Anti-Cancer Drugs 1998, 9, pp. 88–92

Research paper

A new anti-metastatic drug, ND-2001, inhibits lung metastases in rat hepatoma cells by suppressing haptotaxis of tumor cells toward laminin

Yasuhiro Kuramitsu, Jun-ichi Hamada,[1] Tsutomu Tsuruoka,[2] Kiyoshi Morikawa, Hiroshi Kobayashi and Masuo Hosokawa

Laboratory of Pathology and [1]Cell biology, Cancer Institute, Hokkaido University School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060, Japan. [2]Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222, Japan.

We examined the effects of *ex vivo* treatment of tumor cells with sodium D-glucaro-δ-lactam (sodium 5-amino-5-deoxy-D-glucosaccharic acid-δ-lactam; ND-2001). The *ex vivo* treatment of rat hepatoma cKDH-8/11 cells with this new synthetic product of the antibiotic nojirimycin, ND-2001 (50 μg/ml), inhibited the experimentally induced lung metastases of the tumor cells significantly at an inhibition rate of 69.2% (one of 10 animals remained metastasis free). Also, it was elucidated in *in vitro* tumor cell invasion assays that ND-2001 (50 μg/ml) suppressed the invasion activities of cKDH-8/11 cells to Matrigel™ Matrix at an inhibition rate of 69.3%. However, phagokinetic track assays revealed that ND-2001 did not suppress the random motility of cKDH-8/11 cells. However, ND-2001 (50 μg/ml) suppressed the haptotaxis, another important role in tumor invasion, of cKDH-8/11 cells toward laminin (inhibition rate of 77.0%). These results suggest that ND-2001 suppressed the haptotaxis of tumor cells toward laminin directly at the step of invading the basement membrane and brought about the inhibition of lung metastases. [© 1998 Rapid Science Ltd.]

Key words: Haptotaxis, invasion, metastasis, random motility.

Introduction

Cancer metastasis is crucial to patients who expect to recover from cancer. The greatest challenge to clinicians in the treatment of cancer is the threat of distant metastasis, for metastasis is a complex multistep process: proliferation, local invasion, intravasation, migration in blood circulation, lodgement at the capillary bed in distant organs, extravasation and proliferation in the target organ. Each of these steps is complex, involving various cellular functions such as changes in cell mobility, adhesion and release of lytic enzymes in the invaded zone.[1–5]

Nojirimycin A, 5-amino-5-deoxy-D-glucose, is an antibiotic which inhibits tumor metastases.[6] Sodium D-glucaro-δ-lactam (sodium 5-amino-5-deoxy-D-glucosaccharic acid-δ-lactam; ND-2001) is a new synthetic derivative of the antibiotic nojirimycin.[7,8] Tsuruoka *et al.* reported that lung metastases of experimental tumors were inhibited by administration of ND-2001.[9] However, it has not been elucidated how ND-2001 inhibits lung metastases of tumors. Here, we set out a possible mechanism responsible for the inhibition of tumor lung metastases by ND-2001, using tumor cells treated with ND-2001 *ex vivo*.

Materials and methods

Animals

Female Wister King Aptekman/Hok (WKAH) rats, 8–12 weeks old, were supplied by the Experimental Animal Institute of Hokkaido University School of Medicine (Sapporo, Japan). The animals were kept in a room with controlled temperature, humidity and a 12 h light/dark cycle. Food and water were supplied *ad libitum*.

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture, and the Ministry of Health and Welfare, Japan.

Correspondence to Y Kuramitsu, First Department of Biochemistry, Yamaguchi University School of Medicine, Kogushi 1144, Ube 755, Japan. Tel: (+81) 836-22-2213; Fax: (+81) 836-22-2212

Cell lines

KDH-8 is a rat transplantable hepatocellular carcinoma induced by 3'-methyl-4-dimethylaminoazo-benzene in a WKAH rat and maintained *in vivo* by i.p. passage every 5 days.[10] cKDH-8/11 is a sub-clone isolated from the primary culture of KDH-8 tumor cells by limiting dilution and has properties similar to those of the parent *in vivo* line. cKDH-8/11 cells were maintained in a continuous *in vitro* culture in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS).[11,12] RLE is a rat lung endotherial cell line derived from Fisher 344 rats. Those cells were maintained continuously *in vitro* in 1.0% gelatin-coated plastic tissue culture plates (Corning) containing a 1:1 ratio of DMEM and Ham's nutrient mixture F12 (Nissui) medium supplemented with 15% FBS.[13]

Reagents

Sodium D-glucaro-δ-lactam was synthesized via two oxidation steps involving C-1 and C-6 of the antibiotic nojirimycin.[7-9] Figure 1 shows the chemical structure of ND-2001. ND-2001 was kindly provided by Meiji Seika Kaisha (Yokohama, Japan).

Treatment of cKDH-8/11 cells with ND-2001 *ex vivo*

cKDH-8/11 cells (2×10^6) were seeded onto 100 mm culture dishes in 9 ml of RPMI 1640 medium supplemented with 10% FBS for culturing. Then, 1 ml of ND-2001 (1 mg/ml, 500 μg/ml, 250 μg/ml or 100 μg/ml) in RPMI 1640 medium supplemented with 10% FBS was added into the culture (final concentrations of ND-2001 were 100, 50, 25 or 10 μg/ml). As control, only RPMI 1640 medium supplemented with 10% FBS was added as control. The cultures were incubated at 37°C in a CO_2 incubator for 24 h.

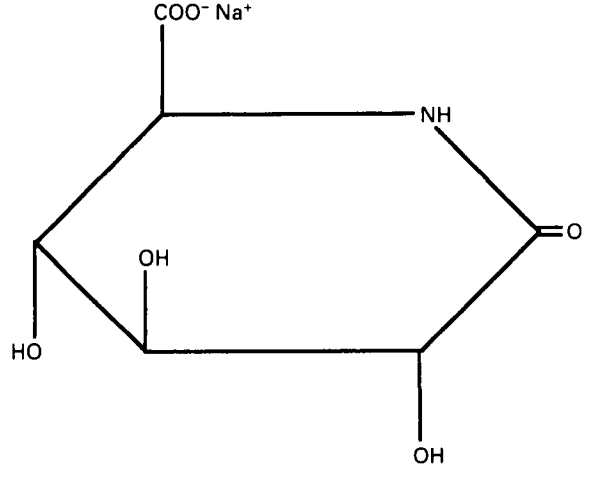

Figure 1. Chemical structure of ND-2001.

Lung metastases of tumor cells after treatment with ND-2001 *ex vivo*

cKDH-8/11 cells treated with or without ND-2001 for 24 h were harvested, washed and suspended in PBS^-. The cells (1×10^5) were implanted i.v. into the tail vein of the rats. The animals were killed 16 days after the tumor implantation for examination of lung metastasis. The colonies metastasized in the lung surface were counted after fixation. The lung was weighed before fixation.

Invasion assay

Invasive activity of the tumor cells was assayed using Transwell™ cell culture chambers (Costar, Cambridge, MA) according to the method previously reported.[14-16] Briefly, the lower surfaces of polyvinylpyrrolidone-free polycarbonate filters with 8.0 μm pores were precoated with 0.1 mg/ml of laminin in PBS^- (5 μg/filter). Matrigel™ Matrix (Becton Dickinson Labware, Bedford, MA) was diluted to 1 mg/ml with cold PBS^-, applied to the upper surface of the filter (10 μg/filter) and dried at room temperature under a hood. The coated filters were thoroughly washed in PBS^- and then dried immediately before use. The tumor cells were harvested with 2 mM EDTA in PBS^-, washed three times with serum-free RPMI 1640 medium and then resuspended in RPMI 1640 medium containing 0.1% bovine serum albumin (BSA) to a final concentration of 10^6 cells/ml. The cell suspensions (100 μl each) were added to the upper compartment and incubated for 3 h at 37°C in a 5% CO_2 atmosphere. We used conditioned medium of RLE cells as a chemoattractant. Three hours later the filters were fixed with 5% glutaraldehide in PBS^- and were stained with Giemsa solution. The cells attached to the upper surface of the filters were wiped with cotton swabs. The cells attached to the under surface of the filter were counted under a microscope.

Phagokinetic track assay

Glass coverslips (18 × 18 mm) were dipped into a 1% BSA solution. After draining the coverslip by holding the

filter edge, we dipped it again into 100% EtOH and rapidly drained it in the hot air stream of a hairdryer. The coverslip was then placed in a 35 mm culture dish and 1.5 ml of the hot gold particle suspension was laid on top of the coverslip. After 45 min of incubation in the particle suspension at room temperature, the coverslip was washed four times with RPMI 1640 containing antibiotics (PSN). cKDH-8/11 cells (1×10^4) were seeded on the coverslip in RPMI 1640 medium supplemented with 2% FBS and PSN. After 24 h of incubation, the cells attached to the coverslip were fixed with 10% formalin solution and the coverslip was mounted on a slide glass. The phagokinetic track area was analyzed by a Cosmozone R500 (Nikon).

Haptotaxis assay

Rat laminin was coated on the under surface of filters of Transwell™ (8 μm pore size; 6.5 mm diameter) chambers (5 μg/filter) and was air-dried on a clean bench overnight. Immediately before use, the filters were washed three times with RPMI 1640 medium. cKDH-8/11 cells treated with or without ND-2001 were detached from the culture dishes with 0.2% trypsin and 2 mM EDTA in PBS^-, and washed twice with RPMI 1640 medium supplemented with 0.1% BSA; they were placed into the lower compartment. We used conditioned medium of RLE cells as a chemoattractant. After 1 h incubation, the filters were fixed with 5% glutaraldehide in PBS^- and were stained with Giemsa solution. The cells attached to the upper surface of the filters were wiped with cotton swabs. The cells attached to the under surface of the filters were counted under a microscope.

Statistical analysis

Significant difference between two values was determined with Student's *t*-test.

Results

Effects of ND-2001 on lung metastases of cKDH-8/11 cells in rats

Table 1 shows that *ex vivo* ND-2001 treatment inhibited artificial lung metastases of cKDH-8/11 cells in WKAH rats. The number of lung metastasis colonies in the rats implanted with cKDH-8/11 cells treated *ex vivo* with ND-2001 (50 μg/ml) was significantly less than that of untreated cKDH-8/11 cells ($p < 0.02$). ND-2001 inhibited the experimental lung metastasis of cKDH-8/11 cells with a inhibition rate of 69.2% (one of 10 animals remaining metastasis-free).

Effects of ND-2001 on *in vitro* invasive activities of cKDH-8/11 cells

Table 2 shows that effects of ND-2001 on *in vitro* invasive activities of cKDH-8/11 cells. ND-2001 treatment (50 μg/ml) suppressed the invasive activities of cKDH-8/11 cells to Matrigel™ Matrix (inhibition rate at 69.3%).

Table 2. Effects of ND-2001 on *in vitro* invasive activities of rat hepatoma cKDH-8/11 cells

Treated with ND-2001[a] (μg/ml)	Invasion assay[b]	
	No. of cells/0.3 mm^2	inhibition rate (%)
0	51.4	0
10	18.2	64.6
50	15.8	69.3

[a]cKDH-8/11 cells were treated with ND-2001 *in vitro* for 24 h.
[b]cKDH-8/11 cells (1×10^5) were placed into the upper compartments of Transwell™ chambers and attracted by RLE conditioned medium. The incubation period for invasion assay was 3 h.

Table 1. Effects of ND-2001 on metastases of rat hepatoma cKDH-8/11 cells in syngeneic WKAH rats

Treated with ND-2001[a] (μg/ml)	Lung metastases			
	Incidence	No. of colonies[b] (mean ± SD)	Inhibition rate (%)	Lung weight (g, mean ± SD)
0	10/10	69.7 ± 24.7	0	1.45 ± 0.27
10	10/10	56.2 ± 52.3	19.4	1.82 ± 1.40
50	9/10	21.5 ± 25.7[c]	69.2	1.24 ± 0.30

[a]cKDH-8/11 cells (1×10^5) were treated with ND-2001 *in vitro* for 24 h and implanted i.v. into WKAH rats.
[b]Rats were killed 16 days after the tumor implantation for examination of lung metastasis colonies.
[c]$p < 0.02$.

Effects of ND-2001 on the random motility of cKDH-8/11 cells

Next we examined the effects of ND-2001 on random motility, which is an important factor of tumor invasion, of cKDH-8/11 cells by phagokinetic track assay. Figure 2 shows that ND-2001 did not suppress the random motility of cKDH-8/11 cells.

Effects of ND-2001 on *in vitro* haptotaxis of cKDH-8/11 cells toward laminin

We examined whether ND-2001 would suppress the haptotaxis of cKDH-8/11 cells toward laminin by a haptotaxis assay. Table 3 shows that ND-2001 suppressed the haptotaxis of cKDH-8/11 cells toward laminin (inhibition rate at 77.0%).

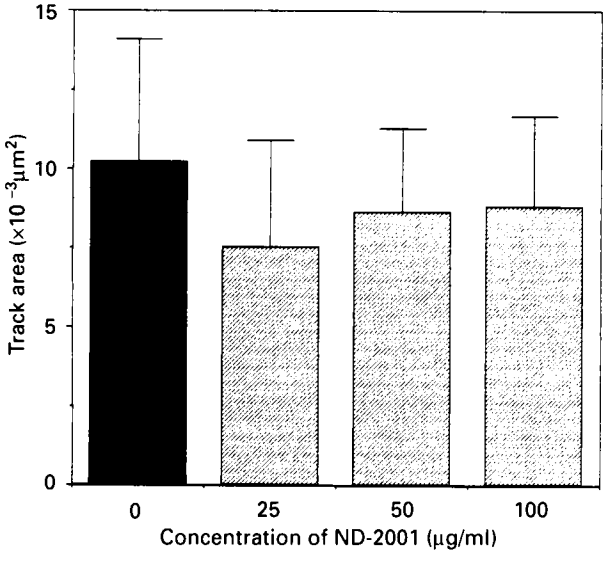

Figure 2. Effects of ND-2001 on the random motility of cKDH-8/11 cells. cKDH-8/11 cells (2×10^6) were seeded onto 100 mm culture dishes in 10 ml of culture medium containing ND-2001 (final concentrations of ND-2001: 100, 50 or 25 μg/ml). The cultures were incubated at 37°C in a CO_2 incubator for 24 h. The random motility of cKDH-8/11 cells was assayed by phagokinetic track assay. The phagokinetic track area was analyzed by a Cosmozone R500 (Nikon).

Table 3. Effects of ND-2001 on *in vitro* haptotaxis of rat hepatoma cKDH-8/11 cells toward laminin

Treated with ND-2001[a] (μg/ml)	Haptotaxis assay[b]	
	No. of cells/0.3 mm^2	Inhibition rate (%)
0	77.0	0
50	17.7	77.0

[a]cKDH-8/11 cells treated *in vitro* with ND-2001 for 24 h.
[b]cKDH-8/11 cells (5×10^4) were placed into the upper compartments of Transwell™ chambers and attracted by RLE conditioned medium. The incubation period for haptotaxis assay was 1 h.

Discussion

The results of the present study indicate that a new anti-metastatic drug ND-2001 had significant potent anti-metastatic effects on rat hepatoma cKDH-8/11 cells. ND-2001 suppressed the invasive activities of cKDH-8/11 cells to Matrigel™ Matrix by 69.3%. Although ND-2001 did not suppress the random motility of cKDH-8/11 cells, it inhibited the haptotaxis of the cells toward laminin by 77.0%. These results suggest that ND-2001 suppressed the haptotaxis of tumor cells toward laminin directly at the step of invading the basement membrane and thus inhibits lung metastases.

Cancer cells need to successfully undergo multiple steps for the development of metastasis. Cancer cell invasion, i.e. active migration from their original tissues and into nearby tissues, is one of the most critical steps in metastasis. After leaving their primary tissues, cancer cells cross the matrix barriers, enter the blood circulation, lodge in a distant organ's capillary bed, extravasate into the organ's parenchyma and proliferate again there.[17,18] Therefore it is important to inhibit cancer cell invasion to prevent cancer metastasis. Motility of cancer cells, production of extracellular matrix-degradative enzymes and haptotaxis toward the extracellular matrix of cancer cells are important for cancer cell invasion. ND-2001 inhibited the haptotaxis of the cells toward laminin, although it did not inhibit any of the random motility, the production or activity of type IV collagenase, or chemotaxis toward laminin and fibronectin (data not shown) of the cKDH-8/11 cells. At present we are not able to elucidate what kind of surface molecules of cKDH-8/11 cells were modified by ND-2001 to inhibit metastasis. However, we speculate that ND-2001 directly modified the sensitivity of the cells for a haptoattractant.

Our present study suggests that ND-2001 might be a new anti-metastatic drug. For clinical application, however, we must examine the therapeutic effect of ND-2001 *in vivo* treatment as well. Furthermore we need to examine effects of ND-2001 on human cancer cells *ex vivo* and *in vivo*.

Acknowledgments

We wish to thank Meiji Seika Kaisha, Ltd, for kindly providing ND-2001. We also thank Ms M Yanome for preparing the manuscript.

References

1. Fidler IJ, Gersten DM, Hart IR. The biology of cancer invasion and metastasis. *Adv Cancer Res* 1978; **28:** 149-250.
2. Liotta LA, Stetler-Stevenson WG, Steeg PS. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991; **64:** 327-36.
3. Liotta LA, Stetler-Stevenson SW. Metalloproteinases and cancer invasion. *Semin Cancer Biol* 1990; **1:** 99-106.
4. Liotta LA. Tumor invasion and metastasis: role of the basement membrane. *Am J Pathol* 1984; **117:** 339-48.
5. Nicolson GL. Organ colonization and cell-surface properties of malignant cells. *Biochem Biophys Acta* 1982; **695:** 113-76.
6. Tsukamoto K, Uno A, Kubota Y, Shimada S, Hori Y, Imokawa G. Role of asparagine-linked carbohydrates in pulmonary metastasis of B16-F10 murine melanoma cells: implication through glycosylation inhibition by nojirimycin. *Melanoma Res* 1992; **2:** 33-9.
7. Tsuruoka T, Niwa T, Shomura T, *et al.* Synthesis of D-glucaro-δ-lactam, an oxidation product of Nojirimycin. *Sci Rep Meiji Seika Kaisha* 1973; **13:** 80-4.
8. Seftor REB, Seftor EA, Grimes WJ, *et al.* Human melanoma cell invasion is inhibited *in vitro* by swainsonine an deoxymannojirimycin with a concomitant decrease in collagenase IV expression. *Melanoma Res* 1991; **1:** 43-54.
9. Tsuruoka T, Fukuyasu H, Azetaka M, *et al.* Inhibition of pulmonary metastases in experimental tumors by D-glucaro-δ-lactam sodium salt (ND-2001). *Abstr 2nd Joint Meet AACR/JCA* 1992; B-3.
10. Kuramitsu Y, Nishibe M, Ohiro Y. A new synthetic lipid A analog, ONO-4007, stimulates the production of tumor necrosis factor-α in tumor tissues, resulting in the rejection of transplanted rat hepatoma cells. *Anti-Cancer Drugs* 1997; **8:** 500-8.
11. Kuramitsu Y, Ohiro Y, Matsushita K, Obara M, Kobayashi M, Hosokawa M. The mechanism of locally enhanced production of tumor necrosis factor-α in tumor tissues by the administration of a new synthetic lipid A analog, ONO-4007 in hepatoma-bearing rats. *Anti-Cancer Drugs* 1997; **8:** 886-93.
12. Sugiura C, Itaya T, Kondoh N, *et al.* Xenogenization of tumor cells by transfection with plasmid containing *env* gene of Friend leukemia virus. *Jpn J Cancer Res* 1988; **79:** 1259-63.
13. Nakajima M, Welch DR, Belloni PN, Nicolson GL. Degradation of basement membrane type IV collagen and lung subendothelial matrix by mammary adenocarcinoma cell clones of differing metastatic potentials. *Cancer Res* 1987; **47:** 4869-76.
14. Yamada K, Kennedy DW, Yamada SS, Gralnick H, Chen W-T, Akiyama S. Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. *Cancer Res* 1990; **50:** 4485-96.
15. Welch DR, Lobl TJ, Seftor EA, Wack PJ, *et al.* Use of the membrane invasion culture system (MICS) as a screen for anti-invasive agents. *Int J Cancer* 1989; **43:** 449-57.
16. Albini A, Iwamoto Y, Kleinman HK, *et al.* A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987; **47:** 3239-45.
17. Nicolson GL. Cancer progression and growth: relationship of paracrine and autocrine growth mechanisms to organ preference of metastasis. *Exp Cell Res* 1993; **204:** 171-80.
18. Nicolson GL. Metastatic tumor cell interactions with endothelium, basement membrane and tissue. *Curr Opin Cell Biol* 1989; **1:** 1009-19.

(Received 14 August 1997; revised form accepted 16 October 1997)