Phosphatase-Inhibitory Activity and Activation of Murine Macrophages by New 5′-Nucleotidase Inhibitors, NPF-86IA, NPF-86IB, NPF-86IIA and NPF-86IIB

Toshiharu MATSUO,[a] Keijiro UCHINO,*[,a] Toshikatsu TOUKAIRIN,[a] Masaya IWAMOTO,[a] Yasuhiro TONOSAKI,[a] Tetsu AKIYAMA,[b] Hiroshi OGAWARA[c] and Akira FUKUCHI[a]

Central Laboratory, Nippon Flour Mills Co., Ltd.,[a] 2114–2, Nurumizu, Atsugi, Kanagawa 243, Japan, Institute for Virus Research, University of Kyoto,[b] Kawaramachi, Seigoin, Sakyo-ku, Kyoto 606, Japan and Department of Biochemistry, Meiji College of Pharmacy,[c] Nozawa-1, Setagaya-ku, Tokyo 154, Japan. *Received November 11, 1988*

New 5′-nucleotidase-inhibitory polyphenols named NPF-86IA, NPF-86IB, NPF-86IIA and NPF-86IIB were isolated from the seeds of *Areca catechu* L. The ability of the inhibitors to precipitate gelatin was investigated by microturbidimetry. These inhibitors produced weak turbidity. As 5′-nucleotidase is a kind of phosphatase, we examined the effects of these inhibitors on alkaline and acidic phosphatases. While they showed moderate inhibitory effects on the activity of acidic phosphatases, they did not have any significant effect on the activity of alkaline phosphatase. Therefore, they showed a higher inhibitory effect on the 5′-nucleotidase than the other phosphatases. Murine macrophages were directly stimulated by the 5′-nucleotidase inhibitors.

Keywords 5′-nucleotidase; inhibitor; alkaline phosphatase; acidic phosphatase; superoxide; *Areca catechu*; polyphenol; antitumor activity

5′-Nucleotidase (EC 3.1.3.5, 5′-ribonucleotide phosphohydrolase) is located primarily in the plasma membrane.[1-3] Most of the 5′-nucleotidase activity of this type is expressed by ecto-enzymes.[4-7] Many inhibitors of ecto-enzymes show antitumor and immunopotentiator activities.[8] For example, bestatin, diketocoriolin B and forphenicine were isolated as inhibitors of aminopeptidase, alkaline phosphatase and Na^+, K^+-adenosine triphosphatase (ATPase), respectively[9] which are located mainly in cell surfaces or membranes. The primary action of these inhibitors seems to occur at the cell surface. Bestatin binds to cells, especially to macrophages, diketocoriolin B acts on B-lymphocytes and increases the number of antibody-forming cells, and forphenicine acts on macrophages and increases antibody formation. Other inhibitors of 5′-nucleotidase may therefore show such activities.

Recently, we have isolated four new 5′-nucleotidase inhibitors, named NPF-86IA, NPF-86IB, NPF-86IIA and NPF-86IIB from the seeds of *Areca catechu* L.[10] These compounds were found to be polyphenolic substances and showed antitumor activity, prolonging the life span of mice inoculated with Ehrlich ascites carcinoma.[11] However, these inhibitors did not show any significant cytotoxicity against various mammalian cells in culture.[11] The primary action of these compounds therefore seems to involve some change at the cell surface.

As the compounds described above inhibit 5′-nucleotidase, a kind of phosphatase, we tested their inhibitory activity against alkaline and acidic phosphatases. In addition, we examined the activation of macrophages by these compounds. This paper describes the results.

Materials and Methods

Animals and Chemicals C3H/He mice, propagated at Shizuoka Agricultural Cooperative Association (Hamamatsu, Japan) were used. Acidic phosphatases of potato and wheat germ and alkaline phosphatases from porcine placenta, chicken intestine, calf intestine and *Escherichia coli* were obtained from Sigma Chemical Co. *p*-Nitrophenyl phosphate and ferricytochrome C were from Nakarai Tesque Inc.

Gelatin Precipitation Test (Tannic Activity) A microturbidimetric method was utilized to evaluate the inhibitors in a gelatin precipitation test.[12]

Enzyme Assay 5′-Nucleotidase inhibitory activity was determined by the method described previously.[13] Inhibition of alkaline and acidic phosphatases was assayed as described previously[14,15] using *p*-nitrophenyl phosphate as a substrate.

Preparation of Peritoneal Macrophages Method A: C3H/He mice (8 weeks old) were injected intraperitoneally with 10 mg of inhibitor. Four days after the injection, peritoneal exudate cells (PEC) were harvested in Hanks balanced solution, plated on fetal calf serum (FCS)-coated plastic discs (70 mm), and kept for 30 min. PEC were washed with RPMI 1640 medium to remove nonadherent cells.

Method B: C3H/He mice were injected intraperitoneally with 1.5 ml of 10% Proteose peptone solution. After 3 d, the mice were killed and their PEC were harvested in Eagle's MEM. The cells were cultured on plastic discs at 37 °C for 2 h and washed vigorously three times with Eagle's MEM. The adherent cells were further incubated with or without the inhibitors for 48 h and then used directly for the measurement of superoxide anion (O_2^-).

Measurement of Superoxide Anion O_2^--Generating activity was measured by the method of Inokuchi *et al.*[16,17] The adherent cells were treated with a solution containing 0.05% ethylenediaminetetraacetic acid (EDTA) and 10% FCS in phosphate-buffered saline (PBS). The resulting single cell suspension was diluted in Krebs–Ringer solution. The reaction mixture (2.5 ml) contained 2 mM glucose, 1 mM, $CaCl_2$, 80 μM ferricytochrome C, and 0.5 ml of the above cell suspension.

Results

Gelatin Precipitation Ability As some polyphenols are known to precipitate proteins (tannic activity),[18] the four inhibitors from *A. catechu* were tested for their abilities to precipitate gelatin. They were found to precipitate gelatin very weakly (Table I).

Inhibitory Activities against 5′-Nucleotidase and Alkaline and Acidic Phosphatases As shown in Tables II and III, NPF-86IA, NPF-86IB, NPF-86IIA and NPF-86IIB strongly inhibited 5′-nucleotidase activities but not alkaline phosphatase activities at 10 μg/ml. The acidic phosphatases were inhibited moderately by these compounds. Tannic

TABLE I. Gelatin Precipitation Test (Tannic Activity)

Compound	Tannic activity (%)
NPF-86IA	30
NPF-86IB	23
NPF-86IIA	<10
NPF-86IIB	<10
Tannic acid	100

TABLE II. Effect on 5′-Nucleotidase Activity

Source of enzyme	IC_{50} (μg/ml)				
	NPF-86IA	NPF-86IB	NPF-86IIA	NPF-86IIB	Tannic acid
Snake	0.096	0.194	0.070	0.075	>200
Rat liver	20.2	23.3	18.5	17.5	>200

TABLE III. Effects on Various Phosphatases

	Inhibition (%)			
	NPF-86IA	NPF-86IB	NPF-81IA	NPF-86IIB
Acidic phosphatase				
Potato	25	13	64	47
Wheat germ	50	35	70	52
Alkaline phosphatase				
Porcine placenta	0	0	0	0
Chicken intestine	0	0	0	0
Calf intestine	0	0	0	0
Escherichia coli	0	0	0	0

a) Inhibitor concentration, 10 μg/ml.

TABLE IV. O_2^- Production by 5′-Nucleotidase Inhibitors (Method A)

Compound	Dose (mg/kg)	O_2^- production (nmol/45 min/mg protein)	O_2^- production ratio (%)
Control	0	38.4±3.3	100
NF-86I	10	69.5±1.5	181
NF-86II	10	107 ±5.6	279

NF-86I and NF-86II are mixtures of A and B components of each fraction, respectively.[10]

TABLE V. O_2^- Production by 5′-Nucleotidase Inhibitors (Method B)

Compound	Concentration (μg/ml)	O_2^- production (nmol/60 min/mg protein)	O_2^- production ratio (%)
Control	0	77.5± 2.3	100
NPF-86IA	100	215 ±12.5	277
NPF-86IB	100	208 ±14.4	268
NPF-86IIA	100	189 ±10.7	244
NPF-86IIB	100	203 ±13.8	262

acid did not show any effect on 5′-nucleotidase activity at 200 μg/ml.

Superoxide-Generating Activity of Peritoneal Macrophages Two methods were used to estimate O_2^--generating activity of macrophages. Intraperitoneal injection of NF-86I and NF-86II at 10 mg/kg enhanced O_2^- production 1.8 to 2.8 fold, respectively (Table IV). When murine peritoneal exudate macrophages were treated *in vitro* with 100 μg/ml of the 5′-nucleotidase inhibitors, these inhibitors enhanced the O_2^--generating activity 2.4- to 2.8-fold (Table V). These results suggest that murine macrophages were directly stimulated by the 5′-nucleotidase inhibitors.

Discussion

5′-Nucleotidase is known to be associated with the plasma membrane of mouse peritoneal macrophages[19] and rat liver cells,[20,21] and was shown to be an ecto-enzyme in guinea-pig neutrophils.[22] Consequently, 5′-nucleotidase is used extensively as a marker for the plasma membrane in a variety of cells and tissues (review[23]). Inhibitors of 5′-nucleotidase are therefore suspected to bind to the cell surface and modify the functions of immunoresponsive cells. Recently, we found three inhibitors, named nucleoticidin,[13] melanocidin A and melanocidin B[24] from microbial metabolites. These substances were polysaccharide in nature and showed antitumor activities. The antitumor effects were not direct but host-mediated. Several investigators have reported the biological properties including immunostimulating activities of polysaccharides such as lentinan,[25] schizophyllan,[26] PS-K[27] and mannan.[28]

We further screened 5′-nucleotidase inhibitors and isolated NPF-86IA, NPF86-IB, NPF86-IIA and NPF-86IIB from the seeds of *Areca catechu* L. These inhibitors were not polysaccharides but polyphenolic substances. Some polyphenols such as tannins have a characteristic ability to precipitate proteins. Their molecular weight usually range from 500 to 3000. In contrast, the average molecular weights of the inhibitors, NPF-86IA, NPF-86IB, NPF86-IIA and NPF86-IIB, were estimated to be 5620, 5000, 29400 and 8610 dalton (Da), respectively. The ability of these inhibitors to precipitate gelatin was investigated by microturbidimetry. These polyphenolic inhibitors gave weak turbidity (Table I). In view of these findings, it is considered that these inhibitors can not be classified as tannins, even if they are polyphenolic in nature. There are very few reports on the biological properties of polyphenols of this type although Takeuchi *et al.* recently reported antitumor activity[29] of polyphenols from the bark of *Ptero carpus indicus* W.

In order to check the possible specificity of the samples as inhibitors of 5′-nucleotidase, we examined the effects on alkaline and acidic phosphatases, as 5′-nucleotidase is a kind of phosphatase. These compounds showed moderate inhibitory effects on the activity of acidic phosphatases, but none of them had any significant effect on the activity of alkaline phosphatase at 10 μg/ml (Table III). It is concluded that the compounds had greater inhibitory effects on 5′-nucleotidase than on alkaline phosphatases and thus seem to be rather specific inhibitors of 5′-nucleotidase. 5′-Nucleotidase and alkaline phosphatases are ecto-enzymes.[30] Studies on the inhibitory activities of these compounds against ecto-enzymes in intact cells, such as Ehrlich, L_{1210} and HL-60 cells, are in progress.

Next, we examined the activation of macrophages by estimating O_2^--generating activity in order to cast light on the mechanism of the antitumor effect. The results suggest that murine macrophages were directly stimulated by the 5′-nucleotidase inhibitors. As described in the previous paper,[10] the inhibitors did not show any cytotoxicity against various mammalian cells. It is likely, therefore, that

the inhibitors show antitumor activity through potentiation of the immunity of host animals.

References

1) W. H. Evans and J. W. Gurd, *Biochem. J.*, **133**, 189 (1973).
2) J. R. Riordan and M. Slavik, *Biochim. Biophys. Acta*, **373**, 356 (1974).
3) A. Zachowski, W. H. Evans and A. Paraf, *Biochim. Biophys. Acta*, **644**, 121 (1981).
4) J. W. Gurd and W. H. Evans, *Arch. Biochem. Biophys.*, **164**, 305 (1974).
5) J. W. DePierre and M. L. Karnovsky, *J. Cell Biol.*, **56**, 275 (1973).
6) A. C. Newby, J. P. Luzio and C. N. Hales, *Biochem. J.*, **146**, 625 (1975).
7) E. G. Trams and C. J. Lauter, *Biochim. Biophys. Acta*, **345**, 180 (1974).
8) H. Umezawa, *Jpn. J. Antibiot.*, **30**, suppl., s138 (1977).
9) H. Umezawa, "Small Molecular Immunomodifiers of Microbial Origin," Japan Scientific Societies' Press, Tokyo, 1981.
10) K. Uchino, T. Matsuo, M. Iwamoto, Y. Tonosaki and A. Fukuchi, *Planta Medica*, **54**, 419 (1988).
11) M. Iwamoto, T. Matsuo, K. Uchino, T. Tonosaki and A. Fukuchi, *Planta Medica*, **54**, 422 (1988).
12) H. I. Oh and J. E. Hoff, *Food Sci.*, **44**, 87 (1979).
13) H. Ogawara, K. Uchino, T. Akiyama and S. Watanabe, *J. Antibiot.*, **38**, 153 (1985).
14) O. A. Bessey, O. H. Lowry and M. J. Brock, *J. Biol. Chem.*, **164**, 321 (1946).
15) A. Chersi, A. Bernardi and G. Bernardi, *Biochim. Biophys. Acta*, **129**, 12 (1966).
16) A. Nakagawara, K. Kayashima, R. Tamada, K. Onoue, K. Ikeda and K. Inokuchi, *Gann*, **70**, 829 (1979).
17) R. Kumashiro, Y. Hiramoto, T. Nhozuka, Y. Abe, H. Inoue, R. Tamada, M. Shiraishi, R. Karino and K. Inokuchi, *Clin. Immunol.*, **16**, 689 (1984).
18) K. H. Gustavson, "Chemistry of Vegetable Tannins," Academic Press, New York, 1966.
19) Z. Werb and Z. A. Cohn, *J. Biol. Chem.*, **247**, 2439 (1972).
20) E. Essner, A. B. Novikoff and B. Masek, *J. Biophys. Biochem. Cytol.*, **4**, 711 (1958).
21) M. G. Farquhar, J. J. M. Bergeron and G. E. Palade, *J. Cell Biol.*, **60**, 8 (1974).
22) J. W. DePierre and M. L. Karnovsky, *J. Biol. Chem.*, **249**, 7111 (1974).
23) J. W. DePierre and M. L. Karnovsky, *J. Cell Biol.*, **56**, 275 (1973).
24) H. Ogawara, K. Uchino, T. Akiyama and S. Watanabe, *J. Antibiot.*, **38**, 587 (1985).
25) G. Chihara, J. Hamuro, Y. Y. Maeda, Y. Arai and F. Fukuoka, *Cancer Res.*, **30**, 2776 (1970).
26) N. Komatsu, S. Okubo, S. Kikumoto, K. Kimura, G. Saito and S. Sakai, *Gann*, **60**, 137 (1969).
27) S. Tsukagoshi and F. Ohasi, *Gann*, **65**, 557 (1974).
28) S. Oka, N. Kumano and K. Kurita, *Gann*, **63**, 365 (1972).
29) S. Takeuchi, Y. Kono, Hadiman, T. Mizutani, K. Maruyama, R. Nakayama, A. Hiraoka, Y. Suzuki, R. Watanabe, A. Kawarada and S. S. Adisewojo, *Agric. Biol. Chem.*, **50**, 569 (1986).
30) T. Aoyagi, K. Nerome, J. Suzuki, T. Takeuchi and H. Umezawa, *Biochem. Biophys. Res. Commun.*, **60**, 1178 (1974).