Synthesis and Antitumor Activities of Glycine-exchanged Analogs of Spicamycin

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A series of SPM VIII analogs were synthesized to investigate the effect of the amino acid moiety on the antitumor activity. The L-threonine analog and the glycylglycine analog of SPM VIII showed much higher cytotoxity to P388 murine leukemia cells (IC_{50} 5.8 nM and 0.11 nM, respectively) than SPM VIII (IC_{50} 25 nM). However, replacement of the glycine moiety with other amino acids greatly reduced the antitumor activity against COL-1 human colon cancer xenograft model. This study indicated that the glycine moiety of SPM VIII is crucial for the antitumor effect.

Spicamycin (**1**)[1,2] was isolated from the culture broth of a *Streptomyces alanosinicus* 879-MT_3 as a differentiation inducer of mouse myeloid leukemia cells (M1) and human promyelocytic leukemia cells (HL-60). And it showed antitumor activity against P388 leukemia in mice. Spicamycin (**1**) is a mixture of several related components that differ only in their alkylacyl moieties (C12~C18), and it showed potent antitumor activity against human breast cancer MX-1 and human stomach cancer SC-9 in the human tumor xenograft model[3]. In the mixture of spicamycin, the dodecanoyl compound (SPM VIII, **2**, Fig. 1) exhibited the highest antitumor activity[3,4]. Other fatty acid analogs of spicamycin were synthesized and evaluated, and KRN5500 (**3**) showed marked activity against human xenograft models[5,6].

The purpose of the present study was to synthesize a series of spicamycin analogs to investigate the effect of the amino acid moiety on the antitumor activity with a hope of identifying a derivative having an increased potency. This report describes syntheses and antitumor activities of the glycine-modified analogs of SPM VIII.

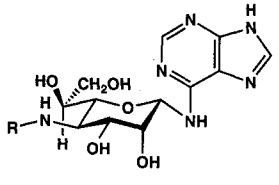
Chemistry

Spicamycin amino nucleoside (SAN, **4**, [6-(4-amino-4-deoxy-L-*glycero*-β-L-*manno*-heptopyranosylamino)-9*H*-purine[7]) was prepared by the hydrolysis of spicamycin (**1**) with 10% HCl in methanol under reflux for 24 hours[3,5]. The dodecanoyl amino acids derivatives (**6a**~**6e**, **6g**, **6h**) were obtained from dodecanoylchloride and appropriate amino acids[3]. In the case of the L-lysine derivative, we used N^{ε}-Cbz-L-lysine for synthesis. The L-methionine analogs were partially racemized under this condition. Treatment of dodecanoyl amino acids with *N*-hydroxysuccinimide (HOSu) and dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) gave active esters, which were allowed to react with SAN (**4**) in DMF in the presence of triethylamine to give glycine exchanged analogs (**7a**~**7e**, **7g**, **7h**). The glycylglycine derivative (**7i**) was obtained from dodecanoylglycine[3] and SAN-Gly (**5**) by the active ester method using HOSu and DCC. Protecting group of N^{ε}-Cbz-L-lysine was treated with HBr in AcOH to give (**7f**). The structure of the analogs, **7a**~**7i** are shown in Table 1.

Results and Discussion

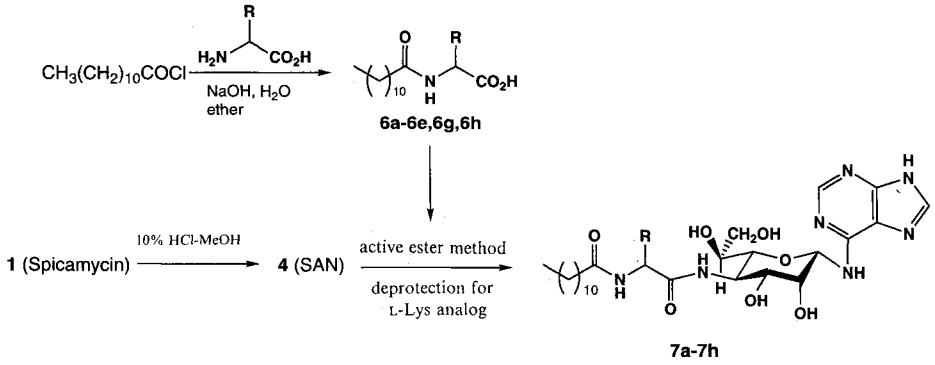
Nine spicamycin derivatives were evaluated for *in vitro*

Fig. 1. Structure of spicamycin (**1**) and related compounds (**2**~**5**).



R; $(CH_3)_2CH(CH_2)_nCONHCH_2CO$-
$CH_3CH_2CH_2(CH_2)_nCONHCH_2CO$-, n=8-14
(Spicamycin, **1**)
$CH_3(CH_2)_{10}CONHCH_2CO$- (SPM VIII, **2**)
$CH_3(CH_2)_8CH=CHCH=CHCONHCH_2CO$- (KRN 5500, **3**)
H- (SAN, **4**)
H_2NCH_2CO- (SAN-Gly, **5**)

Scheme. 1. Syntheses of glycine modified analogs.



Scheme. 2. Synthesis of glycylglycine analog (**7i**).

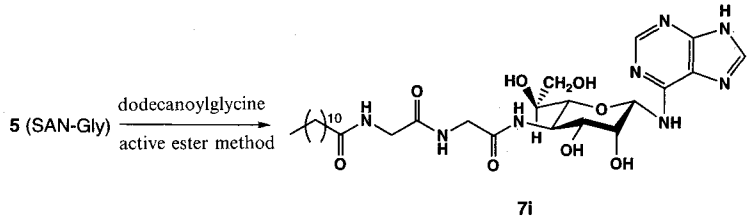


Table 1. Cytotoxic effects of glycine modified spicamycin analogs on P388 murine leukemia cells.

Compound	Amino acid	IC_{50} (μg/ml)
SPM VIII	Gly	0.025
7a	L-Leu	2.50
7b	D,L-Met	1.80
7c	L-Ala	0.023
7d	β-Ala	1.45
7e	L-Lys-Nω-Cbz	<100
7f	L-Lys	36.0
7g	L-Ser	0.025
7h	L-Thr	0.0058
7i	Gly-Gly	0.00011

P388 cells were exposed to glycine exchange spicamycin analogs for 3 days and the cell number was counted.

Table 2. Antitumor activities of glycine modified spicamycin analogs against COL-1 human colon cancer.

Compound	Intravenous dose (mg/kg/day)						
	24	12	6	3	1.5	0.75	T.I.[a]
SPM VIII	Toxic[b]	**94**[c]	**94**	**74**	40		4
7a	−36						0
7b	24						0
7c		Toxic		Toxic		7	0
7d	Toxic		37				0
7e	36						0
7f	Toxic		27				0
7g	Toxic		Toxic		21		0
7h	Toxic		Toxic		23		0
7i			Toxic	**73**			1

Fragments of COL-1 human colon cancer were implanted subcutaneously into athymic nude mice. When the tumor size reached 100 ~ 300 mm^3, glycine exchanged spicamycin analogs were given iv daily for five days.

[a] Therapeutic index (T.I.) = maximum tolerated dose/minimum effective dose.

[b] One or more animals died.

[c] Results were expressed as the values of maximum tumor growth inhibition rate (T.G.I.R) through the experimental span; five mice per group.

The bold value was evaluated as "effective" according to the criteria described in "Experimental".

cytotoxicity against P388 murine leukemia cells and *in vivo* antitumor activities against human tumor xenograft model[4]. The results are summarized in Tables 1 and 2.

The lipophilic amino acids analogs (L-leucyl (**7a**), D,L-methionyl (**7b**) showed significant reductions of cytotoxicity than that of SPM VIII. These compounds did not show *in vitro* activities. The cytotoxicity of L-alanyl analog (**7c**) is nearly equal to that of SPM VIII. However, the β-alanyl analog (**7d**) reduced the cytotoxicity. The *in vivo* activity of L-alanyl (**7c**) and β-alanyl (**7d**) derivatives showed no therapeutic effect but toxicity. The basic (L-lysyl) amino acid derivative (**7f**) and charge protected one (**7e**) showed less cytotoxicity. Replacement of glycine moiety with amino acid containing hydroxyl group caused enhanced cytotoxicity. The IC_{50} value of the L-serine derivative (**7g**) was equal to that of SPM VIII and the L-threonine derivative (**7h**) was effective as SPM VIII at a concentration 4 times lower than SPM VIII. In spite of the potent cytotoxicity, these derivatives did not show the sufficient activity *in vivo*. The cyto-

toxicity of glycylglycine analog (**7i**) was shown to be 20-fold more active than SPM VIII. However, the glycylglycine derivative (**7i**) exhibited the tumor inhibitory effect *in vivo* only at 3 mg/day. The therapeutic index of (**7i**) was inferior to that of SPM VIII. None of these amino acid analogs showed better *in vivo* activity than SPM VIII. Thus, glycine is the best amino acid for antitumor activity in this series.

In the study of the mode of action of spicamycin analog[6] (KRN5500), we reported that KRN5500 inhibit the protein synthesis in tumor cell. In addition, we showed that KRN5500 metabolite, SAN-Gly, inhibited the protein synthesis in reticulocyte lysate, whereas KRN5500 did not. These findings suggested that the amide bond between fatty acid and glycine moieties would be hydrolyzed with the cellular membrane enzyme to generate the SAN-Gly. SAN-Gly inhibit the protein synthesis of tumor cell and show the cytotoxic effect. Since these analogs showed no antitumor activity, corresponding intracelluar metabolites might be less active than SAN-Gly against the inhibition of protein synthesis. Although **7g**, **7h** and **7i** had potent cytotoxicities, these compounds showed considerable reductions in antitumor activity against COL-1 human xenograft model. It is speculated that potent cytotoxicities of these compounds induce reductions of *in vivo* activity, which are also observed in the analog study of fatty acid moiety[3].

Experimental

General

MP's were determined with a Yanagimoto micro hot-stage apparatus and not corrected. Specific rotations were obtained with a Jasco DIP-140 spectropolarimeter. IR spectra were recorded on a Jasco A-3 spectrophotometer. UV spectra were recorded on a Hitachi U-3200 in MeOH solution and NMR spectra on a Jeol GX-500 spectrometer. The FD mass spectra were obtained with a Hitachi M80 mass spectrometer.

Spicamycin Amino Nucleoside (4)

A solution of 1.0 g of **1** in 100 ml of 10% HCl-MeOH was allowed to stir under reflux for 24 hours. The solution was concentrated *in vacuo* to the minimum volume and diethyl ether was added. The resulting precipitate was centrifuged and washed with diethylether to give a white powder. This powder was dissolved in water and washed with *n*-butanol. The aqueous layer was neutralized with silver carbonate and the formed precipitate was filtered off. The filtrate was concentrated and the residue was further purified by column chromatography on silicagel ($CHCl_3$-MeOH 2:1) to give **2** (68% yield); MP 180~183°C (decomp.), $[\alpha]_D^{25}$ +1.2° (*c* 0.25, MeOH), UV λ_{max} 264 nm, IR ν_{max} 3300, 1640 cm^{-1}, FD-MS 327 (M+H), ^1H NMR (CD_3OD) δ_H 3.43 (1H, dd, *J*=10.0, 10.0 Hz, H-4'), 3.62 (2H, m, H-7'), 3.72 (1H, dd, *J*=10.0, 10.0 Hz, H-5), 3.80 (1H, m, H-6'), 3.89 (1H, dd, *J*=10.0, 3.1 Hz, H-3'), 4.07 (1H, dd, *J*=3.1, <1 Hz, H-2'), 5.72 (1H br s, H-1'), 8.20 (1H, s, H-8), 8.40 (1H, s, H-2).

Dodecanoyl L-Alanine (6a)

To a stirred solution of L-alanine (1.0 g, 0.013 mol) in NaOH (5.2 g, 0.13 mol) aqueous solution was added dodecanoyl chloride (2.84 g, 0.013 mol) and the mixture was stirred for 30 minutes at room temperature. The reaction mixture was adjusted to pH 3 by conc. HCl. The resulting precipitate was collected and washed with water and diethylether to give **6a** (3.27 g, 98% yield) as a white powder.

Other dodecanoyl-amino acids were prepared by a similar procedure.

6-[4-Deoxy-4-(dodecanoyl-L-alanyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (7a)

To a stirred solution of **6a** (180 mg, 0.66 mmol) in DMF was added *N*-hydroxysuccinimde (71 mg, 0.61 mmol) and *N*,*N*'-dicyclohexylcarbodiimide (139 mg, 0.67 mmol). After 12 hours, the reaction mixture was filtered. To a stirred solution of **4** (200 mg, 0.61 mmol) in DMF was added the filtrate and triethylamine (0.7 ml, 7.65 mmol). After 24 hours, the solvent was removed and chromatographed on silica gel with chloroform-methanol (6:1) elution. Evaporation of the appropriate fractions afforded **7a** (80 mg, 29% yield) as a white powder.; MP 165~166°C, $[\alpha]_D^{25}$ +6.4° (*c* 0.1, MeOH), FD-MS 580 (M+H), IR ν_{max} 3300, 1640 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.89 (3H, t, *J*=7.0 Hz), 1.20~1.40 (16H, m), 1.39 (3H, d, *J*=8.0 Hz), 1.55~1.65 (2H, m), 2.24 (2H, t, *J*=7.0 Hz), 3.60~3.80 (5H, m), 4.00 (1H, dd, *J*=<1, 2.5 Hz), 4.11 (1H, dd, *J*=10.3, 10.3 Hz), 4.25~4.35 (1H, m), 5.68 (1H, br s), 8.14 (1H, br s), 8.30 (1H, s).

Preparation of compounds, **7b**, **7c**, **7d**, **7e**, **7g** and **7h** followed a similar process as discribed above using appropriate amino acids.

6-[4-Deoxy-4-(dodecanoyl-β-alanyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (7b)

MP 198~200°C, $[\alpha]_D^{25}$ −12.8° (*c* 0.1, MeOH), FD-MS 580 (M+H), IR ν_{max} 3300, 1650 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.89 (3H, t, *J*=7.0 Hz), 1.20~1.40 (16H, m), 1.55~1.63 (2H, m), 2.19 (2H, t, *J*=6.9 Hz), 2.45 (2H, t, *J*=6.7 Hz), 3.47 (2H, t, *J*=6.7 Hz), 3.60~3.75 (5H, m), 4.00 (1H, dd, *J*=<1, 2.5 Hz), 4.12 (1H, dd, *J*=10.4, 10.4 Hz), 5.66 (1H, br s), 8.15 (1H, br s), 8.31 (1H, s).

6-[4-Deoxy-4-(dodecanoyl-L-leucyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (7c)

MP 203~204°C, $[\alpha]_D^{25}$ +5.2° (*c* 0.1, MeOH), FD-MS 622 (M+H), IR ν_{max} 3400, 1650 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.89 (3H, t, *J*=6.7 Hz), 0.94 (3H, t, *J*=

6.7 Hz), 0.98 (3H, t, J=6.7 Hz), 1.20~1.36 (16H, m), 1.53~1.73 (5H, m), 2.23 (2H, t, J=7.3 Hz), 3.60~3.80 (5H, m), 3.98 (1H, dd, J=2.5, <1 Hz), 4.10 (1H, dd, J=10.3, 10.3 Hz), 4.39 (1H, t, J=7.9 Hz), 5.67 (1H, br s), 8.15 (1H, br s), 8.31 (1H, s).

6-[4-Deoxy-4-(dodecanoyl-D,L-methionyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (**7d**)

MP 195~196°C, $[\alpha]_D^{25}$ +2.0° (*c* 0.1, MeOH), FD-MS 640 (M+H), IR ν_{max} 3400, 1650 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.89, 0.90 (total 3H, each t, J=6.7 Hz), 1.20~1.40 (16H, m), 1.57~1.67 (2H, m), 1.90~2.00 (1H, m), 2.25, 2.27 (total 2H, each t, J=7.3 Hz), 2.50~2.65 (2H, m), 3.60~3.80 (5H, m), 3.98, 4.01 (total 1H, each dd, J=<1, 2.1 Hz), 4.12, 4.14 (total 1H, each dd, J=10.3, 10.3 Hz), 4.40~4.50 (1H, m), 5.68 (1H, br s), 8.14 (1H, s), 8.30 (1H, s).

6-[4-Deoxy-4-(dodecanoyl-L-N^{ε}-carbobenzyloxy-lysyl)amino-L-*glycero*-β-L-*manno*-heptopyranosyl-amino]-9*H*-purine (**7e**)

MP 214~215°C, $[\alpha]_D^{25}$ +2.0° (*c* 0.1, MeOH), FD-MS 771 (M+H), UV λ_{max} 264 nm, IR ν_{max} 3400, 1650 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.88 (3H, t, J=6.7 Hz), 1.24~1.85 (18H, m), 2.24 (2H, t, J=7.6 Hz), 3.13 (2H, t, J=7.2 Hz), 3.60~3.80 (5H, m), 3.95 (1H, dd, J=<1, 2.5 Hz), 4.10 (1H, dd, J=10.3, 10.3 Hz), 4.20~4.24 (1H, m), 5.65 (1H, br s), 7.20~7.40 (5H, m), 8.15 (1H, br s), 8.31 (1H, s).

6-[4-Deoxy-4-(dodecanoyl-L-threonyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (**7g**)

MP 191~192°C, $[\alpha]_D^{25}$ −8.8° (*c* 0.1, MeOH), FD-MS 610 (M+H), IR ν_{max} 3400, 1640 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.89 (3H, t, J=7.3 Hz), 1.20~1.40 (16H, m), 1.58~1.68 (2H, m), 2.25~2.35 (2H, m), 3.60~3.80 (5H, m), 3.99 (1H, dd, J=<1, 2.5 Hz), 4.15 (1H, dd, J=10.4, 10.4 Hz), 4.16~4.22 (1H, m), 4.30 (1H, d, J=4.3 Hz), 5.67 (1H, br s), 8.16 (1H, br s), 8.32 (1H, s).

6-[4-Deoxy-4-(dodecanoyl-L-serinyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (**7h**)

MP 188~189°C, $[\alpha]_D^{25}$ −9.2° (*c* 0.1, MeOH), FD-MS 596 (M+H), IR ν_{max} 3400, 1650 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.89 (3H, t, J=6.7 Hz), 1.20~1.40 (16H, m), 1.58~1.66 (2H, m), 2.28 (2H, t, J=7.9 Hz), 3.60~3.85 (7H, m), 4.00 (1H, dd, J=<1, 2.5 Hz), 4.14 (1H, dd, J=10.4, 10.4 Hz), 4.39 (1H, t, J=5.5 Hz), 5.67 (1H, br s), 8.14 (1H, br s), 8.31 (1H, s).

6-[4-Deoxy-4-(dodecanoyl-glycylglycyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (**7i**)

This compound was obtained from SAN-Gly (**5**)[5] and dodecanoyl glycine[3] by active ester method. MP 180~181°C, $[\alpha]_D^{25}$ −9.2° (*c* 0.1, MeOH), FD-MS 623 (M+H), IR ν_{max} 3400, 1650 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.88 (3H, t, J=7.1 Hz), 1.20~1.40 (16H, m), 1.60~1.70 (2H, m), 2.30 (2H, t, J=7.1 Hz), 3.60~3.75 (5H, m), 3.85 (2H, s), 3.88 (1H, d, J=15.4 Hz), 3.92 (1H, d, J=15.4 Hz), 4.00 (1H, dd, J=<1, 2.5 Hz), 4.15 (1H, dd, J=9.3, 9.3 Hz), 5.70 (1H, br s), 8.14 (1H, s), 8.32 (1H, s).

6-[4-Deoxy-4-(dodecanoyl-L-lysyl)amino-L-*glycero*-β-L-*manno*-heptopyranosylamino]-9*H*-purine (**7f**)

To a chilled solution of 20% HBr in AcOH (5 ml) was added **7e** (115 mg, 0.15 mmol) and the mixture was stirred at 5°C for 25 minutes. Fifty ml of ether was added to the solution to precipitate the product which was collected by filtration and dried under rteduced pressure. The crude product was chromatographed on silica gel with $CHCl_3$ - MeOH - H_2O (4 : 1 : 0.05) to give **7f** (59 mg, 62%) as pale yellow solid. MP 190~191°C, $[\alpha]_D^{25}$ −14.4° (*c* 0.1, MeOH), FD-MS 637 (M+H), IR ν_{max} 3400, 1650 cm^{-1}, ^1H NMR (CD_3OD) δ_H 0.88 (3H, t, J=6.7 Hz), 1.20~1.45 (16H, m), 1.45~1.57 (2H, m), 1.57~1.66 (2H, m), 1.66~1.80 (3H, m), 1.83~1.92 (1H, m), 2.26 (2H, t, J=7.3 Hz), 2.94 (2H, t, J=7.3 Hz), 3.60~3.82 (5H, m), 4.04 (1H, dd, J=<1, 2.5 Hz), 4.14 (1H, dd, J=10.3, 10.3 Hz), 4.28 (1H, t, J=6.7 Hz), 5.68 (1H, br s), 8.35 (1H, br s), 8.44 (1H, s).

In Vitro Cytotoxicity

P388 murine leukemia cells were kindly provided by Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Tumor cells were maintained and suspended in RPMI 1640 Medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY), penicillin (100 units/ml, Meiji Seika Kaisha Ltd., Tokyo, Japan) and streptomycin (100 μg/ml, Meiji Seika Kaisha Ltd., Tokyo, Japan), and 2-mercaptoethanol (5×10^{-5} M). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For drug treatment experiments, 5×10^3 tumor cells/well in 96-well plates (U96 Micro Well, Nunc Inter. Med., Roskilde, Denmark) containing 0.1 ml growth medium were cultured in the graded concentrations of spicamycin analogs. After 72 hours incubation, the number of cells in each well was counted with Model ZM Coulter Counter (Coulter Electronics Limited, England). Four wells were used for each drug concentration. The IC_{50} was determined by plotting the logarithm of the drug concentration against the growth rate of the treated cells.

In Vivo Antitumor Activity

For all experiments, 6~8-week-old female, athymic nude mice (BALB/c nu/nu Slc, Japan SLC Inc., Shizuoka, Japan) were used. The animals were kept under specific pathogen free conditions using laminar air flow racks and were fed sterile food and water *ad libitum*.

Human colon cancer COL-1 were kindly supplied by the Central Institute for Experimental Animals, Kanagawa, Japan, and maintained in athymic nude mice. Chemotherapuetic experiments were performed as de-

scribed by INABA *et al*[8]. Fragments of xenografts were implanted sc into the right subaxillary region of athymic nude mice. When the tumors had grown to a palpable size ($100 \sim 300\,mm^3$), the mice were randomly allocated to several experimental groups consisting of five animals each and spicamycin derivatives at each dose were given intravenously by daily injection for five days. Control mice were given 10 ml/kg vehicle. From the start of the injections, the tumor volume (V) was calculated once or twice a week for 3 weeks as follows; $V = abc/2$, where a and b are the long and short diameter and c is the height of the tumor mass in mm. Relative tumor volume (RV) is expressed as $RV = V_n/V_0$, where V_n is the tumor volume on day n and V_0 is the initial tumor volume at the time treatment was commenced (day 1). T.G.I.R. was determined as follows; $T.G.I.R. = (1 - T/C) \times 100$ where T is the mean of RV in treated mice and C is the mean of RV in control mice.

Evaluation as "effective" was based on the maximum T.G.I.R. (%) for an experimental span of 50% or more showing statistical significance as determined by the Mann-Whitney's U-test ($P < 0.05$, one sided). A toxic dose was defined as one causing the death of one or more mice in a group. The therapeutic index (T.I.) was determined as follows; T.I. = maximum tolerated dose/minimum effective dose.

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