Communications to the Editor

LACTACYSTIN, A NOVEL MICROBIAL METABOLITE, INDUCES NEURITOGENESIS OF NEUROBLASTOMA CELLS

Sir:

Neurotrophic factors (NTF) are known to be essential for the survival and functional maintenance of nerve cells[1~3]. The decrease in availability of NTF is considered to cause dysfunction of the nervous system, resulting in various nerve diseases including senile dementia such as ALZHEIMER's disease. This consideration led us to speculate that NTF-like substances can be useful to treat patients with nerve diseases. During the course of our screening for microbial metabolites which induce differentiation of the mouse neuroblastoma cell line Neuro 2A, a novel compound designated lactacystin was isolated from the cultured broth of a *Streptomyces* strain. It was found that when Neuro 2A cells were treated with lactacystin (1.3 μM), a

Fig. 1. Absolute structure of lactacystin.

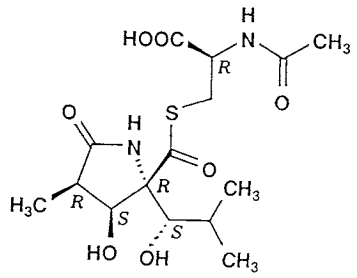
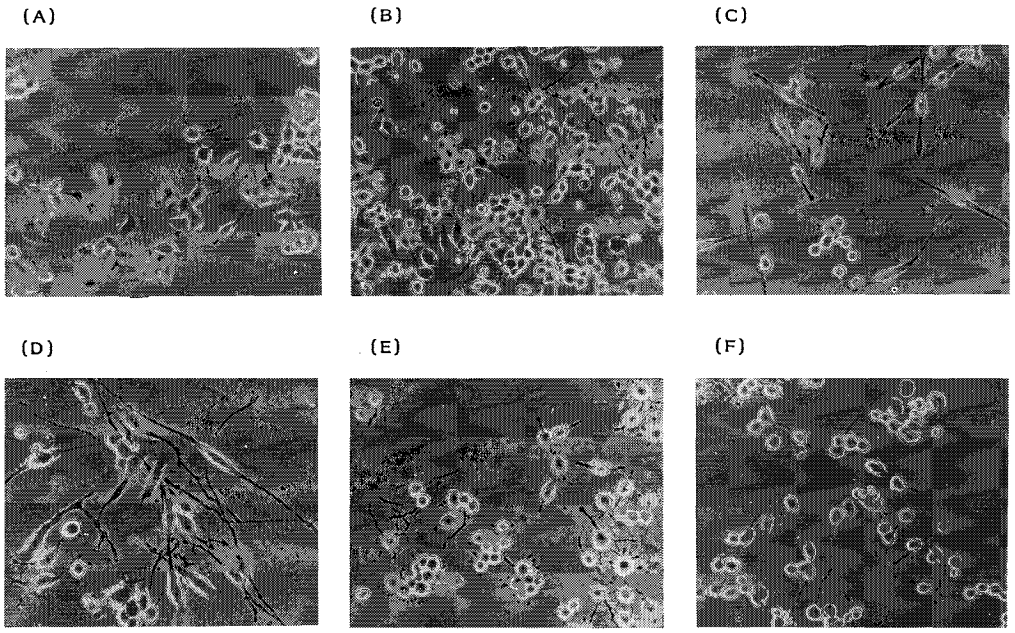


Fig. 2. Photomicrographs of Neuro 2A cells treated with lactacystin, dibutyryl cAMP and β-NGF.

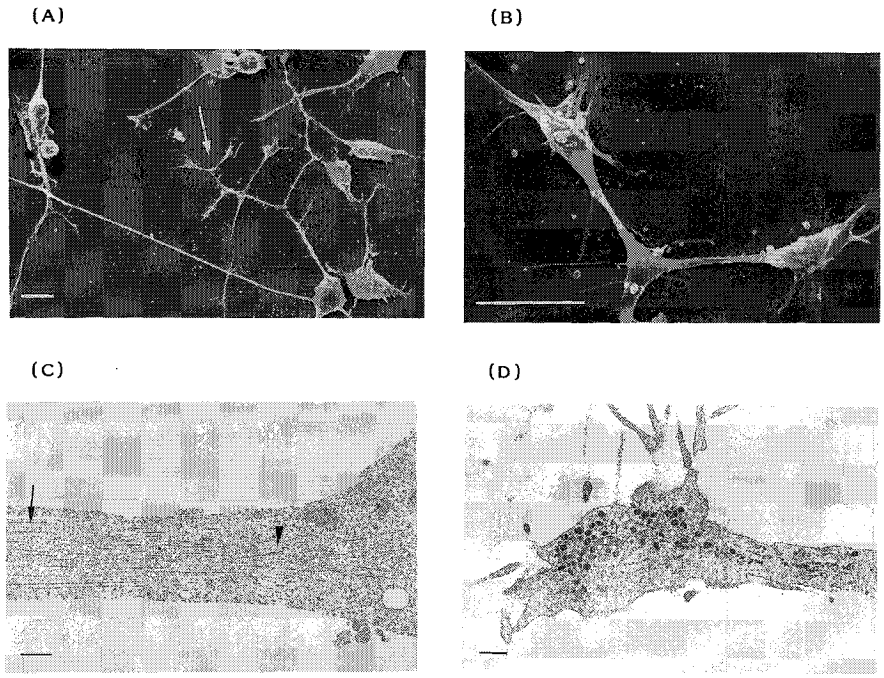


The cells were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and cultured at 37°C in 5% CO_2 humidified atmosphere in a Falcon plastic culture flask containing minimum essential medium supplemented with 10% fetal bovine serum and 1% non-essential amino acid mixture. The cells were plated at a density of 1.5×10^5 cells/ 60 mm i.d. dish and incubated at 37°C. After subculture for 1 day, the following treatments were done: (A) Control (after 1 day); (B) control (after 4 days); (C) 1 day after lactacystin (1.3 μM) treatment; (D) 4 days after lactacystin (1.3 μM) treatment; (E) 1 day after dibutyryl cAMP (1 mM) treatment; (F) 1 day after β-NGF (200 ng/ml) treatment. The control cells (1 day and 4 days) remained in compact form and exhibited minimal neurite extension, almost less than the cell length, and the cell population bearing the minimal extension was less than 8%. Note that most of the cells responded to 4-day treatment of lactacystin by elaborating long neurites.

neurite-like structure was generated from the cell body and the intracellular cAMP level was transiently enhanced. Here we report the structure of lactacystin and its biological activity in neuritogenesis of Neuro 2A cells.

In the screening work, we used Neuro 2A cells which is a stable transformed mouse neuroblastoma cell line. The cultured broths which caused generation of a neurite-like structure from the cell body were selected. Among about 6,000 soil isolates (mainly actinomycetes and fungi), only one culture, *Streptomyces* sp. OM-6519, was picked out, and the active substance was isolated from the cultured broth and characterized. It was purified by column chromatography on activated carbon and Dowex-1X4 (OH^-) and by HPLC (Senshu-Pak $N(CH_3)_2$-3251). From 30 liters of the culture supernatant, 19 mg of colorless needles was obtained: MP 237~238°C (dec), $[\alpha]_D^{25}$ +71.3° (*c* 0.5, methanol). The molecular formula, $C_{15}H_{24}N_2O_7S$, was established by elemental analysis and HRFAB-MS (*m/z* 377.13 $(M+H)^+$). The structure and absolute configuration was determined by NMR and X-ray crystallographic analyses and is shown in Fig. 1. The new compound was designated lactacystin because it has a thioester structure connecting γ-lactam and *N*-acetylcysteine moieties. These results will be reported in the succeeding paper[4].

Fig. 3. Neurite and growth cone structures of Neuro 2A cells cultured in the presence of 1.3 μM lactacystin for 4 days.



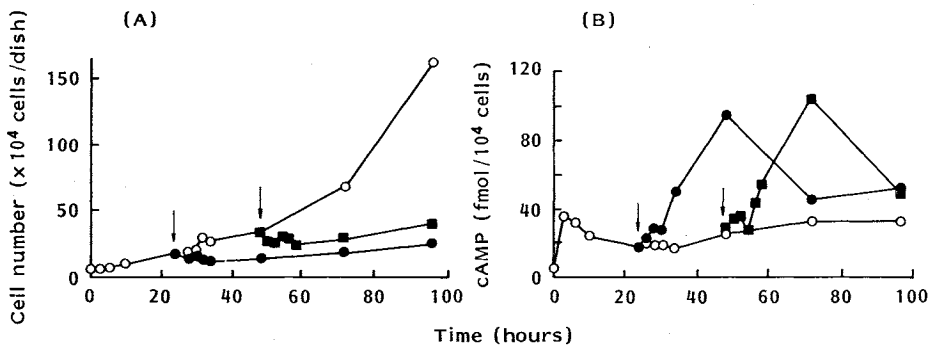
Scanning electron micrographs (A and B) revealed solid structure and tight adhesion of the neurites and growth cones with several microvilli. The micrograph B shows the area indicated with arrow in the micrograph A. Transmission electron micrograph shows that neurite extension (C) contains parallel array of microtubules (arrow) and intermediate filaments (arrow head). Some extensions contained mitochondria and membrane structure (data not shown). It was observed that the growth cone (D) is enriched with large cored vesicles and contains straight microtubles and in some cases microtuble loops. Bar; 10 μm in A and B, 0.5 μm in C and D. Methods: The cells for scanning electron microscopy were cultured on a cover glass set in Falcon plastic dish. The attached cells on a glass were rinsed in phosphate buffered saline (PBS), fixed with glutaraldehyde, post-osmicated, dehydrated in a graded alcohols, critically point dried, mounted on stubs and coated with gold-palladium in a sputtering diode coating. The treated cells were examined with JSM 5300. The cells for transmission electron microscopy were cultured on Falcon plastic dish, washed with minimum essential medium (37°C) and subsequently fixed with 2.5% glutaraldehyde-PBS at 25°C for 30 minutes. The resulting specimens were subjected to the processes of postfixation and Epon embedment as described previously[14].

Lactacystin (1.3 μM) caused characteristic changes in the morphology of Neuro 2A cells. It elicited the formation of short solid bipolar projections of 0.5~0.7 μm i.d. at both sides of the cell body within 1 day after the treatment (Fig. 2C). The prolonged treatment for 3~4 days led to a marked morphological change characterized with a neurite-like structure (0.2~0.5 μm i.d.) (Fig. 2D). The morphology of the cells was heterogeneous, with some cells exhibiting bipolar processes, others exhibiting multipolar and/or branching ones. Such morphological changes were dose-dependent. The minimum concentration of lactacystin required for the changes was 1.3 μM. At higher concentrations than 10.4 μM, it showed some cytotoxicity such as cell retraction and release from the substratum. The lactacystin-induced neurite-like processes have some growth cone structures and some varicosities. This type of morphological change differs from those induced by dibutyryl cAMP, a well-known compound inducing morphological changes in cells[5]. Dibutyryl cAMP-induced neurite-like processes had slender structures, small growth cones, and less neurite branchings (Fig. 2E). β-NGF (200 ng/ml) had no significant effect on the morphology (Fig. 2F) as described by MURPHY *et al.*[6].

Examination by scanning electron microscopy revealed that the lactacystin-induced neurite-like processes have a solid structure of varicosities and growth cones, which adheres to the substratum with numerous microvillae (Figs. 3A and 3B). In transmission electron microscopic examination, the neurite-like processes induced by the treatment with lactacystin for 4 days were found to exhibit several characteristics of established neurites; parallel array of microtubules and intermediate filaments, probably neurofilaments (Fig. 3C), and numerous electron density granules (large cored vesicles) with 118± 13 nm i.d. (n=40) in the growth cones (Fig. 3D). The intermediate filament structure was predominantly observed in the neuronal process area, whereas the parallel array structure was observed in both the neuronal process and growth cone area in a similar frequency. These results indicate that the lactacystin-induced neurite-like process has a microtubule-containing structure, and that the growth cone has large cored vesicles, suggesting that the structure of neurite-like extension mimics those observed in established neurites[7~9].

Finally, we examined the effect of lactacystin on the intracellular cAMP levels of Neuro 2A cells since a similar morphological change was also observed with prostaglandin E_1 or adenosine-isobutylmethylxanthine combination (data not shown), which treatments are known to elevate the cAMP level through a reaction of the receptor-adenylate cyclase coupling system[10]. On application of 1.3 μM lactacystin to the medium, although the growth rate of Neuro 2A cells decreased (Fig. 4A), the intracellular cAMP level of the cells increased and

Fig. 4. Effect of lactacystin on the cAMP level and growth rate of Neuro 2A cells.



For assay of intracellular cAMP level, the cells were incubated in 35 mm i.d. culture dish containing 2 ml of the medium with or without the drug, the culture medium was aspirated at an appropriate period and rapidly washed with cold phosphate buffered saline followed by addition of 0.01 N HCl in 95% ethanol and stored at 4°C for 2 hours. The supernatant of the acidic ethanol extract of the cells was transferred to a glass small-tube and dried up in a boiling water bath. The dried-up material was dissolved in 1 ml of 0.003 N HCl and stored at −20°C before being processed. The cAMP was determined by radioimmunoassay using a commercially available RIA kit (Yamasa, Japan). The cell number was counted with a hemocytometer. These experiments were carried out in triplicate. Arrows indicate the addition time of lactacystin; 24 or 48 hours after plating. Note that the lactacystin effect may be independent of the preincubation period.

reached a maximum level of 95~105 fmol/10^4 cells 24 hours after treatment, and then decreased to the basal level (Fig. 4B). The transient increase in cAMP levels was dose-dependent; 2.6 and 5.3 μM lactacystin increased the cAMP level from 29.8 to 120 and 230 fmol/10^4 cells, respectively, 24 hours after treatment. This transient effect was coincident with formation of the bipolar projections. We confirmed the result of SCHWARTZ and COSTA[10] that adenylate cyclase modulators such as prostaglandin E_1, adenosine and isobutylmethylxanthine caused transient accumulation of intracellular cAMP in Neuro 2A cells within 15~30 minutes (data not shown). Thus, the slow response of Neuro 2A cells to lactacystin would not be due to direct activation of adenylate cyclase.

On the other hand, TSUJI *et al.*[11] proposed that the signals for neuritogenesis of Neuro 2A cells may be mediated by protein kinase C and cAMP-dependent protein kinase. In *in vitro* system, lactacystin (1~100 μM) could not affect the protein kinase C activity in rat brain preparation[12]. Furthermore, lactacystin was assessed to have an ability to inhibit proteinases such as thrombin and plasminogen activator since a proteinase inhibitor plays a physiological role in the regulation of neurite outgrowth[13]. Lactacystin inhibited neither thrombin nor plasminogen activator activities. Thus, lactacystin is proved to be a new type of NTF with low MW isolated from *Streptomyces* sp. It is the first microbial metabolite exhibiting neurotrophic activity, and may be used as a useful tool for investigations of differentiation of nervous system and of curing the nerve diseases of nervous system associated with the dysfunction of nervous system due to the decrease of NTF.

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