Neurotropic Pyrimidine Heterocyclic Compounds. I. The Newly Synthesized Pyrimidine Compounds Promote Neurite Outgrowth of GOTO and Neuro 2a Neuroblastoma Cell Lines, and Potentiate Nerve Growth Factor (NGF)-Induced Neurite Sprouting of PC 12 Cells

Akira AWAYA,^{*,a} Hisashi KOBAYASHI,^b Kazutoshi HORIKOMI,^b Shuitsu TANAKA,^d
Ahmed MD. KABIR,^e Keiichi YOKOYAMA,^c Hiroyasu OHNO,^c Koji KATO,^c Takumi KITAHARA,^c
Ikuo TOMINO,^c Shigeru ISAYAMA^c and Shun NAKAMURA^e

New Product Planning Dept.^a and Institute of Biological Science,^b Mitsui Pharmaceuticals, Inc., 5–12, Iwamotocho 3-chome, Chiyoda-ku, Tokyo 101,^a and 1900 Togo, Mobara 297,^b Japan, Synthetic Chemistry & Bioscience Laboratories, Mitsui Petrochemical Industries, Ltd., Iwakuni 740, Japan Department of Natural Science, Saga Medical School,^d Saga 849, Japan and Division of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry,^e 4–1–1, Ogawahigashi, Kodaira, Tokyo 187, Japan. Received September 9, 1992

From the study using cultured human and mouse neuroblastoma cells, we found that a new type of synthetic bicyclic pyrimidine compounds possessing piperazine moiety strongly promoted neurite outgrowth in neuroblastoma cell lines human GOTO and mouse neuro 2a. The most effective compounds of these 2-piperazinopyrimidine derivatives possessing nerve growth factor (NGF)-like activity were 2-piperazino-6-oxo-5,6-dihydro(7H)pyrrolo[2,3-d]pyrimidine and 2-piperadino-6-methyl-5-oxo-5,6-dihydro(7H)pyrrolo[3,4-d]pyrimidine. The piperazinopyrimidine compounds were also shown to potentiate NGF-induced neurite sprouting of rat pheochromocytoma PC12 cells. The compounds were more effective in cell cultures than isopropylaminopyrimidine (isaxonine) which had been previously developed and then withdrawn. We discussed the merit of the method of the screening of neurotropic compounds by neurite sprouting activity in cultured neuroblastoma cells.

Keywords 2-piperazinopyrimidine; neuroblastoma cell; neurite outgrowth; GOTO; neuro 2a; PC12 cells

Introduction

Nagai and our colleagues have reported that some component of gangliosides, particularly GQ1b strongly promoted neurite outgrowth in human and mouse neuroblastoma cell lines.^{1–3)} Thus, we investigated the effect of total gangliosides (tGS), GQ1b and GM1 on the functional recovery of rat sciatic nerves which had been crushed as a model of peripheral nervous disorders.⁴⁾

Further, we have reported that tGS facilitated the survival of cultured rat chromaffin cells⁵⁾ and that tGS induced peculiar morphological features in grafted dopaminergic cells and promoted the recovery of motor imbalance in rats with unilateral lesions in the nigrostriatal dopamine pathway.⁶⁾

On the other hand, nerve growth factor (NGF) is the best characterized neurotrophic factor that is capable of inducing cell differentiation of neural cells and the ability to promote neurite sprouting.⁷⁾ But the application of NGF to the therapy of patients is difficult for various reasons. At present, few pharmaceuticals have actually been applied for clinical therapy or tested for governmental approval for the therapy, in the field of peripheral and/or central nervous disorders, such as gangliosides mixtures,⁸⁾ mecobalamin,⁹⁾ thyrotropin-releasing hormone (TRH)¹⁰⁾ and ORG 2766 peptide,¹¹⁾ etc. These are originally compounds of natural origin and there are some pharmaceutical problems in developing the mixture agents of natural products such as gangliosides.

So we have been exploring and surveying synthetic neurotropic agents of small molecular weight by making the best use of our experience in studying the neurotropic effect of gangliosides *in vitro* and *in vivo*. Thus, new heterocyclic 2-piperazinopyrimidine compounds,¹²⁾ some of which were originally synthesized for the screening of

antibacterials and herbicides in our laboratory^{13–15)} were at first tested for their possible neurotrophic activity by using tissue culture systems of neuroblastoma cell lines.

We found that the pyrimidine compounds were as much potent activators in promoting neurite outgrowth as tGS and were more effective than 2-isopropylaminopyrimidine phosphate, *i.e.*, isaxonine. Isaxonine was previously developed as a neurotropic pharmaceutical,¹⁶⁾ but probably for its toxicological effects, the application of the compound to patients was abandoned and the compound was withdrawn from clinical use at that time.

Moreover, we examined the effect of our 2-piperazinopyrimidine compounds on the NGF-induced neurite sprouting of rat pheochromocytoma PC12 cells, and found that the compounds enhanced the effect of NGF on neurite outgrowth of the cells.

Materials and Methods

Materials The human neuroblastoma cell lines, GOTO from adrenal gland, and NB-1 from a metastatic neck lymph node, were obtained from Dr. M. Sekiguchi (Institute of Medical Science, The University of Tokyo). These two cell lines were both established by Sekiguchi *et al.* (1979). The mouse neuro 2a neuroblastoma cell line was a gift of Dr. Y. Nagai (Faculty of Medicine, The University of Tokyo). Subclone 22a¹⁷⁾ isolated from rat PC12 pheochromocytoma cells was used throughout this study. Mouse NGF (2.5S) was isolated from submaxillary glands of male adult mouse by the method of Mobley *et al.*¹⁸⁾

Rosewell Park Memorial Institute Medium 1640 (RPMI) and Dulbecco's Modified Eagle's Medium (MEM) were from Nissui Seiyaku Co., Ltd., and fetal calf serum (FCS) used for neuroblastoma cell culture was from GIBCO (Gland Island, NY, U.S.A.). Horse serum and fetal bovine serum used for PC12 cell culture were purchased from JRH Biosciences (Lenexa, KS, U.S.A.) and Whittaker Bioproducts, Inc. (Walkersville, MD, U.S.A.), respectively. Tissue culture plates used for PC12 cell culture were obtained from Nippon Becton Dickinson Co., Ltd. (Tokyo, Japan) except for the 24-well multi-dish (Nunc, Nippon Intermed Co., Ltd., Tokyo, Japan).

Culture Conditions for GOTO, NB-1 and Neuro 2a Cell Lines The three

cell lines were usually cultured in a medium containing 45% RPMI and 45% MEM, supplemented with 10% fetal calf serum, sodium penicillin G (100 units/ml), and streptomycin in sulfate (100 mg/ml). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. For experiments analyzing the effects of serum, serum-free medium (50% RPMI and 50% MEM, supplemented with the above antibiotics) was used.

Assay of Effects of Synthesized Compounds on Neurite Sprouting of Neuroblastoma Cell Lines Neuroblastoma cell lines GOTO, and neuro 2a growing in the exponential phase were distributed at a density of 1000 cells/well in a 48-well plate, and were cultured for 1 d. The medium was replaced by a medium containing FCS and antibiotics or FCS-free medium containing antibiotics, and the cells were further cultured with or without synthetic compounds for 24 h. Then, under a microscope, the number of cells possessing outgrown neurites (cells having at least one neurite with a length at least as large as a longer diameter of the cell bodies) was counted visually, and the proportion of the number of these cells to the total number of the cells was calculated and expressed as a percentage. The viability of these cell lines in the cultures with the higher concentration of synthetic compounds decreased during culture periods over 48 h.

Culture Conditions and Assay of Neurite Outgrowth of PC12 Cells Subclone 22a cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO₂ on collagen-coated tissue culture dishes in RPMI 1640 medium supplemented with 7% heat-inactivated horse serum, 5% fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin. The cells were seeded on 24-well multi-dish (0.8 × 10⁴ cells/well) and incubated for 2–4 d before use. The seeded cells were washed twice and incubated with 0.5 ml of serum-free RPMI 1640 medium containing 5 µg/ml transferin, 5 µg/ml insulin, 20 nM progesterone and 30 nM Na₂Se₂O₃. Drugs were added in this medium at each concentration in the presence of 1.5 ng/ml NGF or in its absence. The cells were then cultured for 48 h, washed with PBS twice, fixed with 1% glutaraldehyde in PBS. Evaluation of the presence or absence of neurites on cells or cell aggregates was then made directly under a microscope. On the cell dissociation, the cells were vigorously triturated so that most of them were single cells or small aggregates containing 2–5 cells. A cell (or aggregate) having one or more neurites longer than the diameter of the cell body (or aggregate itself) was counted as a positive. The percentage of neurite-bearing cells (or aggregates) with SEM was shown against the total cell (or aggregate) number 400 per well. During the 6 d of culture with 0.3 mM of 820 as described in the Synthetic Compounds, the cells died in the absence of NGF, but the cells survived and outgrew neurites in the presence of NGF.

Synthetic Compounds All melting points are uncorrected. Infrared (IR) spectra were measured with a Jasco DS-701G. ¹H-NMR spectra were taken at 90 MHz with a JEOL FX-90Q using tetramethylsilane as an internal standard.

2-Piperadino-5-oxo-5,6-dihydro(7H)pyrrolo[3,4-*d*]pyrimidine Hydrochloride (883)¹² To a solution of ethyl 4-chloromethyl-2-(4-benzylpiperazino)pyrimidine-5-carboxylate (2.25 g, 6 mmol) in ethanol (10 ml) were added dropwise 30% aq. NH₄OH solution (10 ml, 59 mmol) at 20°C, and then stirred for 12 h. The resulting mixture was poured into aq. NaHCO₃, and then extracted with CHCl₃. The extract was evaporated and recrystallized from toluene to give pale brown crystals (0.70 g, yield: 38%). A suspension of these crystals (0.7 g, 2.3 mmol) and 10% Pd-C (0.07 g) in AcOH (13 ml) was stirred for 1 h in a hydrogen atmosphere. The catalyst was filtered off and the filtrate was evaporated. The residue was basified with aq. NaHCO₃, and extracted with toluene. The extract was dried over MgSO₄, and evaporated to give 0.3 g (yield: 66%) of pale brown oil. IR (KBr) cm⁻¹: 3210, 3100, 2920, 2880, 1700, 1612, 1260, 1150, 980. NMR (DMSO-*d*₆) δ: 2.80 (4H, brs), 3.24 (1H, brs), 3.78 (4H, brs), 4.23 (2H, s), 8.18 (1H, brs), 8.58 (1H, s). The resulting oil was converted into the hydrochloride (883) in a usual manner.

2-Piperadino-6-methyl-5-oxo-5,6-dihydro(7H)pyrrolo[3,4-*d*]pyrimidine maleate (818)¹² and 2-piperadino-6-ethyl-5-oxo-5,6-dihydro(7H)pyrrolo[3,4-*d*]pyrimidine hydrochloride (858)¹² were also prepared in the same manner as described above. (818: free form) mp 176–177°C. IR (CHCl₃) cm⁻¹: 1685, 1618, 1522, 1350. NMR (CDCl₃) δ: 2.98 (4H, m), 3.18 (3H, s), 3.96 (4H, m), 4.24 (2H, s), 8.70 (1H, s). (858: free form) mp 59°C. IR (KBr) cm⁻¹: 3480, 3300, 2900, 1650, 1618, 1520, 1438, 1236, 1160, 862. NMR (CDCl₃) δ: 1.24 (3H, t, *J* = 7 Hz), 2.12 (1H, brs), 2.95 (4H, brs), 3.62 (2H, q, *J* = 7 Hz), 3.92 (4H, brs), 4.21 (2H, s), 8.66 (1H, s).

2-Piperadino-7-methyl-6-oxo-5,6-dihydro(7H)pyrrolo[2,3-*d*]pyrimidine Maleate (820)¹² A mixture of ethyl 2-(4-benzylpiperazino)-4-chloropyrimidine-5-acetate (200 g, 0.534 mol) and 40% methylamine methanol solution (82.9 g, 1.07 mol) in ethanol (430 ml) was placed in a pressure

vessel, then heated at 120°C for 2 h. The solvent was evaporated, and to the residue water was added, then extracted with CHCl₃. The extract was dried over MgSO₄, and evaporated. The resulting residue was chromatographed on silica gel to give red crystals (126 g, 73%). A suspension of these crystals (126 g, 0.39 mol) and 10% Pd-C (10 g) in ethanol (2000 ml) was refluxed for 4.5 h in a hydrogen atmosphere. The catalyst was filtered off and the filtrate was evaporated. The residue was basified with aq. Na₂CO₃, and extracted with CHCl₃. The extract was dried over MgSO₄, and evaporated to give 64.3 g (yield: 70%) of red crystals. mp 145–147°C. IR (KBr) cm⁻¹: 3340, 1738, 1630, 1580, 1450, 1105. NMR (CDCl₃) δ: 1.86 (1H, brs), 2.94 (4H, m), 3.21 (3H, s), 3.43 (2H, s), 3.82 (4H, m), 7.93 (1H, s). The resulting crystals were converted into the maleate (820) in a usual manner.

2-Piperadino-6-methyl-5-oxo-5,6-dihydro(7H)pyrazolo[3,4-*d*]pyrimidine Hydrochloride (862)¹³ To a solution of ethyl 2-(4-benzylpiperadino)-4-chloropyrimidine-5-carboxylate (5.3 g, 14.6 mmol) in CHCl₃ (10 ml), 2-methylhydrazine (2.0 g, 43.4 mmol) was added dropwise over 15 min. The resulting mixture was stirred at room temperature for 3 h. The solvent was evaporated and washed with AcOEt to give pale brown crystals (3.0 g, 63%). A suspension of these crystals (3.0 g, 9.2 mol) and 10% Pd-C (0.3 g) in ethanol (30 ml) was heated at 60°C for 3 h in a hydrogen atmosphere. The catalyst was filtered off and filtrate was evaporated.

The residue was basified with aq. Na₂CO₃, and extracted with CHCl₃. The extract was dried over MgSO₄, and evaporated to give 1.8 g (yield: 88%) of yellow crystals. mp 272–276°C (dec.). IR (KBr) cm⁻¹: 3600–3300, 1700, 1621, 1565, 1443. NMR (DMSO-*d*₆) δ: 3.20 (4H, m), 3.64 (3H, s), 4.24 (2H, m), 8.70 (1H, s). The resulting crystals were converted into the hydrochloride (862) in a usual manner.

6-Ethyl-2-piperazino-5-oxo-5,6-dihydroprido[4,3-*d*]pyrimidine Hydrochloride (712)¹⁴ To a suspension of sodium hydride (60% in oil, 0.24 g, 6 mmol) in dimethylformamide (DMF) (3 ml) was added 2-(4-benzylpiperazino)-5-ethylaminocarbonyl-4-methylpyrimidine (1.7 g, 5 mmol) in DMF (10 ml). The reaction mixture was heated at 150°C for 1.5 h and then evaporated. Water was added to the residue and the aqueous layer was extracted with AcOEt. The extract was dried over MgSO₄, and evaporated. The residue was recrystallized from ethyl acetate–hexane to give 1.5 g of light yellowish crystals (yield: 87%). A suspension of these crystals (1.5 g) and 10% Pd-C (0.1 g) in AcOH (5 ml) and EtOH (75 ml) was stirred at 45°C for 3.5 h in a hydrogen atmosphere. The catalyst was filtered off and filtrate was evaporated. The residue was basified with aq. K₂CO₃, and extracted with CHCl₃. The extract was dried over Na₂SO₄, and evaporated to give 0.9 g (yield: 83%) of pale yellow crystals. mp 149–152°C. IR (KBr) cm⁻¹: 1655, 1645, 1620, 1575. NMR (CDCl₃) δ: 1.34 (3H, t, *J* = 7.0 Hz), 1.87 (1H, brs), 2.94 (4H, t, *J* = 6 Hz), 3.96 (6H, m), 6.27 (1H, d, *J* = 8 Hz), 7.29 (1H, d, *J* = 8 Hz), 9.22 (1H, s). The resulting crystals were converted into the hydrochloride (712) in a usual manner.

2-Piperadino-6-methyl-5-oxo-5,6-dihydroprido[4,3-*d*]pyrimidine hydrochloride (893)¹⁴ was also prepared in the same manner as described above. (893: free form) Yield: 86%. NMR (CDCl₃) δ: 2.93 (4H, m), 3.50 (3H, s), 3.94 (4H, m), 4.40 (1H, brs), 6.25 (1H, d, *J* = 8 Hz), 7.25 (1H, d, *J* = 8 Hz), 9.23 (1H, s).

Results

The effects of newly synthesized pyrimidine compounds on neurite outgrowth were studied in cultures of human and mouse neuroblastoma cell lines GOTO and neuro 2a. The chemical structures of the tested compounds are shown in Table I.

The compounds were freely soluble in water at 20 mM, and were added to the exponentially growing neuroblastoma cells. After 24 h the number of cells having a neurite were counted and the proportion of the number of the cells having a neurite to the total number of cells was calculated as described in Materials and Methods.

The neurite sprouting activity of the tested compounds are shown in Fig. 1 and the quantitative results are summarized in Table II. The relative activities among these compounds measured with GOTO cells were similar to that measured with neuro 2a cells. In the tested range of the concentrations (each 1 µg/ml, 2.9–4.3 µM and 10 µg/ml,

818

820

858

862

893

isaxonine

712

saline

Fig. 1. The Neurite Sprouting Activity of the Chemical Compounds

The chemicals were added to neuro 2a cells as described in Materials and Methods. Twenty four hours later, the photopictures were taken (magnification $\times 100$).

TABLE I. Physical Properties of Pyrimidines

No.	Type	R	mp (°C)	Formula
883	I	H	> 300	C ₁₀ H ₁₃ N ₅ O·HCl
818	I	CH ₃	193—195	C ₁₁ H ₁₅ N ₅ O·C ₄ H ₄ O ₄
858	I	C ₂ H ₅	Oil	C ₁₂ H ₁₇ N ₅ O·HCl
820	II	CH ₃	180—182	C ₁₁ H ₁₅ N ₅ O·C ₄ H ₄ O ₄
862	III	CH ₃	Oil	C ₁₀ H ₁₄ N ₆ O·HCl
893	IV	CH ₃	> 300	C ₁₂ H ₁₅ N ₅ O·HCl
712	IV	C ₂ H ₅	277—279	C ₁₃ H ₁₆ N ₅ O·HCl
Isaxonine	V		124—125	C ₁₇ H ₁₁ N ₃ ·H ₃ PO ₄

TABLE II. Neurite Outgrowth Activity of 2-Piperazinopyrimidine Compounds in Neuroblastoma Cell Lines

	Neuroblastoma cell lines	Compound No.	Concentration		The number of cells having neurites Mean ± S.D. (%) (n=3)
			μg/ml	μM	
Exp. 1	Human GOTO	818	10	28.6	21.3 ± 0.4 ^a
		820	10	28.6	25.3 ± 1.1 ^a
		893	10	35.5	16.3 ± 0.8 ^b
		712	10	33.8	15.0 ± 1.0 ^c
		Isaxonine	10	42.5	12.5 ± 0.9
Exp. 2	Mouse Neuro 2a	Saline	—	—	8.4 ± 0.6
		818	1	2.9	19.8 ± 2.7 ^c
			10	28.6	17.2 ± 2.7 ^c
		858	1	3.5	15.7 ± 1.2 ^c
			10	35.2	18.4 ± 0.3 ^b
		820	1	2.9	27.7 ± 2.8 ^a
			10	28.6	25.0 ± 1.0 ^a
		862	1	3.7	19.6 ± 1.1 ^b
			10	36.9	15.5 ± 2.2
		893	1	3.6	17.2 ± 2.7 ^c
			10	35.5	15.4 ± 2.2
		712	1	3.4	15.9 ± 2.0
			10	33.8	13.7 ± 1.0
		Isaxonine	1	4.3	12.1 ± 1.3
		Saline	—	—	7.9 ± 0.8

The chemical compounds were added at various concentrations to the medium as indicated in the Table. After 24 h the numbers of neurite-bearing cells were scored as described in Materials and Methods. The data are shown as mean ± S.D. (n=3) in percentage of neurite-bearing cells. Statistical difference between each compound and isaxonine in student's *t*-test. ^a *p* < 0.001, ^b *p* < 0.01, ^c *p* < 0.05.

28.6—36.9 μM) of the compounds, our pyrimidine derivatives having piperazine moiety at the 2-position seemed to have stronger neurite promoting activity than isaxonine having isopropylamino residue at the same position (Table I).

In separate experiments using mouse neuro 2a cells, the activities of **883** (CH₃ group of **818** is replaced with a hydrogen atom) at 1 μg/ml and 10 μg/ml were similar to that of **818**, and stronger than that of isaxonine (data not

TABLE III. The Enhancing Effect of 2-Piperazinopyrimidine Compounds on NGF-Induced Neurite Outgrowth of PC12 Cells

	Addition (concentration mM)	The percentage of neurite bearing cells
Ex. 1	None	36.1 ^c (34.5, 37.8) ^b (100) ^a
	820 (0.3)	36.5 (41.3, 31.8) (101)
	820 (0.5)	50.1 (48.0, 52.3) (139)
	820 (1.0)	63.5 (60.0, 67.0) (176)
	820 (2.0)	19.1 (20.0, 18.3) (53)
Ex. 2	None	23.2 ± 2.6 ^d (100) ^a
	820 (0.5)	34.5 ± 3.6 (149)
	818 (0.5)	33.4 ± 2.1 (144)
	893 (0.5)	7.6 ± 0.7 (33)
	712 (0.5)	5.0 ± 1.2 (53)
Ex. 3	None	23.3 ± 1.4 ^d (100) ^a
	883 (0.5)	42.6 ± 3.7 (183)
	818 (0.5)	40.3 ± 2.2 (173)
	Isaxonine (0.5)	24.6 ± 0.8 (105)

The 2-piperazinopyrimidine compounds were added to the culture medium as indicated in the Table together with NGF (1.5 ng/ml). After 48 h the number of neurite bearing cells was scored from 400 cells as described in Materials and Methods. The number of neurite bearing cells were negligible (less than 9) in the absence of NGF irrespective of the presence of the chemical compounds. ^a In the parentheses the enhancing effect of the chemical compounds are shown as the relative percentages to the values obtained in the absence of the chemical compounds. ^b In the parentheses the values of two determinations are shown. ^c The values are the averages of the two determinations. ^d The values are shown as mean ± S.E. (n=3).

shown). The tested compounds were hydrochloride or maleic acid salts of piperazinopyrimidine amines. The activity of **820** (HCl form) was not different from that of the maleic acid form, and the activity of maleic acid alone was negligibly small (data not shown).

Our new compounds (I, II and III in Table I) are 6- and 5-member-ring condensed pyrimidine heterocyclics. Although the difference between the activity of 5-member-ring condensed pyrimidine heterocyclics such as **883**, **818**, **858**, **820** and **862** and that of 6-member-ring condensed pyrimidine heterocyclics such as **893** and **712** was not so large, **818** and **820**, particularly **820**, seemed to have the most potent activity (Table II).

Next, we examined the effect of our pyrimidine compounds on the NGF-induced neurite formation of pheochromocytoma PC12 cells. A typical result of the neurite sprouting activity of the tested compound and NGF is shown in Fig. 2 and the quantitative results are summarized in Table III. In PC12 cell cultures, these pyrimidine compounds alone did not show NGF-like neurite outgrowth activity. And even in the presence of NGF these pyrimidine compounds did not increase the cell numbers having neurites in the range of the concentration used in the neuroblastoma cell cultures, so the concentration of solution of these compounds was raised to about 1 mM (Table III). As a representative, **820** was at first tested in the presence or absence of serum in PC12 cell cultures. The effect of **820** as a potentiator of NGF-induced neurite formation was more potent in the serum-free medium (data not shown). In between at 0.3 mM and at 0.5 mM, **820** began to increase the cell number having neurites, and at 1 mM **820** showed the maximum enhancing activity of the NGF effect, and then at 2 mM **820** was toxic to the cells (Table III).

In the following tests, the activities of these pyrimidine compounds at 0.5 mM were compared to each other in the presence of NGF. **820** and **883** seemed to be as effective as **818** and were a little more potent than **818**. On the other

Fig. 2. The Enhancing Effect of Chemical Compounds (**818** and **820**) on Neurite Outgrowth in PC12 Cells Induced by NGF

The chemicals (0.3 mM) and/or NGF (1.0 ng/ml) were added as indicated in the figure and photopictures were taken 24 h later (magnification $\times 100$).

hand, isaxonine was not so active and 6-6 condensed heterocyclics such as **893** and **712** seemed to be quite toxic to the cells at the concentration of 0.5 mM.

The enhancing effect of each pyrimidine compound on the NGF-induced neurite outgrowth of PC12 cells was very similar to the neurite outgrowth activity of each compound in neuroblastoma cell cultures. In toxicological studies, the LD₅₀ values of **883**, **818**, **858** and **820** were above 1000 mg/kg *p.o.*, and those of **862**, **893** and **712** were below 750 mg/kg *p.o.* This result may be well reflected in the cell toxicity of **893** and **712** at a higher concentration (0.5 mM).

Discussion

Previous to this study, we tested the antidepressive activity of newly synthesized bicyclic pyrimidine compounds possessing piperazine moiety described in this paper which were thought to be analogues of antidepressants such as MK-212¹⁹⁾ and quipazine²⁰⁾ (Chart 1). But as antidepressants as well as antibacterials and herbicides, these pyrimidine compounds were not effective at all or not so potent (data not shown). In spite of this fact, in *in vitro* cell culture study, we found substantial neurite promoting activity of the compounds in human and mouse neu-

Chart 1

roblastoma cell lines, and also a marked enhancing effect of the compounds on the NGF-induced neurite outgrowth of PC12 cells. On the other hand, the neurite sprouting activity of 2-piperazinopyrimidine heterocyclics having considerable antidepressive activity was found to be fairly smaller than those of **818**, **820** and **712** (data not shown).

These pyrimidines are condensed 6-5 and 6-6 bicyclic compounds having acid-amide joining and further piperazine derivatives substituted at the 2-position of the pyrimidine ring. The chemical structures of these pyrimidines are quite different from isaxonine which was so far only a synthesized compound known to have neurite outgrowth activity. Sulfhydryl (SH) and isopropylamino derivatives of **820** at the 2-position in place of piperazine were known to lose their activity in our study (data not shown). The functional group of the piperazine moiety seemed to be essential to their activity.

To survey the significance of the activity of 5- and 6-member-ring (having $\text{--}\overset{\text{O}}{\underset{\text{R}}{\text{C}}}\text{--}\text{N--}$) condensed 2-piperazino-

pyrimidine, the activity of the simplest compound, 2-piperazinopyrimidine having no condensed ring and that of 2-piperazinoquinazoline having a condensed benzene ring were tested, and they were found to be not so potent as compared to those of 6-5 condensed pyrimidine heterocyclics, particularly that of the **820** compound (data not shown). Therefore, coexistence of 2-, 5- and 6-substitution of pyrimidine ring, i.e., 2-piperazine and 5- and 6-membered ring having $\text{--}\overset{\text{O}}{\underset{\text{R}}{\text{C}}}\text{--}\text{N--}$ seemed to have some role in the

strengthening of neurite promoting activity of the pyrimidine ring, though we did not test pyrimidine itself.

Although a more detailed study of the structure-activity relationship is required, 6-5 bicyclic pyrimidine derivatives exhibited more potent activity than 6-6 bicyclic pyrimidine derivatives. With regard to the substitution by the alkyl group of RN of amide, in both 6-5 and 6-6 bicyclic pyrimidines, there might not be a difference of potency between the hydrogen atom, and CH_3 and C_2H_5 group. In other culture-tests, isopropyl derivatives were known to be a little more toxic and less active than CH_3 and C_2H_5 derivatives (data not shown). Comparing an isomer **820** with **818**, **820** seemed to be more active than the other.

Regarding the culture condition of GOTO and neuro 2a cells, the presence or absence of serum did not influence the results in general (data not shown). On the other hand, in PC12 cell cultures, the presence of serum showed a tendency to interfere with the enhancing effect of **820** on NGF-induced neurite sprouting of the cells (data not shown).

As described above, quite similar activities of these pyrimidine compounds were found both in neuroblastoma cell cultures and in PC12 cell cultures. An underlying

mechanism, however, of the effect of the compounds on both cell lines remains to be clarified.

In our present study, new types of chemical compounds are demonstrated to have potent neurotropic activity. Some compounds were further examined in peripheral and central nervous disorder models of mice and rats *in vivo*; we found that they promoted the regeneration of nerve and improved the recovery of nerve injuries (unpublished data^{21,22}). This finding indicates that the screening of compounds in neuroblastoma cell cultures by neurite sprouting activity is a convenient and time-saving method for the selection of candidates of new potent neurotropic agents.

We are currently exploring candidates of more active substances for pharmaceuticals by screening a lot of analogous and derivative compounds related to these pyrimidines, and are studying the precise mode of action of these substances.

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