THE ANTIPROLIFERATIVE ACTION OF DEOXYSPERGUALIN IS DIFFERENT FROM THAT INDUCED BY AMINE OXIDASE

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The amine oxidase activities contained in calf serum and human serum were detected at levels of 90.8 and less than 0.1 nmol O_2/minute/ml serum, respectively, by measuring oxygen consumption coupled with spermidine oxidation. Deoxyspergualin (NKT-01) and spergualin (SGL) containing spermidine in their structure were also oxidized in calf serum at the rate of 3.6 and 11.6 nmol O_2/minute/ml serum, respectively. To investigate whether amine oxidase is essential for NKT-01 and SGL to exhibit their antiproliferative activities or not, the *in vitro* activities of NKT-01, SGL and polyamines against L1210 cells were examined in the presence of calf or human serum. Polyamines exhibited antiproliferative activity only in the presence of calf serum, while NKT-01 and SGL inhibited cell growth in the presence of both calf and human serum. In the presence of calf serum the activity of NKT-01 was inhibited by aminoguanidine, an amine oxidase inhibitor. Aminoguanidine did not inhibit the activity of NKT-01 in the presence of human serum. The activity of NKT-01 was shown at much lower concentrations in the presence of human serum than that in the presence of calf serum, and was strongly dependent on incubation time. The *in vivo* activities of NKT-01, SGL and SGL derivatives correlated with their *in vitro* activities in the presence of human serum. These results suggest that the *in vivo* antitumor activities of NKT-01, SGL and SGL derivatives may be attributed to a mechanism different from those of amine oxidase-oxidized product and represent a novel growth inhibitory action.

Deoxyspergualin (NKT-01) is a novel derivative of the antitumor antibiotic spergualin (SGL) produced by a strain of *Bacillus laterosporus*[1,2]. NKT-01 and SGL have striking antitumor activity *in vitro* and *in vivo*[1,3~6]. The cytotoxic effect is thought to be due to the amine oxidase-oxidized product[7], although the molecular mechanism is still unclear. However, we found the action of NKT-01 and SGL different from that of amine oxidase-oxidized products. In this paper, we report the antiproliferative effect of NKT-01 which is not influenced by amine oxidase.

Materials and Methods

Materials

Human and calf sera were purchased from Flow Laboratories Inc., U.S.A., and fetal calf serum from Gibco Laboratories Inc., U.S.A. Aminoguanidine bicarbonate was obtained from Aldrich Chemical Co., Inc., U.S.A. Putrescine·2HCl, spermidine·3HCl and spermine·4HCl were obtained

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from Nakarai Chemicals Ltd., Japan. NKT-01 and SGL were prepared at Takara Shuzo Co., Ltd., Japan[8], and other SGL derivatives were prepared at Nippon Kayaku Co., Ltd., Japan.

Assay of Amine Oxidase

Amine oxidase activity was determined by measuring oxygen consumption with a Clark electrode[8]. Fifty μl of substrate was added to an electrode cell containing 700 μl of serum and 650 μl of 0.1 M sodium phosphate buffer, pH 7.2, and the rate of oxygen uptake was recorded with a Gilson K-ICT-O oxygraph.

Cell Preparation

The L1210 leukemia cell line was maintained by intraperitoneal passage of ascites in DBA/2 mice. For experiments, the L1210 cells were obtained from the peritoneal cavity of mice on day 5 or 6 after inoculation.

Determination of Growth Inhibitory Activity

L1210 cells were cultured at an initial density of 5×10^4 cells/ml/well in RPMI 1640 medium supplemented with 10% serum. After preincubation of 37°C for 2 hours in a CO_2 incubator, the drugs were added and the cultures were incubated for a further 48 or 72 hours. The inhibition of growth was estimated from cell numbers before and after treatment with drugs.

Determination of *In Vivo* Antitumor Activity

L1210 leukemia cells (10^5) were inoculated ip in CDF_1 mice on day 0. The drug was administered ip on days 1 through 9. Antitumor activity was expressed by the T/C (%) value based on mean survival time (survival time in a drug-treated group/that in a control group $\times$ 100).

Results and Discussion

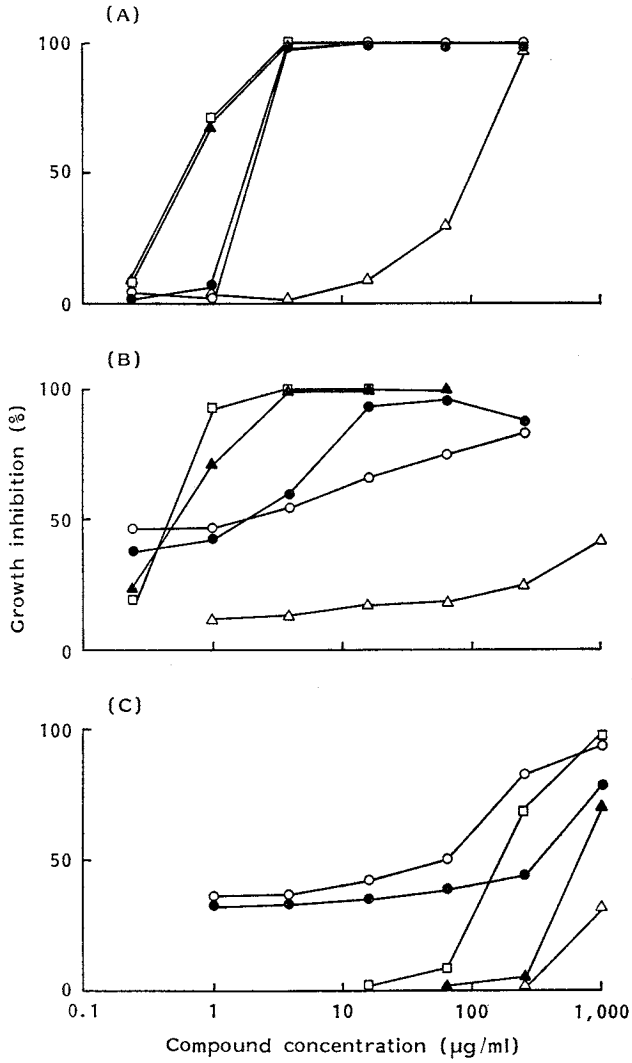
Since the cytotoxicity of SGL is reported to be dependent on the amine oxidase level in the serum used for culturing cells[7], we investigated the correlation between SGL cytotoxicity and the level of this enzyme. As shown in Table 1, when spermidine was used as substrate, the amine oxidase activities contained in calf serum, fetal calf serum and human serum were detected at the levels of 90.8, 1.0 and less than 0.1 nmol O_2/minute/ml serum, respectively. Calf serum thus contained the highest level of amine oxidase activity and the oxidation of NKT-01, SGL and three other polyamines, putrescine, spermidine and spermine, were measured in this serum. Spermine and spermidine were oxidized rapidly and putrescine was slightly oxidized. NKT-01 and SGL were oxidized at 1/25 and 1/8 the rate of spermidine, respectively.

As shown in Fig. 1, the growth inhibitory activities of spermine, spermidine and putrescine against L1210 cells were strongly dependent upon the level of amine oxidase in the serum. These activities also correlated with their rates of oxidation in calf serum. The antiproliferative activities of NKT-01 and SGL on the growth of L1210 cells were also dependent on the level of amine oxidase in the serum. The activities of NKT-01 and SGL were lower than that of spermidine or spermine in the presence of calf or fetal calf serum, but greater in the presence of human serum. The dose response curves of NKT-01 and SGL in the presence of human serum was

Table 1. Amine oxidase activity in various sera and oxidation rates of deoxyspergualin (NKT-01), spergualin (SGL) and polyamines by calf serum.

Serum	Substrate	Amine oxidase activity (nmol O_2/minute/ml serum)
Human	Spermidine	<0.1
Fetal calf	Spermidine	1.0
Calf	Spermidine	90.8
	NKT-01	3.6
	SGL	11.6
	Putrescine	0.2
	Spermine	172

Fig. 1. The growth inhibitory activity of deoxyspergualin (NKT-01), spergualin (SGL) and polyamines against L1210 leukemia cells in the presence of calf (A), fetal calf (B) or human (C) serum.
Incubation time was 48 hours.
○ NKT-01, ● SGL, △ putrescine, ▲ spermidine, □ spermine.



different from those in the presence of calf serum. In the presence of human serum, the response curve had a very flat slope and the effective concentration was below 1/100 of that in the presence of calf serum, although the cell growth was incompletely inhibited even at 100 μg/ml of NKT-01.

Next, we examined whether the activity of amine oxidase was related to the inhibitory activity of NKT-01. In the presence of calf serum, the inhibitory activity of NKT-01 was reduced by aminoguanidine, an amine oxidase inhibitor, in a dose dependent fashion. In contrast, this effect of aminoguanidine was not observed in the presence of human serum (Fig. 2). These results indicate that in medium containing human serum, the antiproliferative action of NKT-01 has a different mechanism from the amine oxidase-mediated cytotoxicity seen in the presence of calf serum.

The time dependency of the growth inhibitory activity of NKT-01 is shown in Fig. 3. The maxi-

Fig. 2. Effect of aminoguanidine on the growth inhibitory activity of deoxyspergualin (NKT-01) against L1210 leukemia cells in the presence of human (A) or calf (B) serum.
Incubation time was 72 and 48 hours in the presence of human and calf serum, respectively.
Aminoguanidine (μg/ml): ○ 0, ● 5, △ 10, ▲ 20, □ 40.

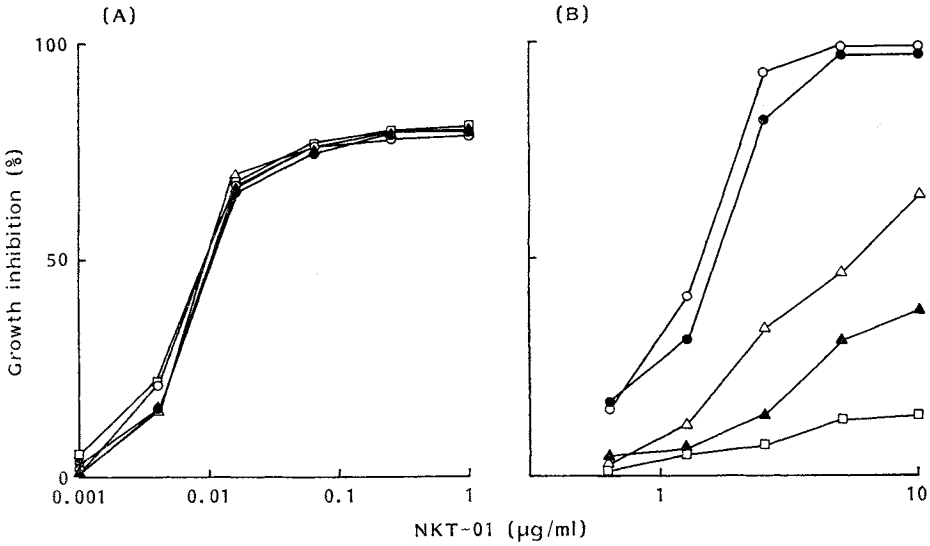
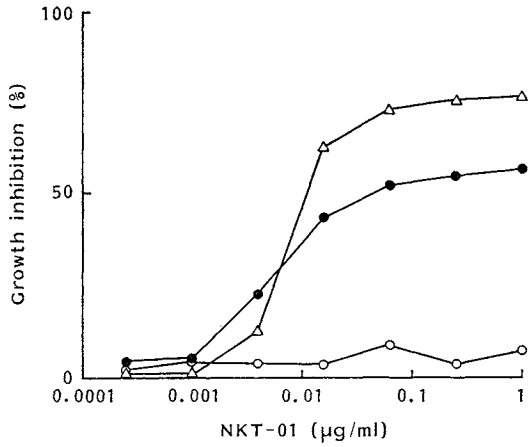


Fig. 3. Time dependency of the inhibition of L1210 leukemia cell growth by deoxyspergualin (NKT-01) in the presence of human serum.
Incubation time (hours): ○ 24, ● 48, △ 72.

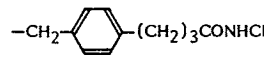
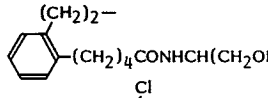
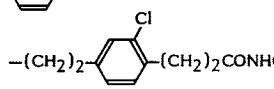


mum growth inhibition in the presence of human serum was strongly dependent upon incubation time, and even after 72 hours of incubation, the inhibition rate did not reach more than 90%. These results indicate that the action of NKT-01 in the presence of human serum is cytostatic rather than cytocidal.

NKT-01 and SGL have excellent *in vivo* antitumor activities against transplantable leukemias in mice such as L1210, P388, P815 *etc.*[4,6]. Therefore, we investigated the correlation between the *in vitro* and the *in vivo* activities of SGL derivatives against L1210 cells. As shown in Table 2, the *in vitro* activities in the presence of human serum correlated with the *in vivo* activities, but activities in the presence of fetal calf serum did not. This observation suggests that the *in vivo* antitumor activity

Table 2. Correlation between the *in vitro* activity and the *in vivo* activity of SGL derivatives against L1210 leukemia.

$$H_2NCNH\text{-}X\text{-}NH(CH_2)_4NH(CH_2)_3NH_2$$
$$\parallel$$
$$NH$$

Compound	Structure X	IC_{30}[a] (μg/ml)	IC_{50}[b] (μg/ml)	MED[c] (mg/kg)
NKT-01	$-(CH_2)_6CONHCH(OH)CO-$	<0.1	1.6	0.25
SGL	$-(CH_2)_4CH(OH)CH_2CONHCH(OH)CO-$	<0.1	1.7	0.78
1		<0.1	36	0.78
2		12.5	1.0	>12.5
3		7.0	101	>6.25

[a] Drug concentration in human serum required for 30% inhibition of cell growth.
[b] Drug concentration in fetal calf serum required for 50% inhibition of cell growth.
[c] Minimum effective dose at which T/C is beyond 150%.

of NKT-01 may be due to the novel, amine oxidase-independent action which we demonstrated in the presence of human serum using aminoguanidine.

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