

15-Deoxyspergualin (NKT-01) is a synthetic derivative of spergualin (SGL), an antibiotic discovered from a culture filtrate of *Bacillus laterosporus*.[1,2] NKT-01 and SGL exhibit antitumor activity against various leukemias both *in vitro* and *in vivo*.[3~5] They also showed immunosuppressive activity and efficacy in organ transplantation in animals.[6~8]

In spite of vigorous efforts, the details of the mechanisms of antitumor and immunosuppressive action remain unclear. Regarding the antitumor activity against highly sensitive tumors such as L1210 (IMC) leukemia, involvement of tumor-specific cytotoxic T-lymphocytes (CTL) was clearly demonstrated.[9] The CTL are induced at lower doses of SGL and exhibit immunological antitumor effect to sensitive tumors *in vivo* in addition to direct cytocidal action. The direct action of SGLs was shown to be extremely dependent on the treatment period in growth inhibition experiments of cultured tumor cells as well as on the treatment schedule *in vivo*.[3~5,10]

In the present study, we investigated the nature of the direct action of NKT-01 *in vivo*. We estimated the cell-killing in terms of (a) release of ^{125}I from inoculated ^{125}I-iododeoxyuridine-labeled P388 leukemia cells in mice after treatment with NKT-01, and (b) colony formation of P388 cells withdrawn from the peritoneal cavity of NKT-01-treated mice as the experimental endpoints. In adidition, the effect of NKT-01

on cell cycle traverse *in vivo* was examined by using flow cytometry. We further describe the nature of action of NKT-01 by assessing the combination effect of NKT-01 with various types of antitumor agents in simultaneous and sequential treatment schedules *in vivo*.

Materials and Methods

Drugs

NKT-01 was prepared by Takara Shuzo Co., Ltd. (Ohtsu, Japan), dissolved in physiological saline, and stored in the dark at 4°C before use. Other antitumor drugs were purchased from commercial sources and dissolved in saline or distilled water just before use.

Mice and Tumors

$CD2F_1$ (BALB/c × DBA/2) and C57BL/6 mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). P388 leukemia is maintained by weekly transplantation in DBA/2 mice and inoculated into $CD2F_1$ mice for *in vivo* experiments. M5076 fibrosarcoma is maintained and used for experiment by sc implantation into C57BL/6 mice.

Labeling of P388 Leukemia Cells with ^{125}I-Iododeoxyuridine

P388 cells collected from peritoneal cavity were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 5 μM 2-mercaptoethanol and 60 μg/ml kanamycin in a spinner culture bottle at 37°C in 5% CO_2. After 1 day preculture, exponentially growing cells (8.8×10^4/ml) were labeled by an addition of ^{125}I-5-iodo-2'-deoxyuridine (^{125}IUdR) (New England Nuclear Research Products (Boston, MA)) at the concentration of 2.5 nCi/ml for 1 day. Labeled cells were washed 3 times and suspended in HANKS' balanced salt solution. Viability of the cells was determined by the trypan blue dye exclusion method, and incorporation of ^{125}IUdR into cells was measured with a Beckman model Gamma 8000 gamma spectrometer.

Determination of Cell Death

$CD2F_1$ mice were given 0.1% NaI-supplemented water to minimize accumulation of ^{125}I in thyroid gland, and inoculated ip with 2×10^7 labeled P388 cells in 0.5 ml. Administration of NKT-01, cisplatin (CDDP) or 5-fluorouracil (5-FU) was started 4 hours after the inoculation. Remaining ^{125}I in mice was monitored daily by counting the whole body radioactivity by inserting live mice into a plastic holder for the gamma spectrometer.[11] Measured radioactive counts of ^{125}I were compensated for radioactive decay of the isotope, and compared to the initial count.

Colony Formation of P388 Leukemia Cells

Mice were inoculated with 2×10^7/0.5 ml of P388 cells and treated with NKT-01 once a day for 5 days. The day after the final injection, P388 cells were collected from the peritoneal cavity and washed twice by centrifugation. The cells were suspended in culture medium and then mixed with noble agar solution at a final concentration of 0.1%. After incubation for 18 days at 37°C in 5% CO_2, numbers of colonies formed were counted. Surviving fraction was calculated by comparing the colony forming efficiency of cells from NKT-01-treated mice with that from control mice.

DNA Histograms

P388 cells were prepared for analysis of cell cycle traverse according to KRISHAN with slight modifications.[12] Briefly, P388 cells were collected from the peritoneal cavity and washed with phosphate-buffered saline. After fixing with 70% ethanol at −20°C, cells were washed, and incubated with 1 mg/ml ribonuclease-A (Sigma Chem. Co. (St. Louis, MO.)) in phosphate buffer (0.2 M, pH 7.0) at 37°C for 30 minutes. Thus treated cells were washed again and stained with 15 μg/ml propidium iodide at 4°C at least for 2 hours. DNA histograms were obtained by measurement of fluorescence of propidium iodide intercalating to cellular DNA with a flow cytometry (Coulter EPICS V).

Combination Chemotherapy

$CD2F_1$ mice were inoculated with P388 cells, and treated with NKT-01 and other antitumor drugs using various treatment schedules. ILS (%) was calculated from survival time of mice as follows;

$$\text{ILS (\%)} = \left(\frac{\text{median survival time of treated mice}}{\text{median survival time of control mice}} - 1 \right) \times 100$$

Combination effects were estimated by a combination index (CI), which was calculated as a ratio of ILS (%) in combination to the sum of ILS (%)'s of two single-drug-treated groups. When CI equals to 1, it means the combination effect is just additive. CI value more, or less than 1 means synergistic, or antagonistic effect, respectively.[13]

In the combination experiments of sc inoculated M5076 fibrosarcoma (10^6 cells), CI was based on the tumor growth delay, which was determined from time to reach 500 mm^3 in tumor volume. Tumor volume was calculated as follows;

$$\text{tumor volume} = L \times W^2/2,$$

where L and W are length and width of tumor mass, respectively.

Results

Mode of Cell-killing of NKT-01

To examine the features by cell-killing of NKT-01 *in vivo*, we monitored direct cell death by NKT-01 by the ^{125}I release method, and compared with those by CDDP and 5-FU. Excretion of ^{125}I from mice inoculated with $^{125}IUdR$-labeled P388 cells was determined after starting the drug administration (Fig. 1). Gradual decrease in remaining radioactivity in control mice was observed, which seemed to be due to excision repair of $^{125}IUdR$-incorporated DNA. No difference in excretion of ^{125}I from mice was observed between the control mice and NKT-01-treated mice within 4 days after tumor inoculation. Excretion of $^{125}IUdR$ due to cell death by NKT-01 was observed from day 5, and reached about half of control level on day 7. However, the life prolonging effect of NKT-01 could not be explained only by this small degree of cell-killing detected by ^{125}I excretion. These results suggest that NKT-01 acts on tumor cells in cytostatic manner in the early period and then repeatedly treated tumor cells with NKT-01 were gradually killed and excreted. In contrast to NKT-01, rapid decrease in remaining ^{125}I was observed immediately after CDDP treatment, and remaining radioactivity on day 7 decreased to about 10% of control level. Although 5-FU exhibited stronger life prolonging effect (shown in parentheses in Fig. 1) than single injection of CDDP and comparable activity to 5-day treatment of CDDP, the excretion of $^{125}IUdR$ was delayed and the remaining level on day 7 was larger than that of mice treated with CDDP by either treatment schedule. Therefore, these results indicate that cell cycle non-specific cytotoxic agents such as CDDP kill tumor cells immediately, while the cell cycle specific antimetabolites such as 5-FU exhibit cytotoxic effects after some delay. From this viewpoint, to demonstrate its cell-killing action, NKT-01 requires extraordinarily longer period as compared with other cell cycle specific drugs such as 5-FU.

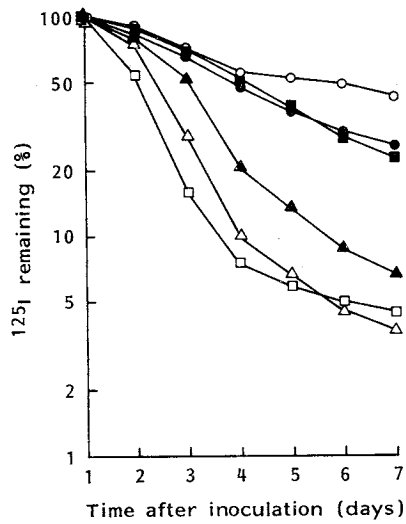
To determine the actual cell viability after 5-day treatment with NKT-01, clony forming ability of P388 cells treated with NKT-01 *in vivo* was determined in soft agar. As shown in Table 1, 5-day treatment with NKT-01 strongly reduced cellular viability to form colonies. Therefore, it was confirmed that cells were committed to death after the 5-day treatment (day 6), even though cells were not killed in the early phase (up to day 4 as shown in Fig. 1).

Combination Effect of NKT-01 with Other Antitumor Agents

The mechanisms of action of NKT-01 were thought to be different from other antitumor agents such

Fig. 1. Effect of NKT-01, CDDP and 5-FU on the rate of ^{125}IUdR excretion from mice inoculated with ^{125}IUdR-labeled P388 leukemia cells.

○ Control (100)*, ● NKT-01: 1.6×5 (123), ■ NKT-01: 25.6×5 (143), ▲ 5-FU: 20×5 (193), □ CDDP: 5×1 (152), △ CDDP: 1×5 (195).



NKT-01 was administered daily for 5 days (q1d×5) at the doses of 25.6 and 1.6 mg/kg/day. CDDP (5 mg/kg/total) was administered either by q1d×5 or single injection, and 20 mg/kg/day of 5-FU was administered by q1d×5.

* Numbers in the parentheses indicate life prolongation of tumor-bearing mice expressed as T/C (%).

as DNA binders, inhibitors of DNA biosynthesis, mitotic inhibitors and so on. Therefore, it was assumed from the general principles of combination chemotherapy that the combination of NKT-01 with other agents might be synergistic. We examined the effect of NKT-01 combined simultaneously with other drugs on P388 leukemia (10^6 cells) implanted ip into $CD2F_1$ mice. NKT-01 and 8 other drugs combined were administered daily for 5 days, since all drugs were known to show efficacy against

Table 1. Surviving fraction estimated from colony formation of P388 leukemia cells harvested from NKT-01-treated mice.

Drug[a]	Dose (mg/kg/day)	PE[b] (%)	SF[c] (%)
Control	0.0	41 ±7.2	100 ±18
NKT-01	25.6	0.30±0.11	0.73± 0.27
	1.6	1.0 ±0.35	2.4 ± 0.85

[a] NKT-01 was injected once a day for 5 days, and P388 leukemia cells were withdrawn 1 day after the final injection. Each group consisted of four mice, and colony formation was determined by tripricated culture of harvested peritoneal cells from each mouse.

[b] PE; plating efficiency = (number of colonies formed in soft agar)/(plated cell number) × 100.

[c] SF; surviving fraction = (PE of drug treatment)/(PE of control) × 100.

Table 2. Effect of simultaneous combination of NKT-01 with various antitumor drugs against P388 leukemia.

Drug	Dose[a] (mg/kg/day)	ILS (%) observed		ILS (%)[b] expected	CI[c]
		Single	Combine		
NKT-01	25.00	58, 66[d]	—	—	—
CDDP	0.50	22	62	80	0.78
Doxorubicin	0.50	68	103	134	0.77
Daunorubicin	1.00	60	103	126	0.82
5-FU	24.00	68	84	126	0.67
Ara-C	20.00	58	81	116	0.70
Methotrexate	1.25	53	71	111	0.64
Vincristine	0.125	60	79	118	0.67
Etoposide	0.50	57	90	123	0.73

[a] NKT-01 and each antitumor drug were administered simultaneously once a day for 5 days.

[b] ILS (%) expected is sum of ILS (%)'s produced by each drug alone and by NKT-01 alone.

[c] CI; combination index = (ILS (%) observed)/(ILS (%) expected). CI value being smaller than 1 means antagonistic effect.

[d] ILS (%) obtained by NKT-01 was 58% and 66% in the combination experiment No. 1 (with CDDP, 5-FU, Ara-C, methotrexate and vincristine), and experiment No. 2 (with doxorubicin, daunorubicin and etoposide), respectively.

P388 leukemia by this treatment schedule. As shown in Table 2, although the ILS in the combination was always larger than any ILS values obtained by the single agent treatments, the CI was less than 1.0 in all combinations examined. This means all the combinations were antagonistic, contrary to expectation. The CI values obtained in combinations with cell cycle non-specific agents (CDDP, doxorubicin and daunorubicin) were larger compared with cell cycle specific agents such as antimetabolites (5-FU, Ara-C and methotrexate), an inhibitor of topoisomerase II (etoposide) and a mitotic inhibitor (vincristine). From these results, it could be postulated that the cytostatic effect of NKT-01 limits the cell cycle progression of the tumor cells and decreased sensitive cell population to other agents passing through S phase to M phase, and then the cell cycle dependent effects of other agents were limited.

Next, from the above viewpoints, we examined sequential combination of NKT-01 in P388 leukemia. The results of the combinations of NKT-01 with cyclophosphamide (CYC), CDDP and Ara-C in various sequences were shown in Tables 3, 4 and 5, respectively. According to the treatment schedule dependency of each drug, single administration schedule was employed for CYC and CDDP, and 5 daily schedule was used for Ara-C and NKT-01. The sequential combinations of NKT-01 followed by these agents were also antagonistic in terms of combination index being less than 1.0, as well as the simultaneous combinations. In contrast, combinations in the reverse sequence, where the treatment with NKT-01 followed the other agents, produced synergistic effects.

To further examine the differential effect of the sequence of the combination, we used a solid tumor system. M5076 fibrosarcoma was inoculated sc and treated with 3 sequences of combination of NKT-01 and Ara-C as shown in Fig. 2. NKT-01 at 25 mg/kg/day and Ara-C at 80 mg/kg/day as a single treatment for 5 days produced almost the same activity on the tumor volume of M5076 fibrosarcoma. Growth inhibitory effect of the combination was superior to those of each single agent treatment in all sequences.

Table 3. Combination effect of NKT-01 and CYC against P388 leukemia.

CYC alone[a]		CYC with NKT-01 (day 1~5)[b]			CYC with NKT-01 (day 5~9)[c]		
Schedule	ILS (%)		ILS (%)	CI		ILS (%)	CI
—	0		76	—		39	—
Day 1	104	Simultaneous	141	0.78	CYC→NKT-01	162	1.13
Day 2	89					150	1.17
Day 3	85		120	0.75		125	1.01
Day 4	76					110	0.95
Day 5	82		122	0.77	Simultaneous	85	0.70
Day 6	76	NKT-01→CYC	116	0.76			
Day 7	67		116	0.81		79	0.75
Day 8	48		107	0.86			

[a] CYC was injected once on the indicated day at the dose of 80 mg/kg.
[b] NKT-01 was injected daily for 5 days starting day 1 (day 1~5) at the dose of 25 mg/kg/day, and CYC was injected once as shown in the first column.
[c] NKT-01 was injected on day 5~9 and combined with CYC.

Table 4. Combination effect of NKT-01 and CDDP against P388 leukemia.

Schedule	ILS (%) observed			ILS (%) expected	CI
	NKT-01[a]	CDDP[b]	Combine		
P388 ⇩ CDDP ↓↓↓↓↓ NKT-01	54	34	70	88	0.79
⇩ ↓↓↓↓↓	54	44	79	98	0.81
⇩ ↓↓↓↓↓	54	54	96	108	0.88
⇩ ↓↓↓↓↓	50	54	109	104	1.05
⇩ ↓↓↓↓↓	45	54	114	99	1.15

[a] NKT-01 (25 mg/kg/day) was injected daily for 5 days as shown by five arrows.
[b] CDDP (2 mg/kg) was injected once on the indicated day as shown by closed arrows.

Table 5. Combination effect of NKT-01 and Ara-C against P388 leukemia.

Schedule	ILS (%) observed			ISL (%) expected	CI
	NKT-01[a]	Ara-C[b]	Combine		
P388 NKT-01 ⇩ ↓↓↓↓↓ Ara-C ⇩⇩⇩⇩⇩	58	93	119	151	0.79
		77	96	135	0.71
Ara-C ⇩⇩⇩⇩⇩ NKT-01 ⇩ ↓↓↓↓↓	58	83	114	141	0.81
		57	99	115	0.86
Ara-C ⇩⇩⇩⇩⇩ NKT-01 ↓↓↓↓↓	35	83	163	118	1.38
		57	113	92	1.22

[a] NKT-01 was injected daily for 5 days at the dose of 25 mg/kg/day.
[b] Ara-C was injected daily for 5 days at the dose of 80 (upper) or 20 mg/kg/day (lower line in each combination schedule).

Fig. 2. Combination effect of NKT-01 and Ara-C on the growth of M5076 fibrosarcoma.

Solid tumor-bearing C57BL/6 mice were treated with 25 mg/kg/day of NKT-01 alone (●), 80 mg/kg/day of Ara-C alone (□), or combination (▲) in 3 treatment schedules. (A) NKT-01 (day 1～5) followed by Ara-C (day 6～10), (B) NKT-01 (day 1～5) and Ara-C (day 1～5) simultaneously, (C) Ara-C (day 1～5) followed by NKT-01 (day 6～10), ○: Control.

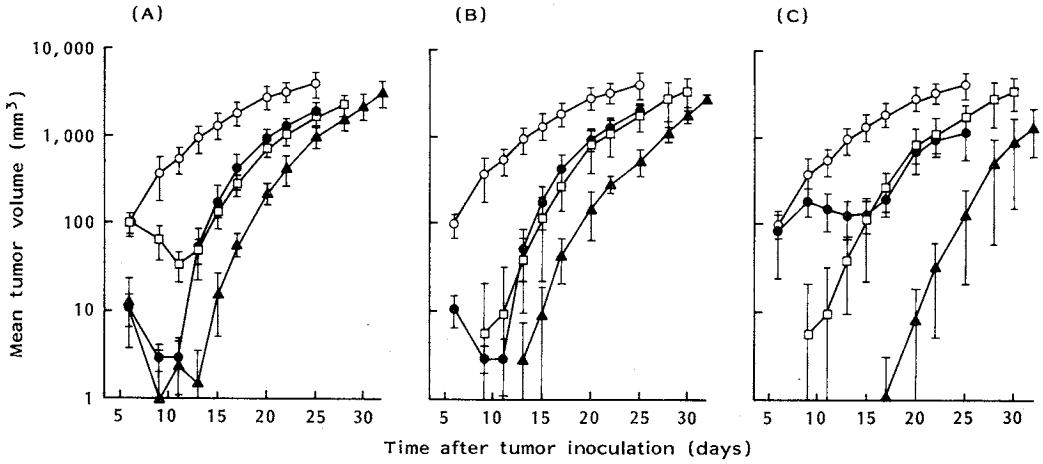
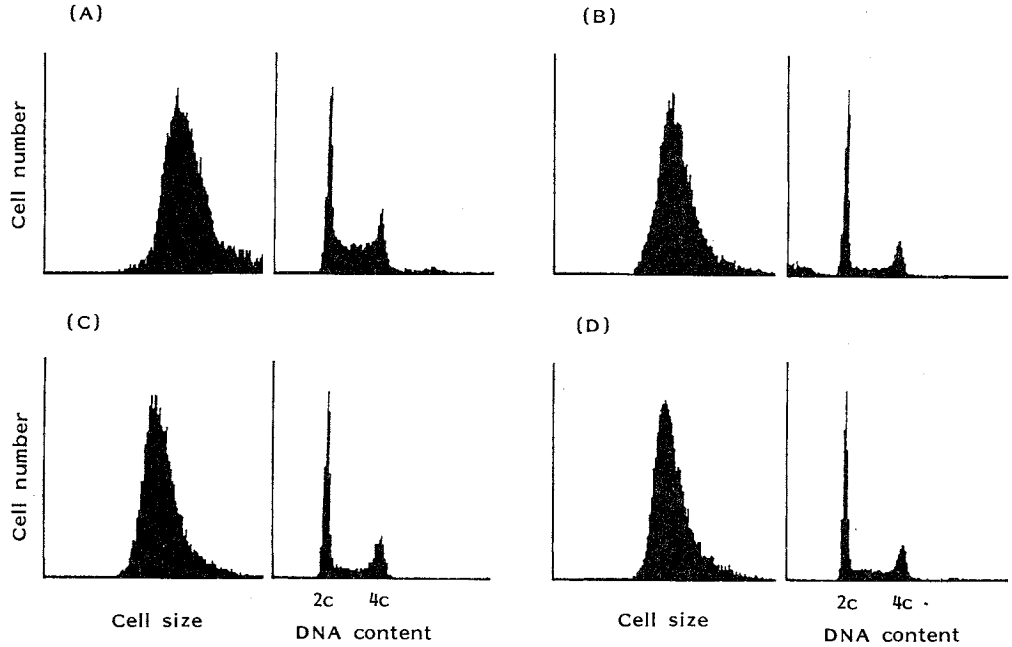


Fig. 3. Effect of NKT-01 on cell cycle progression of P388 leukemia cells.

(A) Control (day 3), (B) control (day 8), (C) NKT-01: 25.6 mg/kg × 3, (D) NKT-01: 25.6 mg/kg × 5.



P388 cells harvested from drug-treated mice were stained with propidium iodide, and the distribution was measured with flow cytometry.

However, tumor growth delay (TGD) in time to reach 500 mm^3 in tumor volume obtained by the simultaneous combination did not surpass the sum of TGD's produced by each single treatment (CI = 0.93). As is the same in the combination of NKT-01 followed by Ara-C (CI = 0.79), while the combination of Ara-C followed by NKT-01 inhibited the tumor growth synergistically over the sum of TGD of 2 single treatments (CI = 1.04). These sequence-dependent results obtained with M5076 fibrosarcoma were consistent with those obtained in the combination experiments using P388 leukemia.

These results in the sequential combination experiments support the assumption described above that tumor cells pretreated or simultaneously treated with NKT-01 were limited in ability to proceed through the cell cycle and showed decreased sensitivity to drugs following NKT-01.

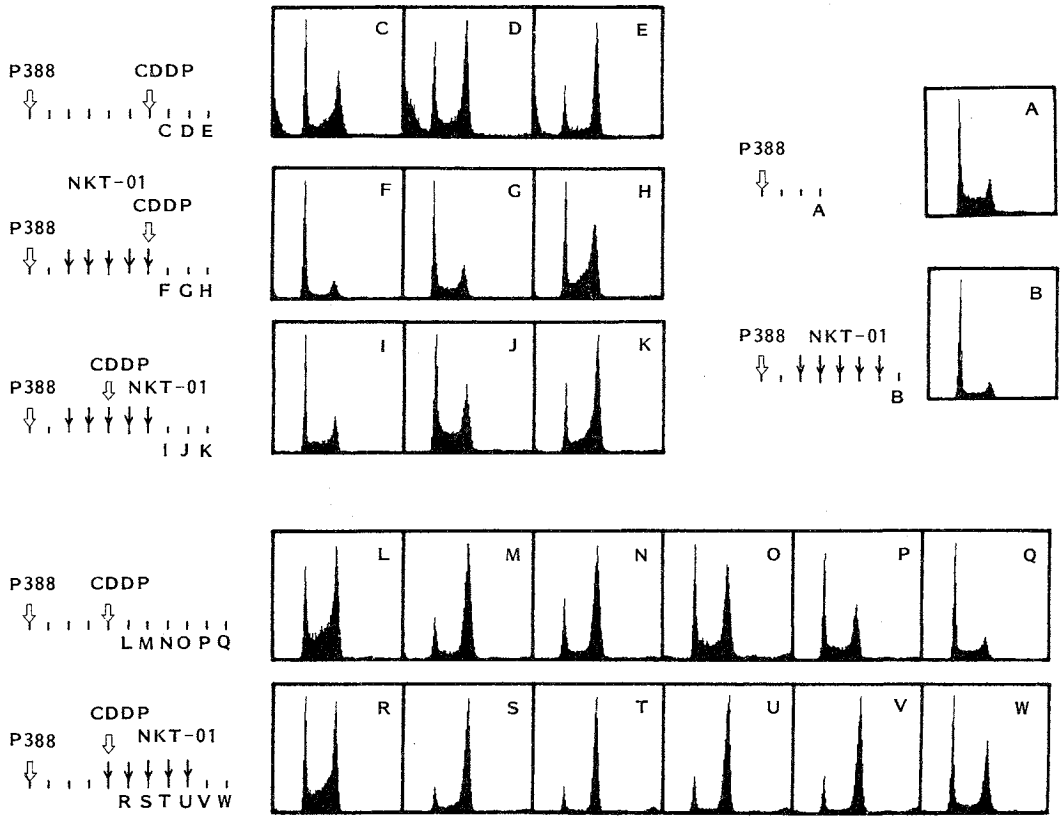
Effect of NKT-01 on Cell Cycle Traverse

In order to examine the effect of NKT-01 on cell cycle traverse, DNA histograms of P388 cells treated with NKT-01 *in vivo* were measured with by flow cytometry after propidium iodide staining. As shown in Fig. 3, NKT-01 decreased the cycling population in S and G_2-M phases and arrested the cells in G_0/G_1 phase (2c). Distribution of cell size was also shifted to smaller size than control cells. No obvious difference was observed between the histograms obtained after 3 injections of NKT-01 and 5 injections. These histograms of DNA content and cell size obtained after treatment with NKT-01 were similar to those of late stage of control (day 8), of which a large population seems not to proliferate any more.

Analysis of Cell Cycle Perturbation in Sequential Combinations of NKT-01 and CDDP

To confirm the implication of the effect of NKT-01 on cell cycle traverse in combination chemotherapy,

Fig. 4. Effect of NKT-01 on cell cycle perturbation of P388 leukemia caused by CDDP.



P388 leukemia cells were withdrawn from peritoneal cavity of tumor-bearing mice treated with 25 mg/kg/day of NKT-01 and/or 2 mg/kg of CDDP in various schedules as shown by arrows.

we further examine the DNA distribution of P388 leukemia cells from the mice treated with NKT-01 combined with CDDP. The change of DNA histograms by 3 sequential combinations as well as by single treatment were examined daily (Fig. 4 A to W). The CDDP-caused synchronization of tumor cells in G_2/M phase was observed from the day after the injection. The arrest was maintained for about 4 days and recovered gradually (Fig. 4 C to E, and L to Q). The G_2 blocking effect of CDDP injected after or in the middle of 5 daily treatment of NKT-01 was not remarkable until 3 days after the injection (Fig. 4 F to K) as compared with that by CDDP injected alone. In contrast, in the case of combination of CDDP followed by NKT-01, G_2 arrest caused by CDDP was observed the day after the administration as seen in single injection. And the effect was prolonged until day 6, whereas cells treated with CDDP alone began to recover from day 4 and showed an almost normal pattern on day 6 as seen in control. Moreover, the effect of CDDP in this sequential combination was so intensified that cell population in G_1 and S phases almost disappeared and that cells containing tetraploid DNA arose which were not obviously seen in the single treatment with CDDP (Fig. 4 R to W).

These results of cell cycle perturbation obtained in each sequential combination were consistent with the therapeutic combination effects in the corresponding sequences.

Discussion

In order to examine the nature of cell-killing of NKT-01 *in vivo*, we estimated direct cell death by means of ^{125}IUdR release and colony formation in soft agar. Apparent cell-killing was not observed in the early phase of NKT-01 treatment, and gradual cell death was detected after a few days. The strong effect of NKT-01 to prolong survival time of tumor-bearing mice could not be explained by this weak cell-killing activity. Since the life prolonging activity is thought to be outcome from both the cytostatic effect and subsequent cell-kill action, the long lasting cytostatic activity in the early period contributes to the exhibition of antitumor efficacy. In the case of CDDP, which cross-links DNA strands and directly kills tumor cells in a cell cycle non-specific manner, rapid excretion of ^{125}I from mice was observed after the injection. The excretion of ^{125}IUdR by 5-FU was delayed due to its cell cycle dependent antimetabolic effect, which mainly inhibits thymidylate synthetase. From this comparative experiment with CDDP and 5-FU, the effect of NKT-01 seems to be cell cycle dependent and to require a long time to cause cell death. This nature of cell-killing of NKT-01 could account for the strong dependency on exposure time in its exhibition of antitumor activity observed both *in vitro* and *in vivo*.[5,10]

NKT-01 arrested the cells in G_0/G_1 phase and reduced the cycling cell population (Fig. 3). HIRATSUKA *et al.* also examined the effect of NKT-01 on cell cycle progression of cultured L1210 leukemia cells.[14] They clearly demonstrated the delay of cell cycle traverse using 5-bromo-2′-deoxyuridine and its fluorescent-labeled antibody with flow cytometry. Usual antitumor agents cause G_2 phase arrest with exceptions of mitotic inhibitors, which arrest cells in M phase. Similar pattern of G_0/G_1 arrest was only clearly reported with tamoxifen, an effective antiestrogen,[15] and difluoromethylornithine, an inhibitor of polyamine biosynthesis.[16] Recent studies on regulation of cell cycling revealed that the conversion of G_0 cells to G_1 phase and progression to S phase are two critical steps for cell proliferation, and that many factors (for example, estrongen for mammary cells) and their signal transduction steps are involved in these steps of cell cycle.[17,18] Therefore, it is possible to postulate that NKT-01 may disturb the effect of such factors including oncogene/tumor suppressor gene products, and/or inhibit their signal transduction pathways.

In addition to antitumor effect, which was focused in the present studies, NKT-01 has been shown to have potent immunosuppressive activities.[7,8] The cytostatic effect and G_0/G_1 blocking activity observed to tumor cells might be involved in the inhibition of immunological response and growth of immune cells as well.

Since cycling cells have generally higher sensitivity than resting cells to usual antitumor agents, especially to cell cycle phase specific agents, the G_0/G_1 arrest and delay of cell cycle traverse caused by NKT-01 decreased the efficacy of subsequent antitumor agents. This was also confirmed by the facts observed in the flow cytometry experiments; occurrence of G_2 blockage by CDDP was delayed by the pretreatment or simultaneous treatment with NKT-01. On the contrary, the combination effect was synergistic in the case that NKT-01 was administered after the injection of other agents. This finding is consistent with the fact that the G_2 arresting effect of CDDP was intensified and lasted for longer period when it was followed by NKT-01. These results also indicate that NKT-01 not only arrests the cells in G_0/G_1 phase but also prolongs cycling time of cells passing through other phases.

Combination effect was thought to be influenced by several factors such as combination sequence, drug dose ratio, treatment schedule of each drug and so on. In this study we showed the critical role of administration sequence in the combination of NKT-01. Other factors were not examined intensively in this study. We used relatively low dose, which was expected to produce about 50% of ILS, rather than the optimal dose, to estimate the combination effect quantitatively, since combination of the optimal doses of two drugs might cure tumor-bearing mice and CI could not be calculated precisely. Also only single administration or 5-day daily treatment was selected for drug administration schedule due to similar practical reason. However, by changing the drug ratio, or schedule of each drug administration, more effective combination effect might be obtained.

Although the details of mechanisms of action of NKT-01 remain to be solved, its effect on cell cycle traverse plays an important role not only in the exhibition of antitumor activity but also in the outcome of the therapeutic effect of the combination. Synergistic combination effects of NKT-01 could be expected when it is administered after treatment with other antitumor drugs. This may be supported by the facts

that NKT-01 has no cross-resistance to other antitumor agents and that NKT-01-resistant tumor cells shows collateral sensitivity especially to cell cycle non-specific antitumor agents.[5]

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