Neurotropic Pyrimidine Heterocyclic Compounds. II. Effects of Novel Neurotropic Pyrimidine Derivatives on Astrocytic Morphological Differentiation

Yutaka KOYAMA,^a Akira AWAYA,^b Nobue ISHIKAWA,^a Shigeki FUJITA,^a Ikuo TOMINO,^c
Keiichi YOKOYAMA,^c Shintaro ARAKI,^c Mitsuyuki TAKESUE,^c Koji KATO,^c
Masaharu ISHIGURO,^c Takumi KITAHARA,^c Noriaki KIHARA,^c and Akemichi BABA*,^a

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University,^a 1-6 Yamada-oka, Suita, Osaka 565, Japan, Institute of Biological Science, Mitsui Pharmaceuticals, Inc.,^b 12-2 Nihonbashi-3 chome, Chuo-ku, Tokyo 103, Japan and Synthetic Chemistry and Bioscience Lab., Mitsui Petrochemical Industries, Ltd.,^c Iwakuni, Yamaguchi 740, Japan. Received July 29, 1996; accepted October 12, 1996

Astrocytes play an important role in supporting nerve regeneration after brain injuries. In this study, the effects of novel neurotropic synthesized pyrimidine compounds on astrocytic morphological differentiation were examined. Treatment of protoplasmic cultured astrocytes with 2-piperidino-5,6-dihydro-7-methyl-6-oxo(7*H*)pyrrolo[2,3-*d*]pyrimidine maleate (MS-430) and the related compounds caused astrocytic process formation in 60 min. The morphology of MS-430-treated cells was similar to that of dibutyryl cAMP (DBcAMP)-treated cells. The astrocytic process formation by MS-430 was observed within 60 min and the maximum effect was obtained at the drug concentration of 0.5–1.0 mM. Rhodamine-phalloidin staining showed that astrocytic cytoskeletal actin was reorganized by MS-430 and DBcAMP. MS-430 did not increase cAMP accumulation in cultured astrocytes. These results suggest that the neurotropic pyrimidines induced astrocytic morphological differentiation through a cAMP-independent mechanism.

Key words 2-piperidinopyrimidine; neurotrophic action; astrocyte; morphological differentiation; stellate cell

The development of novel neurotrophic drugs promoting remodeling of the nervous system after brain injuries is a concern in neuropharmacology. A novel synthesized heterocyclic pyrimidine derivative, 2-piperidino-6-methyl-5-oxo-5,6-dihydro(7*H*)-pyrrolo[3,4-*d*]pyrimidine maleate (MS-818), stimulates the neurite outgrowth of neuroblastoma cell lines and potentiates nerve growth factor (NGF)-induced neurite sprouting of PC12.¹⁾ MS-818 promotes the regeneration (axonal sprouting and elongation) of the perineal nerve of mice after a nerve crushing injury.²⁾ A related compound of MS-818, 2-piperidino-5,6-dihydro-7-methyl-6-oxo(7*H*)pyrrolo[2,3-*d*]pyrimidine maleate (MS-430),³⁾ promotes the functional and histological repair of rat sciatic nerves after a crush injury.⁴⁾ MS-430 also stimulates the neurite outgrowth of neuroblastoma cell lines and potentiates NGF- and epidermal growth factor-induced neurite sprouting of PC12 cells.^{1,5)} However, the mode of neurotrophic actions of these pyrimidine derivatives on nerve regeneration is not known.

Astrocytes support neuronal development and axonal outgrowth by producing several neurotrophic factors and cell adhesion molecules. With brain injuries, astrocytes turn their phenotype into so called reactive astrocytes, which are proliferative and characterized by glial fibrillary acidic protein (GFAP) expression and hypertrophy of cell bodies. Reactive astrocytes have high activity in producing neurotrophic substances.⁶⁾ Thus, they are thought to play important pathophysiological roles in the repair of the nervous system after brain injuries. Differentiation of protoplasmic astrocytes (type 1 astrocytes) is induced by treatment with various agents *in vitro*. Treatments with cAMP analogues,⁷⁾ thyroid hormone and insulin⁸⁾ turn protoplasmic cultured astrocytes into a process-bearing

shape, which resembles that of astrocytes in mature brains. The enzyme activities related to astrocytic functions increase with the morphological changes by these hormones.^{9–11)} To show a possible involvement of astrocytes in the neurotrophic action of three representative pyrimidine compounds, *i.e.*, already reported MS-

MS-210

$C_{13}H_{18}N_4O \cdot HCl$ MW 282.8

MS-430

$C_{16}H_{20}N_4O_5$ MW 348.4

MS-818

$C_{11}H_{15}N_5O \cdot C_4H_4O_4$
MW 349.4

Fig. 1. Chemical Structures of MS-210, MS-430 and MS-818

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* To whom correspondence should be addressed.

818 and MS-430, and a newly synthesized monocyclic pyrimidine derivative related to MS-430, 2-piperidino-4-(2-oxo-pyrrolidino)pyrimidine hydrochloride (MS-210), the effects of these compounds on the cell morphology of rat cultured astrocytes were examined in this study.

MATERIALS AND METHODS

Materials MS-818, MS-430 and MS-210 were synthesized as described before.^{1,3,12,13)}

MS-430 A mixture of ethyl 2-piperidino-4-chloropyrimidine-5-acetate (10.6 g, 32.5 mmol) and 40% methylamine methanol solution (5.0 g, 64.5 mmol) in ethanol (20 ml) was placed in a pressure vessel, then heated at 120 °C for 2 h. The solvent was evaporated, and water was added to the residue and then extracted with chloroform. The extract was dried over MgSO₄, then evaporated. The resulting residue was chromatographed on silica gel to give 4.2 g (yield: 56%) of red crystals. NMR (CDCl₃) δ : 1.68 (6H, m), 3.22 (3H, s), 3.44 (2H, s), 3.80 (4H, m), 7.92 (1H, s). The resulting crystals were converted into a maleate (MS-430: mp 135–136 °C) in the usual manner. NMR (CDCl₃) δ : 1.64 (6H, br s), 3.12 (3H, s), 3.46 (2H, s), 3.7–3.9 (4H, m), 6.22 (2H, s), 7.90 (1H, s).

MS-210 To a solution of 4-chloro-2-piperidinopyrimidine (26.45 g, 134 mm) in *n*-amylalcohol (150 ml) was added 4-aminobutyric acid (19.0 g, 184 mm) and potassium carbonate (25.0 g, 180 mm). The mixture was heated at 160 °C for 8 h and concentrated under reduced pressure. To the residue, chloroform and water were added for extraction. The organic layer was concentrated under reduced pressure. The resulting residue was chromatographed on silica gel to give 28.8 g of 4-(3-carboxypropylamino)-2-piperidinopyrimidine (yield: 66%). 28.8 g of 4-(3-carboxypropylamino)-2-piperidinopyrimidine was dissolved in 100 ml of chloroform. Ten ml of thionyl chloride was added to the solution. The mixture was stirred at room temperature for 5 h. Sodium carbonate solution was added to the mixture. The organic layer was separated and evaporated to give 14.7 g (yield: 55%) of white crystal. NMR (CDCl₃) δ : 1.68 (6H, br s), 1.9–2.4 (2H, m), 2.4–2.6 (2H, m), 3.74 (4H, br s), 4.07 (2H, t, J = 8 Hz), 7.52 (1H, d, J = 7 Hz), 8.22 (1H, d, J = 7 Hz). The resulting crystals were converted into hydrochloride (MS-210: 260–262 °C, dec.) in the usual manner. NMR (CDCl₃) δ : 1.75 (6H, m), 2.20 (2H, m), 2.75 (2H, t, J = 7 Hz), 3.98 (4H, m), 4.06 (2H, t, J = 7 Hz), 8.16 (1H, d, J = 7 Hz).

Preparation of Cultured Astrocytes Astrocytes were prepared from the cerebrum of 1 to 2-d-old Wistar rats as described before.¹⁴⁾ Cells were seeded at 1×10^4 cells/cm² in culture dishes (100 mm diameter, Corning), and grown in 10% bovine fetal serum-containing Eagle's minimum essential medium (MEM). After cultures became confluent by protoplasmic cells, small process-bearing cells on the cell monolayer (mainly type 2 astrocytes) were removed. The remaining cells were trypsinized and seeded on 12 well culture plates (Corning) or glass cover slips coated with 10 μ g/ml poly-D-lysine. Cells cultured for 18–28 d were used. At this stage, about 95% of cultured cells were GFAP positive as determined by immunochemical staining.

Observation of Cell Morphology Culture medium was replaced by serum-free Ham's F-12/Dulbecco-modified MEM = 1:1. Drugs tested were added to the serum-free medium and incubated in a CO₂ incubator for the indicated times. The morphology of cultured astrocytes was observed under a phase-contrast microscope. Cells having a round cell body and two or more processes longer than twice the cell body were scored as stellate cells. Results are expressed as the percentage of stellate cells to the total cell number. For observation of cytoskeletal actin organization, cells on glass cover slips were fixed by 3% paraformaldehyde and 0.1% Triton X-100 in phosphate-buffered saline. Then, fixed cells were incubated with rhodamine-phalloidine (Molecular Probes, Inc., $\times 200$ dilution), which selectively binds to filamentous-actin (F-actin). Cells were rinsed by phosphate-buffered saline and mounted on slide glasses. The cytoskeletal F-actin organization of cultured astrocytes was observed under fluorescence microscopy with a rhodamine filter.

Measurement of cAMP Accumulation After removal of the cultured medium, astrocytes were incubated with or without the drugs tested in the serum-free medium for 10 min. Then, the drug-containing medium was removed and cells were rinsed by phosphate-buffered saline. Cyclic AMP in the drug-treated cells was extracted by 500 μ l of 5% trichloroacetic acid. After the acid extractions were centrifuged, cAMP content in the acid soluble fraction was measured by using a [¹²⁵I]cAMP radioimmunoassay kit (Amersham). The acid insoluble fraction was solubilized in 0.1 N NaOH and the protein content was measured. Statistical analysis was done by Student's *t*-test.

RESULTS

As shown in Fig. 2A, cultured astrocytes from the rat cerebrum had a flat and polygonal shape. In the absence of serum, treatment with 0.5 mM MS-430 for 60 min caused morphological changes accompanied by glial process formation, *i.e.*, stellation, of cultured astrocytes (Fig. 2B). At concentrations of 0.5–1.0 mM, most astrocytes attained a stellate shape (Fig. 3A). MS-818 and MS-210, chemicals related to MS-430, caused astrocytic morphological changes in a dose-dependent manner up to 1.0 mM, although MS-818 was less effective. The number of stellate astrocytes reached a maximum at 1–2 h after the addition of the neurotropic pyrimidines (Fig. 3B). Thereafter, the stellate astrocytes gradually expanded the cytoplasm and most astrocytes became flat shape in 24 h after the drug additions. Treatment with 0.5 mM dibutyl cAMP (DBcAMP) caused astrocytic morphological changes accompanied by process formation in most cells (Fig. 2C and ref. 14). Under phase-contrast microscopy, astrocytes treated with MS-430 had a similar morphology to those treated with DBcAMP. The DBcAMP-treated cells retained the stellate morphology for at least 4–8 h. In further incubations with DBcAMP, enlargement of the cytoplasm of DBcAMP-treated stellate astrocytes was observed. Most DBcAMP-treated astrocytes returned to a flat shape in 24–48 h (data not shown).

Fig. 2. Effects of MS-430 and DBcAMP on Cell Morphology and Cytoskeletal Actin Reorganization of Cultured Astrocytes

A, B, C: Observation of cell morphology. Cultured astrocytes were treated with (A) no drug, (B) 0.5 mM MS-430 and (C) 0.5 mM DBcAMP for 60 min. Cell morphology was observed under a phase-contrast microscopy. Bar = 50 μ m. D, E, F: Observation of cytoskeletal actin organization. Cultured astrocytes were treated with (D) no drug, (E) 0.5 mM MS-430 and (F) 0.5 mM DBcAMP for 60 min. Cytoskeletal actin was stained by rhodamine-phalloidin. Bar = 50 μ m.

Rhodamine-phalloidin staining of protoplasmic astrocytes showed that stress fibers were a prominent cytoskeletal actin structure of protoplasmic cultured astrocytes (Fig. 2D). As has been reported,^{15,16)} when accompanied with DBcAMP-induced morphological changes, astrocytic stress fibers disappeared (Fig. 2F). The treatment of protoplasmic astrocytes with MS-430 reorganized astrocytic stress fibers in 60 min (Fig. 2E). DBcAMP stimulated cAMP accumulation in cultured astrocytes (Table 1). Astrocytic cAMP accumulation was not altered by MS-430 or MS-210. MS-818 was inclined to decrease astrocytic cAMP accumulation, but the decrease was not statistically significant.

DISCUSSION

Glial process formation of protoplasmic astrocytes (type 1 astrocyte) is induced by treatments with norepinephrine,⁷⁾ thyroid hormone and insulin.⁸⁾ The morphology of the process-bearing cultured astrocytes resembles that of astrocytes in the mature brain. Treatment with these agents increased glutamate synthetase activity and the capacity for L-glutamate uptake of cultured astrocytes,⁹⁻¹¹⁾ which are responsible for astrocytic functions. These observations indicate that the differentia-

tion of protoplasmic cultured astrocytes is inducible by various types of stimuli. Cyclic AMP analogues have also been shown to induce astrocytic process formation *in vitro*.¹⁴⁻¹⁶⁾ In the absence of serum, DBcAMP caused morphological changes accompanied by process formation in cultured astrocytes (Fig. 2C). Neurotropic pyrimidines, MS-430 and the related compounds, induced astrocytic morphological changes (Fig. 2) which resembled those of DBcAMP-treated astrocytes under phase-contrast microscopy. The induction of glial processes by the MS compounds suggests that neurotropic pyrimidines caused the morphological differentiation of cultured astrocytes. There seems to be no stereochemical resemblance of the MS-compounds to DBcAMP (Fig. 1). At the concentrations of the neurotropic pyrimidine required to induce astrocytic morphological changes, they stimulated the neurite outgrowth of neuroblastoma and PC12 cell.¹⁾

As for intracellular mechanisms inducing morphological differentiation of cultured astrocytes, the activation of cAMP-dependent protein kinase stimulates glial process formation by reorganizing cytoskeletal actin.¹⁷⁾ In addition to DBcAMP, MS-430 caused astrocytic cytoskeletal actin reorganization accompanied by morphological changes (Fig. 2E). However, the neurotropic pyrimidines did not stimulate cAMP accumulation in cultured astro-

Fig. 3A. Dose-Dependency of Astrocytic Morphological Changes Induced by Neurotropic Pyrimidines

In serum-free medium, cultured astrocytes were treated with MS-210 (●), MS-430 (○) and MS-818 (■) at the indicated concentrations for 60 min. The numbers of process-bearing astrocytes (stellate cells) are expressed as percentages of total cells observed.

3B. Time-Course of Astrocytic Morphological Changes

Cultured astrocytes were incubated with 0.5 mM MS-210 (●), MS-430 (○) and MS-818 (■) for the indicated times.

Table 1. Effects of DBcAMP and Neurotropic Pyrimidines on cAMP Accumulation in Cultured Astrocytes

	cAMP accumulation (nmol/mg protein)
None	0.21 ± 0.07
0.5 mM DBcAMP	5.04 ± 1.16**
0.5 mM MS-210	0.27 ± 0.03
0.5 mM MS-430	0.20 ± 0.04
0.5 mM MS-818	0.11 ± 0.04

Cultured astrocytes were incubated with the indicated drugs for 10 min. None means incubation with no drugs. After the incubations, cAMP was extracted by 5% trichloroacetic acid. Results are the mean ± S.E.M. of 5–6 experiments.

** $p < 0.01$ vs. none by Student's t -test.

cytes (Table 1). Thus, it is likely that the neurotropic pyrimidines affect astrocytic morphological differentiation through a cAMP-independent mechanism. Although the action site is not clarified, we found that the neurotropic pyrimidines, MS-818, the analogue compounds of MS-818, and MS-430 potentiated the effects of NGF and epidermal growth factor (EGF) on neurite outgrowth of the PC12 cell^{1,5)} and the effect of basic FGF on the angiogenesis of a chicken embryo.¹⁸⁾ Gangliosides, which are sialic acid-containing glycosphingolipids and pro-

mote neurite outgrowth *in vitro*,¹⁹⁾ are shown to affect astrocytic morphological differentiation through a cAMP-independent mechanism.^{20,21)} As one possibility, the neurotropic pyrimidines may affect astrocytic morphological differentiation by modulating the action of endogenous gangliosides.

Much effort is being focused on the research and development of novel drugs to improve nerve dysfunction after brain damage such as stroke and head trauma. MS-430 promotes sciatic nerve regeneration in a rat crush injury model.⁴⁾ On brain injuries, astrocytes produce neurotrophic factors and cell adhesion molecules, which support axonal outgrowth and the synaptic regeneration of damaged sites.⁶⁾ The present findings on *in vitro* astrocytic differentiation may suggest that nerve regeneration by the neurotropic pyrimidine, MS-430 in *in vivo* models is induced, in part, by stimulating the functions of the glial cells at the injury site.

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