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Inhibition of Pulmonary Metastases and Tumor Cell Invasion in Experimental Tumors by Sodium D-Glucaro-δ-lactam (ND2001)

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Sodium D-glucaro-δ-lactam (ND2001) inhibited spontaneous pulmonary metastases of the highly metastatic B16 melanoma variant with a maximal inhibition rate of 99.5%, and 6 of 7 animals remained metastasis-free. Likewise, ND2001 inhibited the spontaneous pulmonary metastases of both Lewis lung carcinoma (3LL) with a rate of 98.0% (3 of 5 animals remaining metastasis-free) and rat KDH-8 liver carcinoma with a rate of 82.5% (3 of 7 animals remaining metastasis-free), although it was unable to inhibit the metastases of mouse BMT-11 fibrosarcoma and rat SST-2 breast carcinoma. Pretreatment with ND2001 *in vitro* inhibited the pulmonary metastases of the B16 variant and 3LL cells, which indicates direct action upon the cancer cells. When the invasive activity of cancer cells was measured by the Boyden chamber method, the number of invading B16 variant or 3LL cells was reduced with maximal inhibition rates of 93.0% or 89.9%, respectively, but pretreatment with ND2001 failed to reduce the invasive activity of BMT-11 or SST-2 cells. ND2001 showed neither cytocidal nor antitumor activity. These results suggest that ND2001 inhibited pulmonary metastases at the invasive step into the basement membrane by directly changing some property of the tumor cells.

Key words: Antimetastatic agents — Extravasation — Invasion — Metastasis — ND2001

The initial step in tumor metastasis is the liberation of tumor cells from the primary tumor.[1,2] The most important step thereafter is considered to be the extravasation of tumor cells after adhesion to the endothelial cells of a blood vessel of the target organ. Accordingly, we carried out a screening program to seek chemical agents that might inhibit the latter stage of the metastatic process, and found sodium D-glucaro-δ-lactam (sodium 5-amino-5-deoxy-D-glucosaccharic acid-δ-lactam; ND2001) (Fig. 1).[3] ND2001 was synthesized via two oxidation steps involving C-1 and C-6 of the antibiotic nojirimycin.[4] We examined the inhibitory effect of ND2001 upon metastases by utilizing pulmonary metastasis models of mouse and rat tumors. We also examined both the *in vivo* antitumor activity and the *in vitro* cytocidal activity of ND2001, as well as the effect on the invading action of tumor cells. We found that the effect of ND2001 differs from that of all other antimetastatic agents so far reported.

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[4] Abbreviations: 3LL, Lewis lung carcinoma; EMEM, Eagle's minimal essential medium; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}.

MATERIALS AND METHODS

Cell lines and cell culture A highly metastatic variant of the B16 melanoma (the B16 variant) was isolated by following essentially the procedure described by Fidler.[1] In addition, a clone exhibiting spontaneous metastases was isolated from mouse lungs after the s.c. implantation of the above B16 variant into the footpad; this clone was used only for the experiments dealing with spontaneous pulmonary metastases. The other lines used were 3LL,[4] mouse BMT-11 fibrosarcoma,[5,6] rat KDH-8 liver carcinoma,[7] and rat SST-2 breast carcinoma.[8]

BMT-11 cells were cultured on EMEM supplemented with 8% FCS (Flow Laboratories, McLean, VA, USA). Other cells were cultured on DMEM supplemented with 10% FCS. All the cells were cultured at 37°C in a humidified 5% CO_2-95% air atmosphere.

Animal Seven-week-old male BDF_1 mice were used for the B16 variant implantation, 5-week-old female C57BL/6 mice for 3LL implantation, and 8-week-old female WKA/Hok rats for KDH-8 implantation. These animals were all purchased from Japan SLC Inc. (Shizuoka). Six-week-old female C57BL/6 mice used for BMT-11 implantation were purchased from Clea Japan Inc. (Tokyo). Ten-week-old female SHR rats used for SST-2 implantation were purchased from the Japan Rat Co. Ltd. (Saitama).

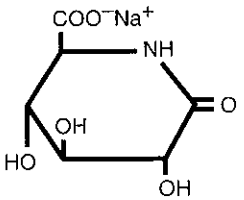

Fig. 1. Chemical structure of ND2001 (sodium D-glucaro-δ-lactam).

Metastasis experiments In experimental metastases, 1×10^5 cells of the B16 variant suspended in PBS were implanted i.v. into the tail of the mice. ND2001, prepared in our laboratories,[4] was administered i.v. to the mice twice a day starting from the day before the implantation (Day −1) to Day 4. The mice were killed on Day 14 and the pulmonary tumor colonies were counted. In the case of spontaneous pulmonary metastases, 5×10^5 cells were implanted s.c. into the right hind footpad, and the drug was administered i.m. once a day on Days 7 through 13, Days 14 through 20, Days 21 through 27 or Days 7 through 27. Pulmonary tumor colonies were counted on Day 35.

In the case of 3LL, 1×10^6 cells were implanted s.c. into the right hind footpad and the metastatic activity was assayed essentially according to the procedure reported by Shiio *et al.*[9] The footpad bearing the tumor was amputated on Day 14 to close off the host's immunity against the tumor and to induce metastases of the tumor cells circulating in the blood.[9, 10] The amputation wound was disinfected by applying povidone iodine (Meiji Seika Kaisha, Ltd., Tokyo). The drug was administered i.v. once a day on Days 14 through 18. Metastasized colonies on the surface of the lungs were counted after they had been fixed in Bouin's solution on Day 23 or 24.

In the case of BMT-11, 1×10^6 cells suspended in EMEM were implanted i.v., and the drug was administered once a day on Days −1, 4, and 5, and twice a day on Days 0, 1, 2, and 3. The wet weight of the lung was measured on Day 15 (Experiment 1) or Day 18 (Experiment 2). In the cases of KDH-8 and SST-2, 5×10^6 cells or 1×10^6 cells were respectively implanted s.c. into the right back. The drug was administered i.v. once a day on Days 1 through 30. The rats were killed on Day 31 for KDH-8 or Day 35 for SST-2. The metastasized colonies on the lung surface were counted after fixation. In the case of KDH-8, the diameter and wet weight of the tumor, and the wet weight of the lung were measured on Day 31. The number of pulmonary tumor colonies was expressed as the mean of all lungs, including those which were negative.

Measurement of *in vitro* growth We examined the effect of ND2001 on the *in vitro* growth of the B16 variant. The cells were cultured, harvested with 0.25% trypsin-1 m*M* EDTA solution from culture dishes and washed twice with PBS. Each well of the 12-well tissue culture plates was filled with 2 ml of 2×10^4 cells suspended in the culture medium, after which either ND2001 or doxorubicin was added. After culturing, the cells were detached at determined times, and suspended in PBS. The number of viable cells was counted by using the trypan blue dye exclusion method.

Pulmonary metastases of tumor cells after treatment with ND2001 The B16 variant or 3LL cells were cultured in the presence of ND2001 for 3 days. The cells were harvested and washed as described above, and suspended in PBS. The tumor cells (1×10^5 cells) were implanted i.v. into the tail vein of the mice, and pulmonary metastases were evaluated on Day 14.

Invasion assay The invasion of tumor cells was assayed essentially according to the Boyden chamber method.[11, 12] Twenty-four-well Transwell cell culture chambers were used (Costar, Cambridge, MA, USA), and 5 μg of Matrigel (Collaborative Research Inc., Bedford, MA, USA) and 10 μg of laminin (Collaborative Research Inc.) were respectively coated on the upper and lower surfaces of the Nucleopore filter (8.0 μm pore size).[12] We placed 600 μl of DMEM supplemented with 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) in the lower compartment. In one experiment, we used 3T3-conditioned medium in the lower compartment in place of the coating of laminin as a chemoattractant.[11] In this instance, 5 μg of Matrigel was coated on the lower surface of the filter in place of laminin. Tumor cells were harvested by treatment with 0.08% trisodium citrate in PBS for 20 min at 37°C, washed twice with DMEM supplemented with 0.1% bovine serum albumin, and suspended in the medium at 1×10^6 cells/ml. One hundred μl of the cell suspension was added to the upper compartment. The Transwell plate was incubated for 4 or 15 h at 37°C in a humidified 5% CO_2-95% air atmosphere. The cells that remained on the upper surface of the filters were removed, and the cells that had migrated to the lower surface were fixed and stained.[11, 12] The numbers of cells in 5 fixed areas (each 0.3 mm^2) were counted under a microscope at a magnification of 200. Each assay was performed in duplicate.

RESULTS

Inhibitory effect of ND2001 against pulmonary metastases of the B16 variant Experimental pulmonary metastases of the B16 variant were inhibited dose-dependently by ND2001 (Table I, Experiment 1). In the spontaneous pulmonary metastasis model, ND2001 also inhibited metastases (Table I, Experiment 2). ND2001 was more effective when administered at an earlier stage. When ND2001 was administered on Days 7 through 27 (total

Table I. Inhibition of Pulmonary Metastases of the B16 Variant by ND2001

Experiment	Implantation	Administration		Pulmonary metastases		
		ND2001 (mg/kg/day)	on Day	No. of mice with metastases/no. of mice used	No. of colonies	
					Mean±SD	%
1	i.v.	0[a)]	−1–4	5/5	217.4±26.8	100
		10	−1–4	5/5	71.6±45.7**	32.9
		30	−1–4	5/5	58.6±28.4**	27.0
		100	−1–4	5/5	18.8±17.9**	8.6
2	Footpad	0[a)]	7–13	6/6	19.3±21.0	100
		30	7–13	2/6	4.5±9.2	23.3
		30	14–20	2/6	2.2±4.4	11.4
		30	21–27	5/6	7.8±5.2	40.4
		30	7–27	1/7	0.1±0.4*	0.5

In experimental metastases (Experiment 1), 1×10^5 cells of the B16 variant suspended in PBS were implanted i.v. into the tail of BDF_1 mice. The drug was administered i.v. to the mice twice a day from the day before the implantation (Day −1) to Day 4. Pulmonary tumor colonies were counted on Day 14. In spontaneous metastases (Experiment 2), 5×10^5 cells of a spontaneous highly metastatic clone derived from the B16 variant were implanted s.c. into the right hind footpad and the drug was administered i.m. once a day on the indicated days. Pulmonary tumor colonies were counted on Day 35.
a) Control (vehicle, 0.9% NaCl).
*: $P<0.05$, compared with control by Student's two-tailed *t* test.
**: $P<0.001$, compared with control by Student's two-tailed *t* test.

Table II. Inhibition of Pulmonary Metastases of 3LL by ND2001

Experiment	Administration (mg/kg/day)	Pulmonary metastases		
		No. of mice with metastases/no. of mice used	No. of colonies	
			Mean±SD	%
1	Vehicle, 0.9% NaCl	4/4	29.5±7.0	100
	ND2001 (1)	6/6	15.2±4.8*	51.5
	ND2001 (3)	5/6	9.8±7.9*	33.2
	ND2001 (10)	3/6	1.0±1.5**	3.4
2	Vehicle, 0.9% NaCl	8/8	40.5±16.3	100
	ND2001 (10)	3/5	2.4±3.4**	5.9
	ND2001 (50)	4/5	1.6±1.1**	4.0
	ND2001 (100)	2/5	0.8±1.3**	2.0

3LL cells (1×10^6 cells) were implanted s.c. into the right hind footpad of C57BL/6 mice. The tumor was amputated on Day 14 and the drug was administered i.v. to the mice once a day on Days 14 through 18. Metastasized colonies on the surface of the lung were counted after fixation on Day 24 (Experiment 1) or Day 23 (Experiment 2).
*: $P<0.01$, compared with control by Student's two-tailed *t* test.
**: $P<0.001$, compared with control by Student's two-tailed *t* test.

treatment time, 21 days), metastasis was observed in only one of 7 mice, while it was observed in all the mice of the untreated group (control). Even so, the tumor volumes in footpads on Day 35, when taken together with the metastases evaluation, did not differ significantly between control and treatment groups (data not shown). This would indicate that ND2001 did not have a direct antitumor effect.

Inhibitory effect of ND2001 against pulmonary metastases of 3LL The colony sizes of pulmonary metastases induced by amputation of the primary tumor of 3LL were similar. The number of pulmonary tumor colonies was reduced dose-dependently by ND2001 at 1–10 mg/kg/day (Table II). The effect of ND2001 at 10 mg/kg/day was strong and comparable to the effect of 50 or 100 mg/kg/day.

Table III. Effect of ND2001 on Growth of Tumor and Pulmonary Metastases in KDH-8

Administration (mg/kg/day)	Tumor		Pulmonary metastases		
	Diameter (mm)	Weight (g)	No. of rats with metastases/no. of rats used	Lung weight (g)	No. of colonies
	Mean±SD (%)	Mean±SD (%)		Mean±SD (%)	Mean±SD (%)
Vehicle, 0.9% NaCl	62.5±3.0 (100)	34.5±6.6 (100)	5/5	3.1±0.8 (100)	277.6±59.1 (100)
ND2001 (30)	51.6±11.8* (82.6)	29.6±17.9 (85.8)	8/8	2.0±0.8* (64.5)	62.8±95.4*** (22.6)
ND2001 (100)	50.3±19.0 (80.5)	32.3±20.1 (93.6)	4/7	2.3±1.6 (74.2)	48.6±122.0** (17.5)
None[a)]				1.3±0.2	

KDH-8 cells (5×10^6 cells) were implanted s.c. into the right back of WKA/Hok rats and the drug was administered i.v. once a day on Days 1 through 30. The rats were killed on Day 31. The diameter and wet weight of the tumor, and the wet weight of the lung were measured. The metastasized colonies on the lung surface were counted after fixation.

a) No cells were implanted (5 rats).

*: $P<0.05$, compared with control by Student's two-tailed *t* test.

**: $P<0.01$, compared with control by Student's two-tailed *t* test.

***: $P<0.001$, compared with control by Student's two-tailed *t* test.

Inhibitory effect of ND2001 against pulmonary metastases of BMT-11 The inhibitory effect against pulmonary metastases of BMT-11 was evaluated by measuring the lung weight. The mean lung weight of the group which had been dosed with ND2001 at 100 mg/kg (n=5) was 78.2% (Experiment 1, 430±190 mg [mean±SD]) or 132.1% (Experiment 2, 370±140 mg) of the control group (n=5) dosed with 0.9% NaCl (Experiment 1, 550±200 mg; Experiment 2, 280±80 mg); this indicated that ND2001 did not inhibit pulmonary metastases of BMT-11.

Inhibitory effect of ND2001 against pulmonary metastases of KDH-8 Pulmonary metastases of KDH-8 were observed in all rats of the untreated group (Table III). By contrast, metastasis-free rats were observed in the group treated with ND2001 at 100 mg/kg/day, and the mean number of colonies of pulmonary metastases was reduced in the 30 and 100 mg/kg/day administration groups. Significant reductions in the wet weight of lung were observed when the 30 mg/kg/day administration group was compared with the control (vehicle administration group). No rats in the treated group died or grew weaker, but 3 rats in the control group and a rat in the 100 mg/kg/day administration group died as a result of tumor growth before Day 31 (these rats were excluded from the evaluation).

On the other hand, there were no significant differences in tumor weight between the control and treated groups, although we did observe a small difference of tumor diameter between the control and 30 mg/kg administration groups (Table III). This indicated that ND2001 did not have a significant effect on the primary tumor growth of KDH-8.

Inhibitory effect of ND2001 against pulmonary metastases of SST-2 While the number of colonies of pulmonary metastases was 485.0±97.8 (mean±SD) (100%) in the control group (n=6), the colony numbers in the groups treated with ND2001 were 417.3±180.7 (86.0%) at 30 mg/kg/day (n=7) and 346.6±202.2 (71.5%) at 100 mg/kg/day (n=7). While the wet weight of the lungs in the control group was 2.8±0.9 g (100%), the weights in the groups treated with ND2001 were 3.1±0.6 g (110.7%) at 30 mg/kg/day and 2.8±0.9 g (100%) at 100 mg/kg/day. The lung weight of rats in which no cells had been implanted (n=5) was 1.5±0.3 g. This indicated that ND2001 had no significant inhibitory effect on the pulmonary metastases of SST-2.

In summary, ND2001 inhibited pulmonary metastases of the B16 variant, 3LL and KDH-8, but did not inhibit the metastases of BMT-11 and SST-2 in 5 metastasis models. Further experiments were conducted to elucidate the cause of these differences in efficacy.

Cytotoxicity of ND2001 The cytotoxicity of ND2001 towards the B16 variant was examined *in vitro*. The growth of the B16 variant cells was strongly suppressed by the antitumor agent doxorubicin, but the growth of B16 cells in the presence of 1 mg/ml of ND2001 was similar to that of the no-addition control during incubation for 3 days, while the number of viable cells on Day 6 did not differ significantly between the culture augmented with ND2001 and the control (Fig. 2).

Pulmonary metastases of tumor cells treated with ND2001 *in vitro* The number of colonies of pulmonary metastases of the B16 variant and 3LL cells was reduced concentration-dependently by treatment with ND2001 *in vitro* (Fig. 3). When we examined the relation between *in vitro* exposure time and pulmonary metastases of the exposed B16 variant using 30 μg/ml of calcium D-glucaro-δ-lactam, which showed no cytotoxity, in 3 mice/group, we found that the mean colony number of pulmonary metastases was 182 in the untreated control, but that the number dropped to 58 (32% of the control)

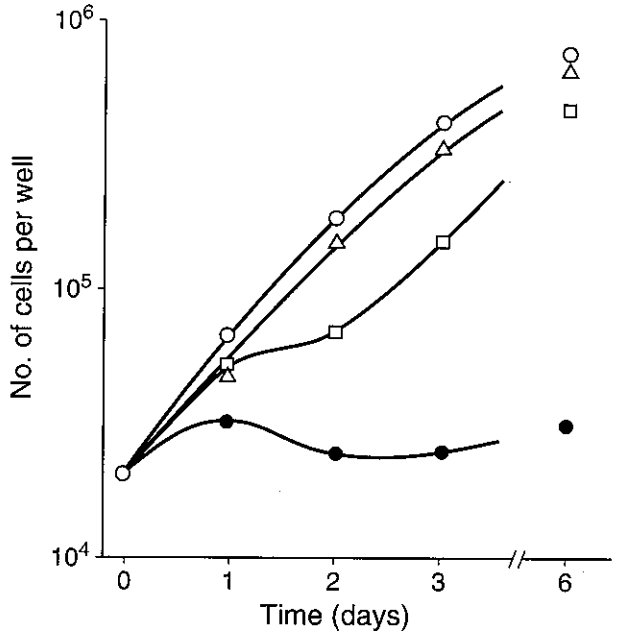

Fig. 2. Effect of ND2001 on *in vitro* growth of the B16 variant. Either ND2001 or doxorubicin was added to the culture of the B16 variant cells (2×10^4 cells/2 ml). The cells were harvested, washed and suspended in PBS. Viable cells were counted by using the trypan blue exclusion method. Symbols: ○, no addition; △, 1 mg/ml of ND2001; □, 3 ng/ml of doxorubicin; ●, 10 ng/ml of doxorubicin.

Table IV. Inhibition of Invasive Activity of Tumor Cells by ND2001

Experiment	Tumor cell line	ND2001 (μg/ml)	Invasion	
			Cell no./mm²	%
1	B16 variant	0	158.7	100
		100	50.0	31.5
2	B16 variant	0	43.0	100
		100	7.3	17.0
3	3LL	0	108.7	100
		1	47.3	43.5
		10	26.7	24.6
		100	22.3	20.5
4	3LL	0	138.3	100
		100	14.0	10.1
5	BMT-11	0	23.3	100
		100	17.3	74.2
6	BMT-11	0	50.7	100
		100	66.7	131.6
7	SST-2	0	4.7	100
		100	4.0	85.1
8	SST-2	0	4.7	100
		100	5.3	112.8

The tumor cells were cultured in the presence of ND2001 for 3 days. Numbers of cells which invaded the reconstituted basement membrane Matrigel in 4 h (Experiments 1–5, 8) or 15 h (Experiment 7) were counted by the procedure described in "Materials and Methods." A laminin coating under the filter surface (Experiments 1, 3–8) or the 3T3-conditioned medium in the lower compartment of the Transwell chamber (Experiment 2) was used as a cell attractant.

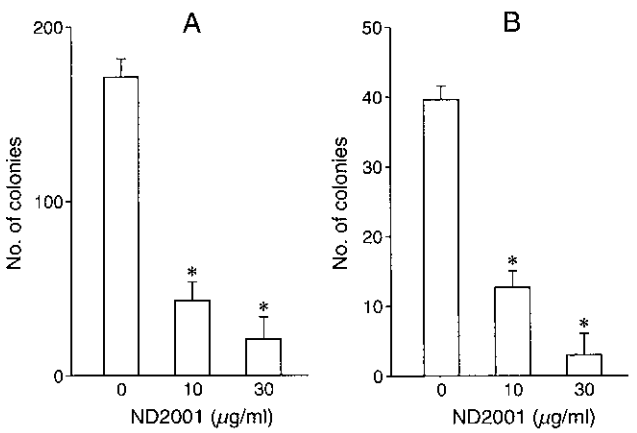

Fig. 3. Inhibition of pulmonary metastases of the B16 variant (A) and 3LL (B) after treatment with ND2001. Each cell line was cultured in the presence of ND2001 at the indicated concentration for 3 days. The cells were harvested, washed and suspended in PBS. Then 1×10^5 cells of the B16 variant or 3LL were implanted i.v. into the tail vein of BDF_1 or C57BL/6 mice, respectively. The pulmonary metastases were evaluated on Day 14. *: $P<0.001$, compared with the control (cells cultured without ND2001) by Student's two-tailed *t* test.

during 6-h exposure, to 42 (23%) during 24-h exposure, to 26 (14%) during 48-h exposure and to 8.7 (4.8%) during 72-h exposure. Even a 6-h treatment with ND2001 was therefore enough to cause a reduction in the metastatic ability of the tumor cells.

Effect of ND2001 on invasive activity of tumor cells The numbers of cells of the B16 variant and 3LL which showed invasive activity were reduced by *in vitro* treatment with ND2001 (Table IV). When we examined the exposure time, we observed an inhibitory effect after the 6-h treatment (data not shown), which suggests that ND2001 induced some change of the cells within a short time. On the other hand, ND2001 did not inhibit the invasion of BMT-11 and SST-2 cells (Table IV), and as KDH-8 cells maintained only *in vivo* did not show any invasive activity, no invasion assay was possible in that case.

DISCUSSION

Although many antitumor agents inhibit metastases by direct action on tumor cells,[13] this effect is due to their cytotoxicity. The results obtained in this study suggest

that ND2001 inhibits pulmonary metastases by a direct action on tumor cells without either inhibiting tumor cell growth or having any significant antitumor effect. ND2001 appears, in this respect, to be a new antimetastatic agent with high specificity that differs from known antitumor agents and immunopotentiators[14, 15] presently used in clinics.

The B16 melanoma variant, 3LL (lung carcinoma) and KDH-8 liver carcinoma, all of which turned out to be sensitive to ND2001, appear to have no common property as regards animal species, organs or tissue types from which the tumors originated. Likewise, there is apparently no common feature between BMT-11 fibrosarcoma and SST-2 breast carcinoma, neither of which was sensitive to ND2001. When an invasion assay system was used to investigate *in vitro* the processes of intravasation and extravasation of tumor cells,[11, 12] however, we found that when ND2001 inhibited pulmonary metastases, it also inhibited the invasion of the tumor cells into the reconstituted basement membrane (Matrigel) set in the Boyden chambers. Conversely, when ND2001 failed to inhibit metastases, the tumor cells did not lose their invasive ability. We therefore consider that the *in vitro* efficacy of ND2001 on sensitive and insensitive tumor cells in the *in vivo* evaluation system is reflected by their *in vitro* efficacy in the invasion assay.

When the sensitive tumor cells were treated with ND2001 *in vitro*, the growing ability of the tumor cells remained intact, but their metastatic ability was reduced. In this case, ND2001 was not administered to mice. It therefore seems likely that the antimetastatic activity of ND2001 is due to direct modification of the cell surface. Furthermore, we deduce from the invasion assay data and the measurement of pulmonary metastases of tumor cells treated with ND2001 *in vitro*, that the target of metastatic inhibition of ND2001 might be a process required for the extravasation of tumor cells. If so, the coincidence of the inhibitory activity on *in vitro* invasion and the *in vivo* antimetastatic activity can be easily understood, since ND2001 acted directly upon the cancer cells.

Agents that inhibit metastasis by inhibiting extravasation have been reported. These agents are modifiers of carbohydrates on the cell surface,[16–18] or agents which interact with adhesion molecules,[19, 20] or inhibitors of cellular matrix degradative enzymes.[21, 22]

Nojirimycin is able to inhibit such processing enzymes of the cell membrane carbohydrate chains as glucosidase I; it is also able to modify the carbohydrate chains of the tumor cell surface[23, 24] and to inhibit metastases.[25] But nojirimycin at higher concentrations has cytotoxicity.[25] Likewise, castanospermine, which inhibits glucosidase I, or swainsonine, which inhibits α-mannosidase II, or KI-8110, which inhibits sialyltransferase, can also inhibit metastases by modifying the cell surface of tumor cells.[16, 17] As a result of the modification of the cell surface, the tumor cells become susceptible to attack by immune cells and their adhesion to the blood vessel is weakened, leading to the suppression of metastases.[17, 26]

ND2001, however, did not show inhibitory activity against the above-mentioned enzymes (unpublished results). On the other hand, although ND2001 did not inhibit cell adhesion of the B16 variant or 3LL cells to Matrigel, it did inhibit cell migration in the haptotactic migration assay[12] in each cell line (unpublished results). Thus it appears to differ from substances that interact with adhesion molecules[19, 20] or inhibitors of extracellular matrix degradative enzymes,[21, 22] and might inhibit cell migration. At the same time, ND2001 is a potent β-glucuronidase inhibitor.[27] We thus suppose that ND2001 possesses a unique mechanism for the inhibition of metastases, although the relationship between metastasis (or the invasion process) and β-glucuronidase is not clear.

If ND2001 is to be considered as suitable for clinical application, we shall need to develop a simple assay for estimating its possible efficacy, since some tumors are expected to be sensitive and others insensitive to ND2001. Moreover, since we do not expect ND2001 to have an antitumor effect, it is not likely to cure metastasized tumors already established. The clinical value of ND2001 might therefore be limited to its use as an agent in adjuvant chemotherapy to prevent metastasis after diagnosis.

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