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Cytostatic Effect of Deoxyspergualin on a Murine Leukemia Cell Line L1210

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The mode of antiproliferative action of deoxyspergualin (NKT-01) was examined. The growth-inhibitory effect on a murine leukemia cell line L1210 following treatment with NKT-01 was time-dependent, and there was little or no effect on the syntheses of DNA and RNA. Thus, the inhibitory activity of NKT-01 was not attributable to the inhibition of DNA and RNA syntheses. The influence of NKT-01 on cell cycle progression was studied by flow cytometric analysis. Bromodeoxyuridine/DNA distribution patterns in cells that were treated for 72 h, showed that the growth inhibition is due to the delay of cell cycle progression but not to cytotoxicity. This finding was also supported by evidence that the treated cells were re-proliferative in fresh medium. In addition, a majority of drug-treated cells was prevented from traversing from the G_0/G_1 phase to the S phase by 144 h or longer exposure to NKT-01. The results suggest that NKT-01 is cytostatic, preventing G_0/G_1-S progression.

Key words: Deoxyspergualin — Leukemia — G_1 arrest

Deoxyspergualin (NKT-01), a derivative of the antibiotic spergualin (SGL),[1] exhibits potent *in vivo* and *in vitro* antiproliferative activity against various malignant cells,[1-3] hematopoietic cells,[4] and mitogen-induced lymphocytes.[5-10] The *in vivo* activity of both NKT-01 and SGL correlated well with their *in vitro* activity in the presence of human serum.[3] The antiproliferative activity of NKT-01 both *in vitro* and *in vivo* was strongly dependent on treatment time; however, the mechanism responsible for its antiproliferative activity remains to be clarified. In the present study, we examined the mode of action of NKT-01 against a murine leukemia cell line in order to gain a better understanding of the nature of this compound.

Murine leukemia L1210 cells, which are much more sensitive than other cell lines to NKT-01 (unpublished data), were used to study the mode of action of NKT-01. The antiproliferative effect of NKT-01 was significantly dependent on the exposure period (Table I). To examine the effects on DNA and RNA syntheses, we analyzed the incorporation of ^{3}H-labeled precursors (thymidine and uridine) into cells that were reseeded in fresh medium without the drug following 22-, 43-, or 67-h treatment with NKT-01. Cell growth was significantly reduced following 43-h treatment, but little or no effect was observed on DNA and RNA syntheses during 67-h exposure, suggesting that the cell cycle was merely extended and that the antiproliferative activity of NKT-01 was not attributable to effects on replication and transcription.

To determine whether or not the growth inhibition is due to cytotoxicity, re-growth of NKT-01-treated cells was examined in drug-free medium. As shown in Fig. 1, NKT-01 inhibitory activity could not be rapidly abolished on removal of the drug at higher concentrations (0.1 and 1.0 μg/ml). The length of lag time up to re-proliferation increased in proportion to the concentration of NKT-01. No recovery was observed for up to 192 h at 1.0 μg/ml. However, the trypan blue dye exclusion test showed that 90% of the cells were still viable for up to 72 h after re-seeding (data not shown). To confirm that NKT-01-treated cells had a lag time up to re-proliferation, the cell cycle traverse rate was estimated from the BrdUrd/DNA distribution. As shown in Fig. 2, the BrdUrd-labeled untreated cells (A), initially in the S phase, moved into the G_2/M and G_0/G_1 phases, and re-entered the S phase approximately 12 h later. In contrast, most of the BrdUrd-labeled NKT-01-treated cells (B) still remained in the G_2/M and G_0/G_1 phases at 12 h. The distribution pattern in untreated cells at 12 h was similar to that in drug-treated cells at 24–27 h. Thus, the progression rate of drug-treated cells was delayed and was about half that of untreated cells. These findings suggest that the inhibitory action of NKT-01 on cell growth was attributable to a delay in the cell cycling rate,

Abbreviations used are: FITC, fluorescein isothiocyanate; BrdUrd, bromodeoxyuridine; TCA, trichloroacetate; PI, propidium iodide.

Table I. Influence of NKT-01 on DNA and RNA Syntheses in L1210 Cells

Treatment time (h)	NKT-01[a] (μg/ml)	Growth inhibition[b] (%)	Radioactivity (dpm/10^6 cells)	
			^{3}H-thymidine	^{3}H-uridine
22	none	0	297363 (100)[c]	62847 (100)
	0.01	−1.4	282566 (95.0)	62356 (99.2)
	0.1	−0.4	258813 (87.0)	58770 (93.5)
	1.0	0.4	262352 (88.2)	50949 (81.1)
43	none	0	327796 (100)	79080 (100)
	0.01	35.2	302597 (92.3)	61114 (77.3)
	0.1	43.9	261448 (79.8)	60175 (76.1)
	1.0	44.1	266015 (81.2)	57100 (72.2)
67	none	0	325545 (100)	69079 (100)
	0.01	50.1	336448 (103)	64835 (93.9)
	0.1	62.1	308596 (94.8)	65900 (95.4)
	1.0	69.6	288717 (88.7)	55353 (80.1)

a) NKT-01 was prepared at Takara Shuzo Co., Ltd., Japan.[1]
b) Growth inhibition was estimated from cell numbers before and after treatment with NKT-01.
c) Numbers in parentheses show relative values with respect to the control. [Method]: A murine leukemia cell line L1210 was cultured in RPMI1640 medium containing 10% human serum (Flow, USA) and 5 μM 2-mercaptoethanol, in an atmosphere of 5% CO_2 at 37°C. L1210 cells were seeded at a cell concentration of 3×10^4 cells/ml in a 75-cm^2 flask. After 22-, 43-, or 67-h treatment with NKT-01, cells were washed with fresh medium and reseeded in the absence of the drug, at a cell density of 1×10^5 cells/well in a 96-well Millititer plate (Millipore, USA). ^{3}H-Thymidine (1 μCi/ml) or ^{3}H-uridine (1 μCi/ml) was added to the appropriate wells and further incubation was carried out for an hour. The cells were washed with PBS(−) and then lysed with 5% TCA and 1% pyrophosphate for an hour at room temperature. The TCA-precipitates were washed twice with the same solution and collected on the filter of each well by aspiration. Each filter was then punched out and placed in a scintillation vial. The incorporation of ^{3}H-precursors into the TCA-precipitates was measured with a scintillation counter.

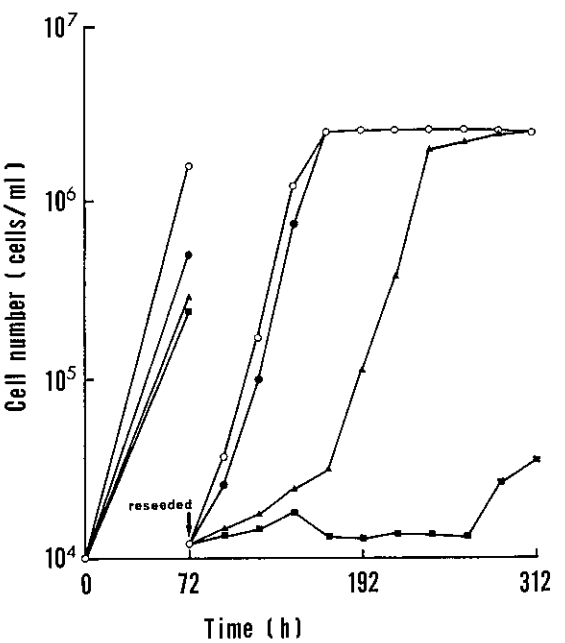

Fig. 1. Regrowth of L1210 cells treated with NKT-01 after removal of the drug. Cells were treated with NKT-01 at 0 (○), 0.01 (●), 0.1 (▲) and 1.0 (■) μg/ml and were re-cultured in drug-free medium for the indicated periods.

but not to cell killing. The effect of NKT-01 is similar to that on hematopoietic cells, in that proliferation activity is suppressed with little toxic effect by the chemical.[4] Thus, it is possible that the suppression of immune responses[5–10] is related to the antiproliferative effect on B- or T-lymphocytes.

Since the antiproliferative effect of NKT-01 was significantly time-dependent, and could be abolished by transferring the cells to fresh (drug-free) medium, cells were continuously treated with NKT-01 for up to 10 days in order to examine the effects on cell cycle transit. Accumulation at the G_0/G_1 phase was evident in cells treated with NKT-01 at all concentrations (0.1, 1.0 and 10 μg/ml) for 144 h (Fig. 3). Prolonged treatment resulted in an increase of the G_0/G_1 population and a decrease of the S population, with the appearance of cell debris. In addition, the reduction of cell viability was observed from 192 h after the beginning of treatment, and cell viability was decreased to 40% at 240 h (Fig. 4). Thus, the appearance of cell debris correlated well with the decline of cell viability, which was determined by the trypan blue dye exclusion test. This cytocidal effect of NKT-01 appeared to be a consequence of long arrest in the G_0/G_1 phase.

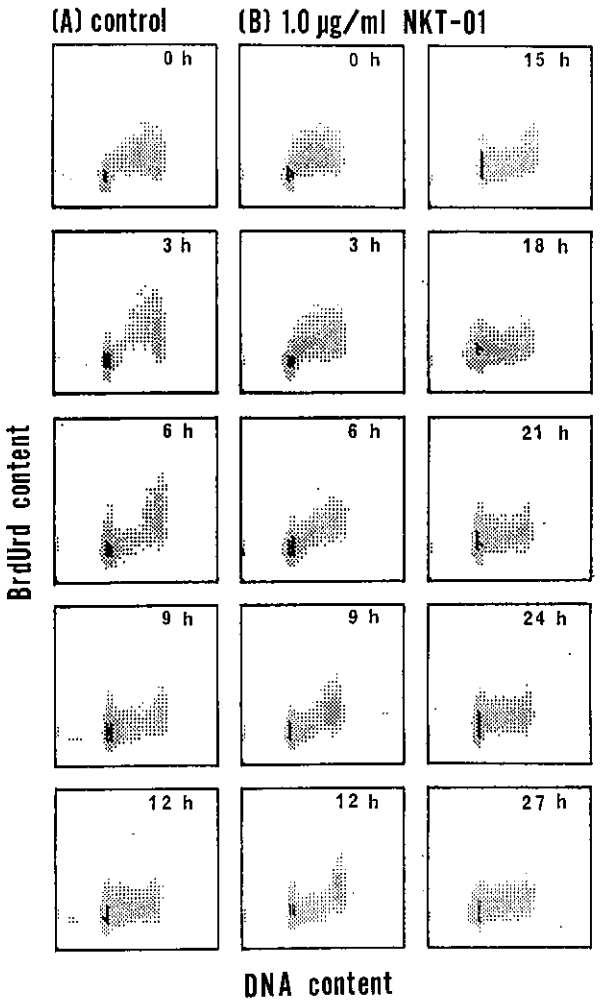

Fig. 2. Bivariate BrdUrd/DNA distribution measured for control (A) and NKT-01-treated cells (B) at 3-h intervals after 30-min treatment with 20 μM BrdUrd. L1210 was treated with NKT-01 at 1.0 μg/ml for 72 h. Control and drug-treated cells were pulse-labeled for 30 min with 20 μM BrdUrd. The cells were refed at a cell concentration of 6×10^5 cells/ml and were fixed in 70% ethanol at -20°C at 3-h intervals. Those cells were then stained with FITC-conjugated anti-BrdUrd antibody (Becton Dickinson, USA) and PI as described by Dolbeare *et al.*[15] Simultaneous analysis of cells for FITC- and PI-fluorescence was performed on an EPICS-V (Coulter Electronics, USA) at a laser excitation wavelength setting of 488 nm. The horizontal axis corresponds to total DNA content (PI fluorescence) and the vertical axis to amount of incorporated BrdUrd (FITC fluorescence).

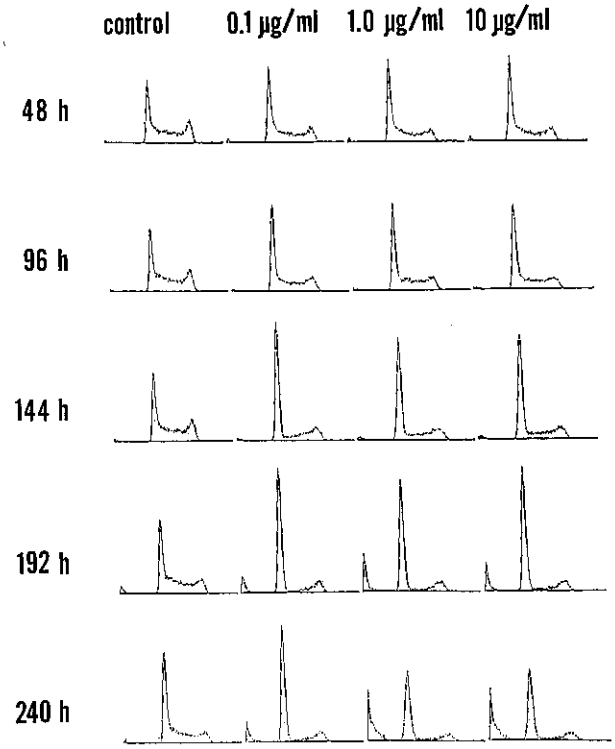

Fig. 3. DNA histograms of L1210 obtained after continuous NKT-01 treatment. L1210 cells were continuously treated with 0.1, 1.0, or 10 μg/ml of NKT-01 on alternate days, for up to 10 days. In advance of the drug treatment, cells to be drug-treated were diluted to a quarter and control cells were diluted 1/20th of their original density, so that confluence would not be reached. For analysis of DNA histograms, cells were fixed in 70% ethanol at -20°C after drug treatment and stained with PI by the method of Krishan[16] and analyzed on an EPICS-V.

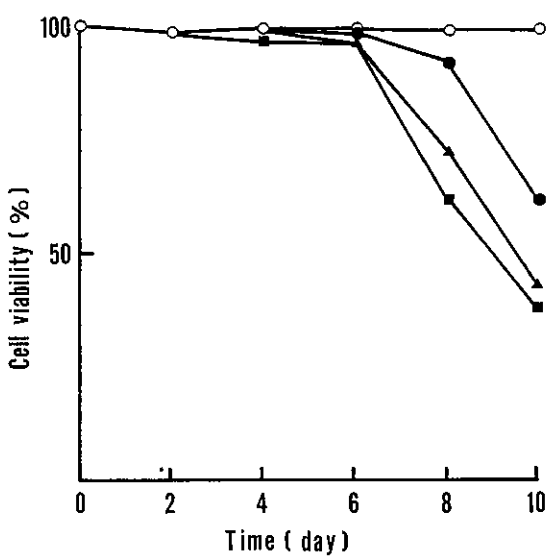

Fig. 4. Effect of NKT-01 on the cell viability of L1210. L1210 cells were treated with 0 (○), 0.1 (●), 1.0 (▲) or 10 (■) μg/ml of NKT-01 as described in Fig. 3. Cell viability was determined by means of the trypan blue dye exclusion test.

In conclusion, the results in the present studies suggest that NKT-01 interferes with a step related to commencement of DNA synthesis, and that the failure to enter the S phase resulted in cell-lethality. Similar results, achieved by induction of G_0/G_1 arrest, have been reported for interferon,[11] prostaglandin A_2,[12] TPA[13] (which suppressed c-*myc*), and GM_1[14] (which inhibited the phosphorylation of PDGF and EGF receptors). Thus, biochemical and molecular interactions between this chemical and possible regulators of cell proliferation should be examined in future studies.

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