EFFECT OF 5′-NUCLEOTIDASE INHIBITORS ON MOUSE IMMUNE SYSTEM AND EXPERIMENTAL MURINE TUMORS

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Nucleoticidin and melanocidins A and B exhibited potent inhibitory activity against 5′-nucleotidases from rat liver membrane and snake venom. Nucleoticidin retarded growth of Sarcoma 180 solid tumor, and melanocidins A and B prolonged the survival period of mice bearing B16 melanoma. These inhibitors enhanced phagocytic activity, interleukin-1 production and superoxide-generating activity of murine peritoneal macrophages. The tumor necrosis factor was also induced by the inhibitors. These results suggested that 5′-nucleotidase inhibitors inhibit tumor growth by modification of the immune system.

In the course of our screening program searching for new inhibitors, we discovered nucleoticidin[1,2], melanocidins A and B[3,4]. These compounds were strong inhibitors of 5′-nucleotidase, a membrane-bound enzyme, and might bind to the cell surface including macrophages. In this paper we report the immuno-potentiating activity of these 5′-nucleotidase inhibitors and their effect on murine tumors.

Materials and Methods

Animals and Chemicals

ICR/CRT, C57BL/6, C3H/He and BALB/c mice, propagated at Chales River Japan Inc., (Atsugi, Japan) were used. C3H/HeJ was provided by the animal center of Institute of Medical Science, University of Tokyo. Vesicular Stomatitis Virus (VSV) was provided by National Institute of Animal Health. Proteose peptone, phytohemagglutinin and lipopolysaccharide (*Escherichia coli* 055: B5) were purchased from Difco Laboratories, concanavalin A (Con A) and ferricytochrome C from Sigma Chemical Company, actinomycin D from Makor Chemicals Ltd., methylrosaniline chloride, 2-methoxyethanol and crystal violet from Tokyo Kasei Kogyo Co., Ltd., heparin from Nakarai Chemicals Ltd. and Bacillus Calmette-Guerin (BCG) from Japan BCG Ind. Other reagents were commercially available products.

Assay of Antitumor Activity

Sarcoma 180: ICR/CRT mice were inoculated subcutaneously with Sarcoma 180 tumor (3×10^5 cells). The inhibitors were administered intraperitoneally for 10 consecutive days from 24 hours after the tumor inoculation. Tumor size was estimated at 15, 21 and 28 days.

B16 Melanoma: $B_6C_3F_1$ mice inoculated intraperitoneally with B16 melanoma (0.5 ml of 10% homogenate). The inhibitors were administered intraperitoneally for 10 consecutive days from 24 hours after the tumor inoculation. The antitumor activity was determined by measuring the increase in mean survival days of mice. (This was done with collaboration with the National Cancer Institute of U.S.A. According to N.C.I., it is said to be significant when T/C (%) is above 125.)

Table 1. Effect of nucleoticidin on Sarcoma 180 solid tumor in mice.

Treatments (×10, ip)		7 days	15 days	21 days	28 days
Control	Tumor size (mm)	—	37.6+19.2	88.3+44.8	164.9+88.2
	Inhibition rate (%)	—	—	—	—
	Body weight (g)	22.6+1.5	21.9+1.3	25.7+2.1	—
1 mg/kg	Tumor size (mm)	—	34.4+14.3	64.6+68.5	113.7+124.2
	Inhibition rate (%)	—	8.5	26.8	31.0
	Body weight (g)	22.1+1.0	21.8+1.0	27.2+1.9	—
5 mg/kg	Tumor size (mm)	—	22.1+14.4	39.6+25.9*	68.3+68.3**
	Inhibition rate (%)	—	41.5	55.2	58.6
	Body weight (g)	20.5+1.5	21.2+1.8	27.1+3.0	—
10 mg/kg	Tumor size (mm)	—	13.5+12.3	25.3+32.1*	54.8+73.7*
	Inhibition rate (%)	—	64.1	85.7	66.8
	Body weight (g)	18.8+1.5	19.3+1.2	24.3+0.9	—

* Significant at $P<0.01$ (Student's t-test).
** Significant at $P<0.02$ (Student's t-test).

Induction of Cytotoxic Macrophages *In Vitro* and Cytotoxicity

One ml of 3% Proteose peptone solution was injected intraperitoneally into C57BL/6 mice. Four days after the injection, peritoneal exudate cells (PEC) were harvested in Eagle's MEM and plated in 96-well microplate (Falcon, 3072) at a density of 2×10^5/well for 1 hour. PEC were washed with Eagle's MEM to remove nonadherent. The adherent cells were incubated for 18 hours in the presence of the inhibitors and/or supernatant of splenocyte culture. Splenocytes obtained from C57BL/6 mice were incubated with 5 μg/ml of Con A for 60 hours and the supernatants collected through Millipore filters were used. Target cells (P815) in the exponential growth phase were labeled with $Na_2{}^{51}CrO_4$. Radiolabeled target cells were plated into each well containing activated macrophages and incubated at 37°C for 18 hours. At the end of incubation, target cells were harvested on the glass filter paper, dried and the residual radioactivity was counted by a liquid scintillation counter.

Superoxide-generating Activity

Peritoneal Exudate Cells (PEC): C3H/He mice (8 weeks old) were injected intraperitoneally with 0.6 mg of the inhibitors. Four days after the injection, PEC were harvested in Hanks balanced solution, plated in fetal calf serum (FCS) coated plastic disc (ϕ 70 mm), and kept for 30 minutes. PEC were washed with RPMI 1640 to remove nonadherent cells. The adherent cells were treated with a solution containing 0.05% EDTA and 10% FCS in phosphate-buffered saline (PBS). The resulting single cell suspension was diluted in 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) saline.

Measurement of Superoxide Anion: The reaction mixture contained 2 mM glucose, 1 mM $CaCl_2$, 80 μM ferricytochrome C, and the cells (1×10^5 cells) in 0.5 ml HEPES-saline. The superoxide-generating activity was measured by the method of INOKUCHI[5,6].

Interferon (IF) Induction

C57BL/6 mice (8 weeks old) were injected intraperitoneally with 0.6 mg of the inhibitors and the sera were collected after 24 hours. The IF level was assayed by the dye-binding method[7,8].

Interleukin (IL)-1 Production

Peritoneal exudate cells (PEC) were harvested from BALB/c mice (8 weeks old) injected 3 days previously with 1.5 ml of 10% Proteose peptone solution intraperitoneally. The mice were killed and their PEC were harvested in Eagle's MEM containing 10 μg/ml of heparin and 5% heat-inactivated FCS. The cells were planted on 24-well microplate at 37°C for 2 hours, washed vigorously three times. The adherent cells were further incubated in 2 ml of RPMI 1640 with or without the inhibitors for 48 hours. Culture fluids were harvested, centrifuged, dialyzed 72 hours against RPMI 1640 medium and sterilized by Millipore filtration. IL-1 assay was carried out by [^{3}H]thymidine incorporation into

Table 2. Life prolongation effect of melanocidins A and B on B16 melanoma bearing mice.

Compounds	Treatments (mg/kg)	Mean survival (days)	T/C* (%)	Body weight (g)**
Melanocidin A	23.00	20.3	114	−0.1
	11.50	22.1	124	−0.2
	5.75	21.0	117	0.1
	2.88	19.9	111	−0.2
Control	0.00	17.8	—	0.9
Melanocidin B	26.00	25.0	140	−0.2
	10.50	20.3	114	0.1
	5.25	20.0	112	0.1
	2.63	20.3	114	0.1
Control	0.00	17.8	—	0.9

* T/C: (Treatment/control ×100) %.
** Treatments − Control.

Fig. 1. Effect on cytotoxicity of P815 cells by peritoneal macrophages (*in vitro*).
a) Melanocidins A and B. ● Control, ○ lentinan 5 μg/ml, ▲ melanocidin A 100 μg/ml, △ melanocidin A 10 μg/ml, ■ melanocidin B 100 μg/ml, □ melanocidin B 10 μg/ml.
b) Nucleoticidin. ● Control, ▲ 10 μg/ml, ■ 100 μg/ml.

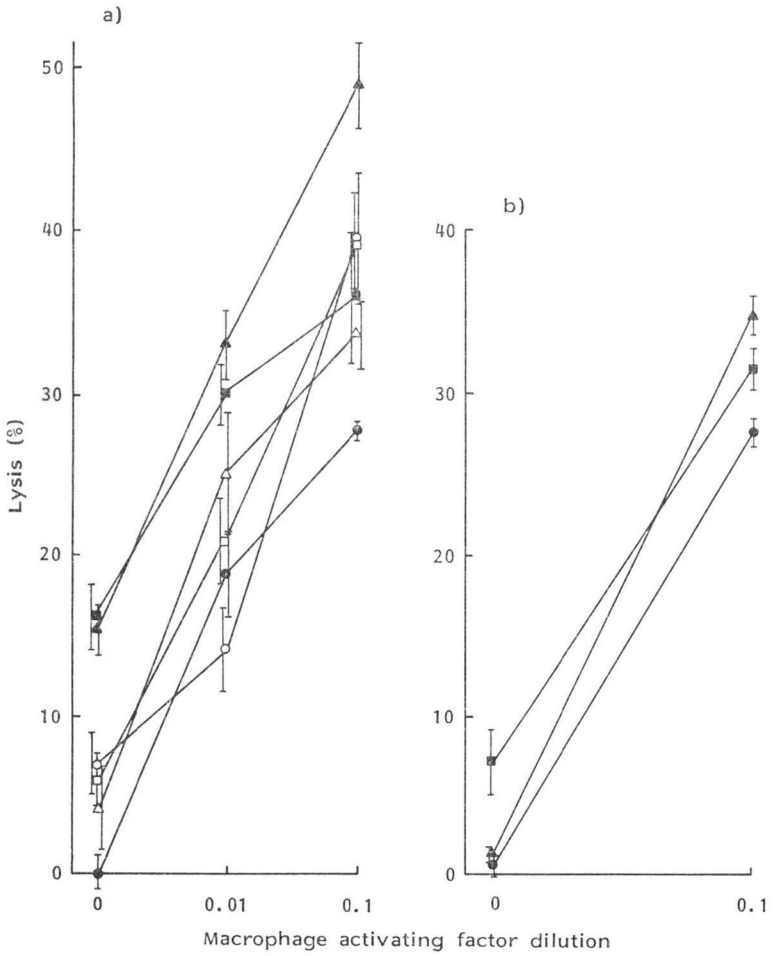

Table 3. O_2^- Production by 5′-nucleotidase inhibitiors.

Compounds	Dose (ip)	O_2^- Production (nmol/minute/2×10^5 cells)	O_2^- Production ratio (%)
Control (saline)	0.3 ml	0.072±0.012	100
Lentinan	0.4 mg	0.195±0.025	271
Nucleoticidin	0.6 mg	0.123±0.021	171
Melanocidin A	0.6 mg	0.138±0.026	192
Melanocidin B	0.6 mg	0.146±0.014	203

Table 4. Release of IL-1 by macrophages incubated with 5′-nucleotidase inhibitors.

Agents added	Concentration (μg/ml)	LAF activity* (cpm [^{3}H]-thymidine incorporation)
None	0	577
LPS	20	5,134**
Nucleoticidin	10	657
	100	4,851***
Melanocidin A	10	210****
	100	1,657***
Melanocidin B	10	343****
	100	2,530***
Lentinan	10	531
	100	407

* LAF activity is presented as [^{3}H]thymidine incorporated by thymocyte cultures, incubated with the tested supernatants in a final dilution of 1/2.
** Significant at $P<0.01$.
*** Significant at $P<0.001$.
**** Significant at $P<0.02$.

murine thymocytes (lymphocyte activating factor: LAF)[9,10]. Thymocytes were cultured for 72 hours at 1.5×10^6 cells/tube (12×105 mm) in the presence of 1 μg/ml phytohemagglutinin and a sample to be assayed for LAF activity. Cultures were pulsed with 1 μCi [^{3}H]thymidine for the final 8-hour incubation.

Tumor Necrosis Factor (TNF) Assay

Preparation of Tumor Necrosis Serum: BALB/c mice (5 weeks old) were injected intraperitoneally with 1.5 mg of living BCG or 0.6 mg each of 5′-nucleotidase inhibitors and 14 days later, injected with 10 μg of lipopolysaccharide (LPS: B5). Two hours after LPS injection, animals were exanguinated and the serum was prepared.

Cytotoxicity: TNF activity was determined by assay of killing of L929 cells, as described by RUFF and GIFFORD[11,12]. Briefly, L929 cells (2.5×10^4/well), tumor necrosis serum and actinomycin D (0.2 μg/well) were incubated for 18 hours in 96-well microplate containing 200 μl medium (Eagle's MEM supplemented with 5% FCS). Then, cells were stained with 0.2% crystal violet for 15 minutes, washed with PBS and treated with 100 μl of 0.5% SDS. The absorbance at 590 nm of the solubilized material was measured.

Results

Antitumor Activity

Nucleoticidin and melanocidins A and B were examined for their antitumor activity against Sarcoma 180 solid tumor in mice. As shown in Table 1, nucleoticidin showed antitumor activity at 5~10 mg/kg. Melanocidins A and B did not show any significant inhibition against Sarcoma 180 solid tumor at the same dose. On the other hand, the survival period of mice bearing B16 mouse melanoma was prolonged by administration of melanocidin B at 26 mg/kg (Table 2).

Nucleoticidin and melanocidins A and B were tested for a direct cytocidal effect on cells such as human epidermoid carcinoma A431 cells and some microorganisms (data are not shown). These compounds did not show such an effect at 500 μg/ml. Furthermore, no toxicity was observed by intraperitoneal administration of 10 mg/kg/day for 10 days. Therefore, it is suggested that their antitumor activities are host-mediated.

Table 5. Cytotoxic activity of tumor necrosis serum treated with 5′-nucleotidase inhibitors and LPS (target: L929 cell).

Treatments			Serum dilution (times)	Cytotoxicity (%)
Priming agents (mg)		Eliciting agents (μg)		
Nucleoticidin	(0.6)	LPS (10)	10×	34.0±3.0
	(0.6)	None	10×	0
Melanocidin A	(0.6)	LPS (10)	10×	43.0±3.0
	(0.6)	None	10×	0
Melanocidin B	(0.6)	LPS (10)	10×	36.0±5.0
	(0.6)	None	10×	0
Lentinan	(0.6)	LPS (10)	10×	39.5±5.5
BCG	(1.5)	LPS (10)	100×	45.0±6.0
None		LPS (10)	10×	0

Effect on Mouse Immune System

In Vitro Activation of Peritoneal Macrophages (Cytotoxicity on P815 Cells)

Murine peritoneal exudate macrophages were treated *in vitro* with 100 μg/ml of 5′-nucleotidase inhibitors and/or macrophages activating factor (MAF: supernatant of splenocyte culture) for the examination of their cytolytic activity against P815 cells. As shown in Fig. 1 a) and b), the 5′-nucleotidase inhibitors gave significant enhancement of cytotoxicity of peritoneal exudate macrophages.

Superoxide-generating Activity by Peritoneal Macrophages

As shown in Table 3, O_2^--generating activity was enhanced after injection of the inhibitors intraperitoneally.

Interferon (IF) Induction

The effect of the inhibitors on the release of IF was examined in C57BL/6 mice. However, IF was not produced for 24 hours after intraperitoneally injection of 0.6 mg each of the inhibitors (data are not shown).

Interleukin (IL)-1 Induction

Monolayers of peritoneal macrophages were incubated with the inhibitors and the level of IL-1 was measured. All inhibitors markedly elevated IL-1 secretion (Table 4).

TNF Induction

The production of the tumor necrosis factor was investigated by using BALB/c mice treated with the inhibitors as a priming agents. TNF activity was measured by assaying killing of L929 cells, and the results are summarized in Table 5. All the inhibitors secreted TNF moderately. Therefore, these 5′-nucleotidase inhibitors are useful reagents as priming agents.

Discussion

Nucleoticidin possessed a strong antitumor activity against Sarcoma 180 and melanocidins A and B showed an antitumor activity against B16 melanoma. However, these effects are not direct but may be host-mediated. This was confirmed demonstrating that murine macrophages were stimulated by the inhibitors to become tumoricidal (Fig. 1), and the enhanced O_2^--generating activity may take a part in the possible direct tumoricidal mechanism of activated macrophages (Table 3). In addition, these inhibitors stimulated the release of IL-1 and TNF through the activation of macrophages (Tables

4 and 5). Melanocidins A and B significantly inhibited IL-1 production at 10 μg/ml. IL-1 may have a multiplicity of divergent biological effect including the augmentation of mitogenesis by immature thymocyte, the differentiation of peripheral T lymphocyte to express cell surface markers and produce lymphokines, and the promotion of antibody production by B lymphocytes. In addition, IL-1 acts not only on leukocytes, but also on cells such as fibroblast, osteoclasts, and endothelial cells. IL-1 can thus rapidly marshal widely disparate host reactions to both local and systemic immunological and inflammatory stimulants. Although the specific mechanism of the antitumor effect of these inhibitors are not fully elucidated, it is felt that augmentation of general immune responses, induction of specific cell-mediated cytotoxicity mediated by macrophages and T lymphocytes are involved in this action.

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