Inhibition of Mouse Tumor Metastasis with Nojirimycin-related Compounds

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The antimetastatic activity of ten compounds structurally related to nojirimycin A was examined using a pulmonary metastatic model of mouse B16 melanoma. Nojirimycin B, deoxynojirimycin, D-gluco-δ-lactam, CP3068 and CP3069 are structural analogues of nojirimycin A, and showed potent or moderate antimetastatic activities. Nojirimycin A, nojirimycin B, deoxynojirimycin and D-gluco-δ-lactam showed potent or moderate inhibitory activities against α-glucosidase, β-glucosidase and β-mannosidase, but CP3068 and CP3069 in which the structures were related to D-gluco-δ-lactam showed no inhibitory activities. CP3041, CP3042, CP3043, CP3045 and CP3048 are analogues of sodium D-glucaro-δ-lactam (ND2001), a carboxy derivative of nojirimycin A, and showed potent or moderate antimetastatic activities. But no analogue was superior to ND2001 concerning with antimetastatic and anti-β-glucuronidase activities. CP3041 and CP3042 showed potent and moderate inhibitory activities against β-glucuronidase, respectively, but CP3043, CP3045 and CP3048 showed little or no activities.

Nojirimycin A, 5-amino-5-deoxy-D-glucose, is an antibiotic produced by several Streptomyces strains[1]. It inhibits various glycosidases activities[2], and mouse tumor metastases[3,4]. Sodium D-glucaro-δ-lactam (ND2001)[5,6], an oxydation derivative of nojirimycin A, shows potent inhibitory activity against β-glucuronidase[7] and mouse tumor metastases[6]. Nojirimycin B[8] and deoxynojirimycin[9,10] are also microbial products, and exhibit anti-glycosidase activities[2,8]. A trihydroxy-pipecolic acid (CP3041), an oxidation product of deoxynojirimycin, was isolated from a plant seed[11] and showed anti-β-glucuronidase activity[12].

The present study was undertaken to examine the antimetastatic activity of structural analogues of nojirimycin A and ND2001, and to clarify the possible relationships of the antimetastatic activity with anti-glycosidases and anti-β-glucuronidase activities.

Materials and Methods

General

Melting points were determined using a YANAKO MP-S3 micro-melting point apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. NMR spectra were recorded by a JEOL GX-400 instrument. Chemical shifts (δ) are reported in ppm from DOH (δ 4.8 ppm) in D_2O or from TMS in other solvents. Coupling constants are given in hertz. Mass spectra were measured with a Hitachi M-80B apparatus.

Preparation of Nojirimycin Derivatives

Nojirimycins A and B, deoxynojirimycin, D-gluco-δ-lactam and sodium D-glucaro-δ-lactam (ND2001) (Fig. 1) were prepared in this laboratory according to the known procedures[1,5,8,9]. Nojirimycins A and B were used as bisulfite adducts.

Syntheses of CP3068 and CP3069

The synthetic pathway of CP3068 and CP3069 starting from (*s*)-(+)-6-hydroxymethyl-2-piperidinone (**1**) is shown in Scheme 1. Optically pure **1** was prepared according to the literature[13].

A solution of **1** (10.0 g, 77.5 mmol) in a mixed solvent of pyridine (40 ml) and methylene chloride (160 ml) was cooled to $-40°C$ and a solution of benzoyl chloride (12.9 ml, 111 mmol) in methylene chloride (80 ml) was added in drops. The reaction proceeded by stirring at $-40°C \sim 0°C$ for 5 hours, and was stopped by adding methanol (14 ml). After standing for 30 minutes, the mixture was washed successively with 10% aqueous sodium carbonate, 5% potassium hydrogen sulfate, and saturated aqueous sodium chloride, was dried over anhydr sodium sulfate, and evaporated to dryness. The residue was subjected to column chromatography on Wakogel C-300 (500 g). After washing with chloroform and chloroform - ethyl acetate 1 : 2 (v/v), the benzoyl ester

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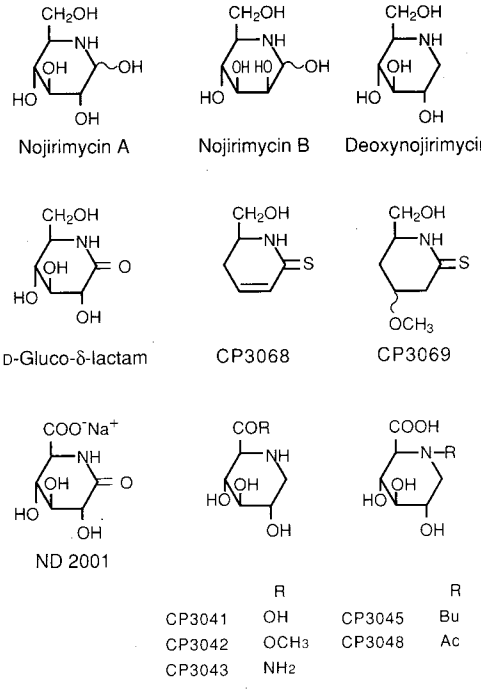
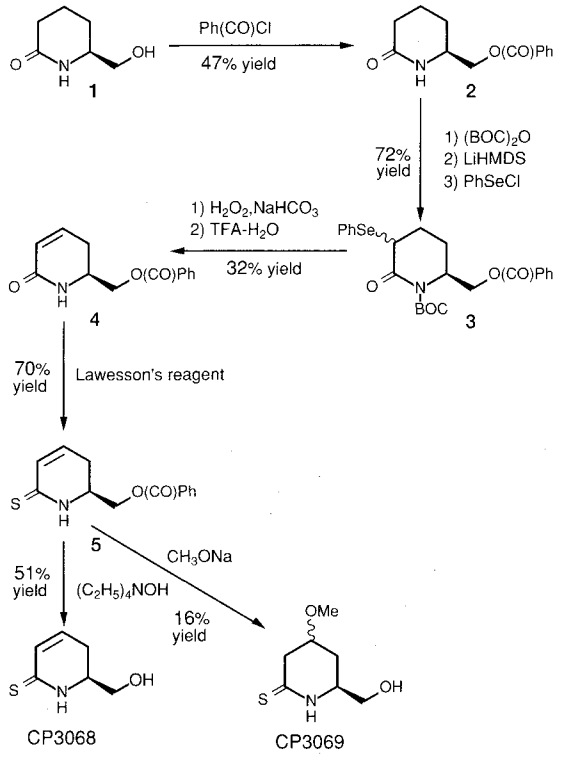
was eluted with chloroform-ethyl acetate 1:3 (v/v) to give 8.46 g of **2**. $[\alpha]_D^{22}$ +65.4°(*c* 1.62, $CHCl_3$), FD-MS *m/z* 234 (MH^+). ^1H NMR ($CDCl_3$), 1.49~2.49 (6H, ring H), 3.84 (1H, m, H-6), 4.11 (1H, dd, *J*=11.3, 8.4 Hz, CH_2O), 4.47 (1H, dd, *J*=4.0 Hz, CH_2O), 6.21 (1H, bs, NH), 7.44~7.48 (2H, m, phenyl H), 7.58~7.62 (1H, m, phenyl H), 8.02~8.04 (2H, m, phenyl H).

Fig. 1. Chemical structures of nojirimycin-related compounds.



Scheme 1. Synthetic pathway of CP3068 and CP3069.



To a solution of the benzoyl ester **2** (8.4 g, 36 mmol) in dry THF (160 ml) was added sodium hydride (net 2.9 g, 72 mmol), and the mixture was stirred at room temperature under nitrogen. After 1 hour, *t*-butoxycarbonyl anhydride (31 g, 142 mmol) dissolved in dry THF (85 ml) was added in drops. After 1.5 hours, acetic acid (0.3 ml) was added under ice-cooling. The reaction mixture was stood for 1 hour, and diluted with chloroform (500 ml). After washing with 5% $NaHCO_3$ and water, followed by evaporation of solvent, the residue was subjected to column chromatography on Wakogel C-300 (240 g). After washing with toluene, it was eluted with a mixture of toluene-ethyl acetate 7:1 (v/v) to give *N*-*t*-butoxycarbonyl benzoyl ester (9.6 g).

This compound (9.6 g, 29 mmol) was dissolved in dry THF (160 ml), and cooled to −78°C under nitrogen. After adding one molar solution of lithium bis (trimethylsilyl) amide in THF (35 ml), the mixture was stirred at −78°C for 20 minutes, and phenylselenenyl chloride (6.1 g, 32 mmol) was added. The mixture was stirred at −78°C for 40 minutes, and then was allowed to rise to room temperature. After 1 hour, acetic acid (3.8 ml) was added. The reaction mixture was stood for 30 minutes, diluted with chloroform (600 ml), washed and concentrated as described above. Column chromatography of the residue using Wakogel C-300 (282 g) gave, following elution with toluene-ethyl acetate 12:1 (v/v) the phenylselenenyl compound **3** (12.6 g) as a mixture of diastereoisomers.

EI-MS *m/z* 489 (MH^+), ^1H NMR ($CDCl_3$), 1.51~1.52 (9H, each s, *t*-butyl H), 7.27~7.66 (7H, m, benzoyl H, Se-phenyl H), 8.00~8.02 (3H, m, benzoyl H, Se-phenyl H).

To a solution of compound **3** (12.6 g, 26 mmol) in a mixed solvent of ethyl acetate (180 ml) and THF (90 ml) were added sodium hydrogen carbonate (6.1 g, 73 mmol), and under ice-cooling 30% hydrogen peroxide (9.7 ml, 85 mmol). After stirring under ice-cooling for 1 hour, sodium thiosulfate (7.7 g) dissolved in water (10 ml) was added. The mixture was stirred at room temperature, then washed and concentrated as described above. Purification by column chromatography on Wakogel C-300 (340 g), developing with toluene-ethyl acetate 5:1 (v/v) gave the α, β-unsaturated compound (6.3 g).

This compound (6.3 g, 19 mmol) was dissolved under ice-cooling in a mixture of trifluoroacetic acid (108 ml) and water (12 ml), and stirred for 1 hour. The reaction mixture was evaporated until dry, and the residue was dissolved in chloroform (240 ml). After washing and concentrating as described above, the residue was purified by column chromatography on Wakogel C-300 (350 g). Development with chloroform-ethyl acetate 2:1 (v/v) gave the de-*t*-butoxycarbonyl unsaturated compound **4** (1.9 g). $[\alpha]_D^{22}$ +22.9° (*c* 1.36, $CHCl_3$), SI-MS *m/z* 232 (M^+), ^1H NMR ($CDCl_3$), 2.37 (1H, ddt, *J*=9.5, 3.0 Hz,

$J_{5a,5b}$=17.6 Hz, H-5a), 2.53 (1H, dt, J=5.0 Hz, H-5b), 4.05 (1H, m, H-6), 4.27 (1H, dd, J=11.7, 7.7 Hz, CH_2O), 4.49 (1H, dd, J=4.4 Hz, CH_2O), 5.96 (1H, dq, J=9.5, 2.0 Hz, H-3), 6.16 (1H, bs, NH), 6.63 (1H, ddd, J=9.5, 5.0, 3.0 Hz, H-4), 7.41~7.58 (2H, m, phenyl H), 7.59~7.61 (1H, m, phenyl H), 8.03~8.05 (2H, m, phenyl H).

To a solution of compound **4** (760 mg, 3.29 mmol) in toluene (16 ml) was added LAWESSON's reagent (2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphethane-2,4-disulfide) (700 mg, 1.73 mmol). The mixture was stirred at 60°C for 10 minutes, cooled to 0°C, and diluted with chloroform (48 ml). After washing and concentration, the residue was purified over Wakogel C-300 (40 g), washed with toluene and developed with toluene-ethyl acetate 7:1 (v/v). Further purification was accomplished using a Kusano prepacked silicagel column CPS-254L-1. Elution with toluene-ethyl acetate 8:1 (v/v) gave (*S*)-(−)-3,4-dehydro-6-benzoyloxymethyl-2-thiopiperidinone (**5**) (472 mg). $[\alpha]_D^{22}$ −39° (*c* 1.40, $CHCl_3$), EI-MS *m/z* 247 (M^+). ^1H NMR ($CDCl_3$), 2.35 (1H, ddt, J=18, *ca.* 10, *ca.* 2 Hz, H-5a), 2.51 (1H, dt, J=*ca.* 6 Hz, H-5b), 4.01 (1H, m, H-6), 4.32 (1H, dd, J=11.5, 7.7 Hz, CH_2O) 4.53 (1H, dd, J=11.5, 4.1 Hz, CH_2O), 6.36 (1H, ddd, $J_{3,4}$=9.6 Hz, H-4), 6.46 (1H, dq, J=*ca.* 2 Hz, H-3), 7.45~7.51 (2H, m, phenyl H), 7.58~7.63 (1H, m, phenyl H), 8.00~8.07 (3H, m, phenyl H, NH).

Compound **5** (57 mg, 1.23 mmol) was dissolved in a mixture of dioxane (570 μl) and water (230 μl), and tetraethylammonium hydroxide (340 μl) was added under ice-cooling. The mixture was stirred at 0°C for 1 hour and evaporated after addition of 1 N HCl (184 μl). The residue was purified over Wakogel C-300 (5.7 g). Washing with chloroform and elution with chloroform-acetone 7:1 (v/v) gave (*S*)-(−)-3,4-dehydro-6-hydroxymethyl-2-thiopiperidinone (CP3068) (14 mg). $[\alpha]_D^{22}$ −266° (*c* 1.75, $CHCl_3$), EI-MS *m/z* 143 (M^+). ^1H NMR (CD_3OD), 2.29 (1H, dddd, $J_{5a,5b}$=18.4 Hz, $J_{5a,3}$=1.9 Hz, $J_{5a,4}$=3.9 Hz, $J_{5a,6}$=9.2 Hz, H-5a), 2.41 (1H, m, $J_{5b,3}$=1.7 Hz, $J_{5b,4}$=4.7 Hz, H-5b), 3.57 (1H, dd, J=13.3, 6.1 Hz, CH_2O), 3.63 (1H, dd, J=5.0 Hz, CH_2O), 3.6 (1H, m, H-6), 6.28 (1H, dt, $J_{3,4}$=9.7 Hz, H-3), 6.37 (1H, ddd, H-4).

To a solution of compound **5** (460 mg, 1.9 mmol) in methanol (9 ml) was added 1 N sodium methylate in methanol (186 μl, 0.19 mmol) under ice-cooling. The mixture was stirred at 0°C for 2.5 hours, and neutralized with Dowex 50W×8 (H^+). The filtrate was evaporated until dry, and the residue was purified over Wakogel C-300 (46 g). Washing with chloroform and elution with chloroform-acetone 6:1 (v/v) gave a mixture of diastereoisomers of 4-methoxy-6-(*S*)-hydroxymethyl-2-thiopiperidinone (CP3069) (53 mg), in addition to CP3068 (90 mg). $[\alpha]_D^{22}$ +33.2° (*c* 1.65, $CHCl_3$), EI-MS, *m/z* 175 (M^+). ^1H NMR revealed that the ratio of two diastereoisomers was 5:1. ^1H NMR of the major isomer (CD_3OD), 1.67 (1H, dddd, H-5a), 2.11 (1H, dddd, H-5b), 2.84 (1H, dd, $J_{3a,3b}$=18.8 Hz, H-3a), 3.07 (1H, bd, H-3b), 3.31~3.36 (4H, m, H-4, OCH_3), 3.51 (1H, dd, $J_{7a,7b}$=10.8 Hz, H-7a), 3.54~3.66 (1H, m, H-6), 3.69 (1H, dd, H-7b).

Syntheses of CP3041, CP3042, CP3043, CP3045 and CP3048

A mixture of ND2001 potassium salt (2.0 g, 8.73 mmol) and sodium borohydride (420 mg, 11.1 mmol) in water (40 ml) was stood at room temperature overnight, and acetone (0.5 ml) was added. The reaction mixture was concentrated to 15 ml, and insoluble products were removed by filtration. The filtrate was passed through a column of Dowex 50W×8 (H^+) (100 ml). The column was washed with water (130 ml), and eluted with 1 N NH_4OH. The eluate was evaporated until dry to give a glassy solid (1.42 g) of crude CP3041. A part of this solid (494 mg) was crystallized from aqueous acetone containing 1 N HCl (2.8 ml) to afford 2(*S*)-carboxy-3(*R*),4(*R*),5(*S*)-trihydroxypiperidine (CP3041, 226 mg). mp, 180°C (decomp), $[\alpha]_D^{22}$ +17.7° (*c* 0.95, H_2O), FD-MS, SI-MS, *m/z* 178 (MH^+). ^1H NMR (D_2O), 3.83 (1H, ddd, $J_{6eq,5}$=5.1 Hz, $J_{6ax,5}$=11.0 Hz, $J_{4,5}$=9.0 Hz, H-5), 3.73 (1H, dd, $J_{3,4}$=9.0 Hz, $J_{2,3}$=10.3 Hz, H-3), 3.55 (1H, t, J=9.0 Hz, H-4), 3.54 (1H, d, H-2), 3.51 (1H, dd, *Jgem*=12.6 Hz, H-6eq), 2.97 (1H, dd, H-6ax). Compound CP3041 isolated from *Baphia racemosa* showed[11] mp, 228~230°C, and $[\alpha]_D^{25}$ +18.30° (H_2O).

To a suspension of a glassy solid of CP3041 (407 mg) in methanol (30 ml) was added 4 N HCl-dioxane (2.5 ml), and the mixture was refluxed for 5 hours. Concentration of the reaction mixture gave crystals of 2(*S*)-methoxycarbonyl-3(*R*),4(*R*),5(*S*)-trihydroxypiperidine (CP3042, 354 mg) as its hydrochloride. mp, 208~211°C, FD-MS *m/z* 191 (M^+), ^1H NMR (D_2O), 4.05 (1H, d, $J_{2,3}$=9.5 Hz, H-2), 3.88 (3H, s, COOMe), *ca.* 3.87 (1H, m, H-5), 3.89 (1H, dd, $J_{3,4}$=8.5 Hz, H-3), 3.63 (1H, t, $J_{4,5}$=$J_{3,4}$ 8.5, H-4), 3.60 (1H, dd, $J_{6eq,5}$=4.9 Hz, *Jgem*=12.8 Hz, H-6eq), 3.06 (1H, dd, $J_{6ax,5}$=10.4 Hz, H-6ax).

A solution of CP3042 hydrochloride (100 mg) in 15% ammonia-ethanol (8 ml) was stood at room temperature overnight, and concentrated until dry. The residue was washed with ethyl ether and dissolved in water by adding 1 N HCl. The aqueous solution (pH 3) was freeze-dried, and the residue was crystallized from H_2O–MeOH–EtOH to give 2(*S*)-amidocarbonyl-3(*R*),4(*R*),5(*S*)-trihydroxypiperidine (CP3043) hydrochloride (48 mg). mp, 220°C (decomp), FD-MS *m/z* 176 (M^+), ^1H NMR (D_2O), 3.85 (1H, d, $J_{2,3}$=10.5 Hz, H-2), *ca.* 3.83 (1H, m, H-5), 3.71 (1H, t, J=*ca.* 10 Hz, H-3), 3.56 (1H, t, J=*ca.* 9 Hz, H-4), 3.55 (1H, dd, J=5, 12 Hz, H-6eq), 3.01 (1H, t, H-6ax).

To a solution of CP3041 (100 mg, 0.56 mmol) in acetonitrile (400 μl) were added butyraldehyde (101 μl, 1.13 mmol) and sodium cyanoborohydride (43 mg, 0.68 mmol), and the mixture was stirred at room temperature for 4 hours. The reaction mixture was adjusted to pH 1 with 1 N HCl, and acetone (200 μl) was added. The

solution was stood for 2 hours, and evaporated until dry. The residue was extracted three times with ethanol (each 1 ml), and the ethanol extract was concentrated and applied to a column of Dowex 50W × 8 (H^+, 10 ml). The column was washed with water (30 ml), and eluted with 1 N NH_4OH to give 2(*S*)-carboxy-3(*R*),4(*R*),5(*S*)-trihydroxy-*N*-butylpiperidine (CP3045, 46 mg).

FD-MS *m/z* 233 (M^+), ^1H NMR (D_2O), 0.89 (1H, t, CH_3), 1.26~1.31 (2H, m, CH_2), 1.45~1.61 (2H, m, CH_2), 2.29 (1H, dd, $J_{6ax,6eq}$ = 11.3 Hz, H-6a), 2.49 (1H, dt, CH_2), 2.71 (1H, dt, CH_2), 2.85 (1H, d, $J_{2,3}$ = 10.0 Hz, H-2), 3.21 (1H, dd, $J_{6eq,5}$ = 5.0 Hz, H-6eq), 3.32 (1H, dd, $J_{3,4}$ = 9.5 Hz, H-3 or H-4), 3.57 (1H, dd, H-4 or H-3), 3.65 (1H, m, H-5).

To a solution of CP3042 hydrochloride (200 mg, 0.88 mmol) in water (200 μl) and methanol (10 ml) were added potassium carbonate (180 mg, 1.30 mmol) and acetic anhydride (330 μl, 3.5 mmol). The mixture was stirred at room temperature for 16 hours, and evaporated until dry. The residue was extracted three times with ethanol (each 2 ml), and the ethanol extract was evaporated until dry. The residue was purified using a Wakogel C-300 column (20 g), developing with $CHCl_3$-MeOH 4:1 (v/v) to give crude *N*-acetylate (202 mg).

A part of this compound (100 mg, 0.43 mmol) was dissolved in a mixture of methanol (1.5 ml) and water (0.5 ml). After adding sodium hydroxide (24 mg, 0.6 mmol), the solution was stirred at room temperature for 4 hours, and evaporated until dry. The residue was dissolved in water (2 ml), neutralized with Dowex 50 W × 8 (H^+), and the filtrate (pH 8) was subjected to column chromatography on DIAION CHP20P (40 ml). Elution with water gave 2(*S*)-carboxy-3(*R*),4(*R*),5(*S*)-trihydroxy-*N*-acetyl-piperidine (CP3048, 72 mg).

^1H NMR in D_2O revealed a mixture of two rotamers in a ratio of 1:2. ^1H NMR of the major rotamer, 2.23 (3H, s, Ac), 3.41 (1H, dd, H-6ax), 3.79 (1H, dd, $J_{3,4}$ = 4.1 Hz, H-3 or H-4), 3.81 (1H, dd, H-6eq), 3.87 (1H, ddd, H-5), 4.33 (1H, dd, H-4 or H-3), 4.82 (1H, m, H-2).

Tumor Cell Lines and Culture

The B16 melanoma high metastatic variant (the B16 variant)[6], Lewis lung carcinoma (3LL), mouse BMT-16 fibrosarcoma[14] and rat SST-2 breast carcinoma[15] were used. BMT-16 cells were cultured on EAGLE's minimal essential medium supplemented with 8% fetal calf serum (Flow Labs, Mclean, VA, U.S.A.), The other cells were cultured on DULBECCO's modified EAGLE's medium supplemented with 10% fetal calf serum. The tumor cells were cultured at 37°C in a humidified 5% CO_2-95% air atmosphere.

Determination of Experimental Lung Metastasis

For experimental metastasis, a test sample was added to the culture of the B16 variant and incubated at 37°C in a humidified 5% CO_2-95% air atmosphere for 72 hours. The concentration of sample was chosen in the range of noncytotoxity. The treated cells were harvested by treating with trypsin-EDTA, and washing according to the procedure described previously[6]. The cells were suspended in DULBECCO's phosphate-buffered saline without Ca^{2+} and Mg^{2+} at 5×10^5 cells/ml. A 0.1 ml portion of the B16 variant cell suspension was implanted iv into a female 6 week-old BDF_1 mouse (Japan SLC Inc. [Shizuoka]). Three mice were used for one group except the case described especially. The mice were cared for 14 days and then pulmonary tumor colonies were counted. The percent inhibition of metastatsis was calculated from the ratio of tumor colony numbers of treated and control groups.

Measurements of Enzyme Activities of Glycosidases and β-Glucuronidase

Enzymes and substrates were purchased from Sigma Chemical Co. (St. Louis. Mo, U.S.A.): α-glucosidase from a baker's yeast, partially purified powder; β-glucosidase from almonds; α-mannosidase from jack beans; β-glucuronidase from bovine liver. Crude enzyme solutions of tumor cells were prepared as described previously[16]. The cultured tumor cells were harvested, washed twice with 10 mM Tris-HCl (pH 7.5) containing 0.14 M NaCl and suspended in 50 mM Tris-HCl (pH 7.5) containing 0.5% Triton X-100. After incubation for 5 minutes at 25°C and then 1 hour at 4°C, the suspension was centrifuged at 10,000 × *g* for 5 minutes at 4°C and the supernatant was used as a crude enzyme of β-glucuronidase.

Glycosidases and β-glucuronidase activities were measured by a modification of the procedures described previously[2,7,8]. The reaction mixture (100 μl) consisted of an enzyme, 0.66 mM *p*-nitrophenyl α-D-glucopyranoside and 100 mM phosphate buffer (pH 6.8) for α-glucosidase activity; an enzyme, 0.33 mM *p*-nitrophenyl β-D-glucopyranoside and 100 mM acetate buffer (pH 5.0) for β-glucosidase activity; an enzyme, 20 mM *p*-nitrophenyl α-D-mannopyranoside and 100 mM acetate buffer (pH 4.5) for α-mannosidase activity; an enzyme, 1 mM phenolphthalein β-D-glucuronide and 0.1 M acetate buffer (pH 5.0) for β-D-glucuronidase activity. Each reaction mixture was incubated for 30 minutes at 37°C, and the reaction was terminated by adding 100 μl of 1 M sodium carbonate. The absorbance of liberated *p*-nitrophenol or phenolphthalein was measured at 405 nm or 540 nm, respectively. The inhibition percentage of a test compound at a given concentration was calculated from the ratio of absorbances in test and control samples.

Results and Discussion

Inhibition of Metastasis by Nojirimycin A Derivatives and Its Analogues

The antimetastatic effects of nojirimycins A and B, deoxynojirimycin, D-gluco-δ-lactam and ND2001 against experimental pulmonary metastasis of the B16 variant are shown in Table 1. The pulmonary metastasis was

most potently inhibited with nojirimycin A, followed by ND2001 and deoxynojirimycin. The inhibition of metastasis was moderate with nojirimycin B and D-gluco-δ-lactam. When the inhibitory rates of metastasis of CP3068 and CP3069, thio analogues of D-gluco-δ-lactam, were compared at the non-cytotoxic concentrations, CP3069 (10 and 30 μg/ml) showed comparable or higher rates of metastasis inhibition than CP3068 (0.3 and 1 μg/ml). The cytotoxicity of CP3068 was reduced at least 30 times by introducing a methoxy group at C-3.

Table 1. Inhibition of experimental pulmonary metastasis of the B16 variant by nojirimycin A derivatives and its structural analogues.

Compound	Concentration (μg/ml)	Number of colonies (Mean ± SD)	Metastasis inhibition (%)
None	—	181.7 ± 18.0	0
Nojirimycin A	10	2.3 ± 2.5	98.7
None	—	119.2 ± 37.3	0
Nojirimycin B	10	51.1 ± 13.1	57.1
None[a]	—	178.2 ± 28.9	0
Deoxynojirimycin	10	34.7 ± 19.0	80.5
None	—	103.8 ± 27.1	0
D-Gluco-δ-lactam	10	61.0 ± 27.8	41.2
None[a]	—	178.2 ± 28.9	0
ND2001[b]	10	14.7 ± 3.8	91.8
None	—	165.7 ± 17.8	0
CP3068	0.3	83.3 ± 18.9	49.7
CP3068	1	65.7 ± 9.7	60.4
None	—	206.5 ± 22.2	0
CP3069	10	99.7 ± 8.3	51.7
CP3069	30	42.7 ± 13.1	79.3

[a] Five mice were used.
[b] Ca salt was used.

The antimetastatic activity of CP3069 was comparable to or even more potent than that of D-gluco-δ-lactam at 10 μg/ml.

Inhibition of Enzyme Activities by Nojirimycin A Derivatives and its Analogues

Nojirimycin A, its derivatives and structural analogues showed the inhibitory activities against various glycosidases and β-glucuronidase as shown in Table 2. Nojirimycin A showed potent inhibitory activity against α and β-glucosidases, but little or no activity against α-mannosidase and β-glucuronidase. Nojirimycin B showed the potent activity against β-glucosidase and α-mannosidase, but moderate activity against α-glucosidase and no activity against β-glucuronidase. Deoxynojirimycin showed moderate activity against α-glucosidase, weak activity against β-glucosidase and α-mannosidase, but no activity against β-glucuronidase. D-Gluco-δ-lactam showed moderate activity against β-glucosidase, but little or no activity against α-mannosidase and β-glucuronidase. On the other hand, ND2001 showed no activity against three glycosidases and the most potent activity against β-glucuronidase. Although both CP3068 and CP3069 are analogues of D-Gluco-δ-lactam, they showed no significant inhibitory activities against the glycosidases and β-glucuronidase examined.

Glycosidase inhibitors such as castanospermine[17], swainsonine[17], nojirimycin A[3,4], and epicyclophellitol[18] have been reported to inhibit experimental pulmonary metastasis. These compounds induced immature carbohydrate chains on the surfaces of tumor cells by inhibiting glycosidase activity, leading to a loss of

Table 2. Inhibition of glycosidases and β-glucuronidase activities by nojirimycin A derivatives and its structural analogues.

Compound	Concentration (mM)	Inhibition (%)			
		α-Glucosidase	β-Glucosidase	α-Mannosidase	β-Glucuronidase
None	—	0	0	0	0
Nojirimycin A	0.1	76.3	87.2	7.4	0
Nojirimycin A	1.0	89.0	93.0	19.0	37.2
Nojirimycin B	0.1	58.7	91.2	71.9	0
Nojirimycin B	1.0	85.3	98.2	90.3	1.9
Deoxynojirimycin	0.1	52.5	23.9	18.0	0
Deoxynojirimycin	1.0	86.2	61.6	54.3	4.5
D-Gluco-δ-lactam	0.1	25.5	65.8	7.6	0
D-Gluco-δ-lactam	1.0	56.8	94.1	31.6	11.9
ND2001	0.1	8.6	4.2	5.4	98.5
ND2001	1.0	9.7	5.3	7.2	99.6
CP3068	0.70[a]	0	11.6	0	7.2
CP3069	0.58[a]	5.4	1.1	1.6	0

[a] 100 μg/ml

metastatic ability. The cells having the changed surface structures could be attacked by the host immune system or could not adhere on host cells[17], but the detailed mechanism involving the sugar chain modification is not known.

From the results in Table 2, nojirimycin A, nojirimycin B, deoxynojirimycin, and D-gluco-δ-lactam may be added in this class of antimetastatic agents. In an attempt to correlate the α-glucosidase and metastasis inhibitions, nojirimycin A showed the most potent activity in both inhibitions. However, CP3068 and CP3069 may belong to other classes of antimetastatic agents, since no inhibitory activity against glycosidase was observed.

Inhibition of Metastasis by ND2001 Analogues

ND2001, which showed potent inhibitions against β-glucuronidase and metastasis, is apparently different from nojirimycin A and its derivatives. A further difference is that ND2001 did not show any significant cytotoxicity at 1 mg/ml[6], whereas nojirimycin A showed cytotoxicity dose-dependently. Nojirimycin A might modify the carbohydrate metabolism that is essential for growth, but ND2001 might not. Because a new mechanism of antimetastatic activity was implied for ND2001, structural analogues of ND2001 listed in Fig. 1 were synthesized. All of these compounds showed antimetastatic activities as shown in Table 3. Both CP3041 devoiding oxo group of ND2001 and CP3043 that is the amide derivative of CP3041 showed potent antimetastatic activities comparable to ND2001. CP3042 (methyl ester of CP3041), CP3045 (*N*-butyl derivative of CP3041) and CP3048 (*N*-acetyl derivative of CP3041) showed moderate antimetastatic activities.

Table 3. Inhibition of experimental pulmonary metastasis of the B16 variant by structural analogues of ND2001.

Experiment	Compound	Concentration (μg/ml)	No. of colonies (Mean$\pm$SD)	Inhibition of metastasis (%)
1	None	—	87.7$\pm$15.5	0
	ND2001	10	20.7$\pm$ 4.0	76.4
	ND2001	30	7.3$\pm$ 4.2	91.7
	CP3041	10	23.0$\pm$15.9	73.8
	CP3041	30	14.0$\pm$ 8.2	84.0
	CP3042	10	47.0$\pm$ 4.4	46.4
	CP3042	30	34.3$\pm$ 9.8	60.9
	CP3043	10	19.7$\pm$ 5.5	77.5
	CP3043	30	16.3$\pm$ 3.2	81.4
2	None	—	68.3$\pm$22.0	0
	ND2001	10	22.3$\pm$ 2.1	67.3
	ND2001	30	9.3$\pm$ 2.5	86.4
	CP3045	10	35.0$\pm$ 4.6	48.8
	CP3045	30	28.0$\pm$ 2.7	59.0
	CP3048	10	32.3$\pm$11.0	52.7
	CP3048	30	25.3$\pm$ 5.9	63.0

Inhibition of Enzyme Activities by ND2001 Analogues

While CP3041 showed potent inhibitory activity against β-glucuronidase, CP3042 inhibited the enzyme moderately as shown in Table 4. CP3043, CP3045 and CP3048 showed little or no inhibitory activity against β-glucuronidase. All the CP compounds examined showed no significant activity against α-glucosidase, β-glucosidase, and α-mannosidase (Table 4).

In an attempt to correlate β-glucuronidase inhibition with the antimetastatic activity, distributions of β-glucuronidase in mouse melanoma B16 and mouse lung carcinoma 3LL, in which metastasis was inhibited by ND2001[6], and in mouse fibrosarcoma BMT-11 and rat breast carcinoma SST-2, in which metastasis was not inhibited by ND2001[6], were examined. The B16 variant showed the highest β-glucuronidase activity (Absorbance of phenolphthalein liberated from the substrate was 0.73 per 10^6 cells), followed by SST-2 (0.20), 3LL (0.03) and BMT-11 (0.00). These results showed that the content of β-glucuronidase is unrelated to the antimetastatic activity. ND2001 showed potent and comparable inhibition against β-glucuronidase of the B16 variant and SST-2. The inhibition rates to the no addition control were 69.9% and 83.6% at 0.1 μg/ml, or 96.4% and 100% at 1 μg/ml for the B16 variant and SST-2, respectively. Therefore, the potent antimetastatic activity of ND2001 could not be ascribed to its potent

Table 4. Inhibition of glycosidase and β-glucuronidase activities by structural analogues of ND2001.

Compound	Concentration		Inhibition (%)			
	μg/ml	mM	α-Glucosidase	β-Glucosidase	α-Mannosidase	β-Glucuronidase
CP3041	100	0.56	6.1	0	0.8	89.4
CP3042	100	0.52	9.6	0	3.1	68.1
CP3043	100	0.57	9.7	2.3	0	19.5
CP3045	100	0.38	0.5	0	5.6	20.9
CP3048	100	0.46	3.2	1.0	1.4	0

anti-β-glucuronidase activity alone.

SLOANE *et al.*[19] and NAKAO *et al.*[20] reported that β-glucuronidase might play important roles for the metastasis of B16 melanoma, as well as proteinases. NISHIMURA *et al.*[21] reported that three derivatives of siastatin B showed antimetastatic activity against the B16 variant, apparently in parallel to the anti-β-glucuronidase activity. Therefore, further work is necessary to clarify the mechanism of antimetastatic action of ND2001 and its related compounds.

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