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Review Article

Discovery and mechanism of action of nonpeptide neuritogenic compounds

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Introduction

Advanced nations are experiencing the aging of society. Senility, including Alzheimer dementia and neuron degenerative diseases, will be a serious problem in these countries. Effective drug development for the neuronal diseases is an urgent issue at the present. Recent studies reveal that neurotrophic factors are effective for the protection of neurons from various hazards including active oxygen and ischemia. Administration of the neurotrophic factor is expected to induce neuronal differentiation in the brain; however, the neurotrophic factor cannot pass the blood-brain barrier because it is a protein. Low-molecular weight organic compounds with the same activity as neurotrophin are expected to pass the blood-brain barrier and are being developed as drugs for brain diseases. This review describes the screening of microbial metabolites which have neuritogenic activity and the mechanism of action of low-molecular organic compounds.

Neurotrophins

Nerve growth factor (NGF) is the most classical neurotrophin which induces differentiation and maturation of the peripheral neurons (1). NGF was originally isolated as a neurotrophic factor necessary for the survival of peripheral neurons, and was then shown to function in the central nervous system (CNS) as a neurotrophic factor (Fig. 1). Cloning of the NGF gene and the NGF receptor gene has already been done (1). Brain-derived neurotrophic factor (BDNF) was isolated as another neurotrophin which maintained the survival of sensory neurons (2, 3). There is over 50% homology between the amino acid sequence of NGF and BDNF. Later, NT-3 (4) was discovered based on its homology with NGF/BDNF. NT-4/5 (5) and NT-6 (6) have also been isolated as members of the NGF gene family.

The research on NGF *in vitr o*is mainly carried out using rat pheochromocytoma PC12 cells, and it is known that two kinds of NGF receptors exist: the high affinity receptor which can bind NGF even if the NGF concentration is low, and the low affinity receptor which can bind NGF when the NGF exists at a high concentration (7). The high affinity receptor, TrkA, is a glycoprotein with a molecular weights of about 140 kD encoded by the proto-oncogene trk, and is known to have tyrosine kinase activity (8). The gene family of trk consists of trk (or trkA), trkB and trkC, which encode receptors for NGF, BDNF and NT-3, respectively (8) (Table I). The low affinity receptor, $p75^{LNGFR}$, belongs to the family of tumor necrosis factor receptor (9).

It is known that neurotrophic factors such as NGF and BDNF markedly suppress neuron degeneration and death. For example, neurotrophins can protect the CNS from various damages, including invasion of active oxygen, emission of glutamic acid, *etc*. Therefore, development of neurotrophins or neurotrophic compounds is important for therapy.

Neuroblastoma is a solid malignant tumor which is frequently observed in infants. According to clinical data

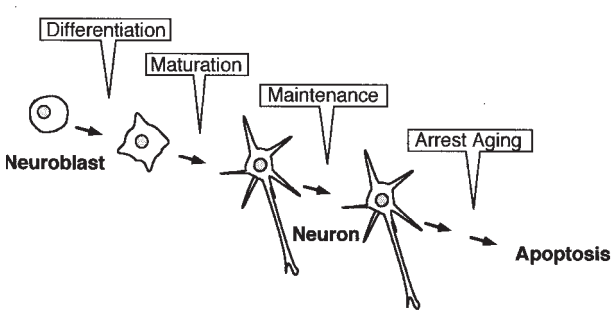


Fig. 1. Biological properties of NGF. NGF triggers neuron differentiation, maintains neuron function and protects neurons from apoptosis.

Table I: Peptide neurotrophins and their receptors. The target cells in which peptide neurotrophins express their biological activity are determined by the receptor present.

	NGF	BDNF	NT-3	NT-4/5
p75 (LNGFR)	+	+	+	+
gp140^{trk} (TrkA)	+	–	±	±
gp140^{trkB} (TrkB)	–	+	±	+
gp140^{trkC} (TrkC)	–	–	+	–

available, the prognosis is comparatively good for a neuroblastoma in which TrkA is expressed, but the prognosis is bad when the expression of TrkA is low. Especially, a neuroblastoma which lacks TrkA and overexpresses *N-myc* gene is highly malignant. The administration of NGF is meaningless for these TrkA-deficient cells. In this case, differentiation-inducing therapy using nonpeptide neurotrophic compounds which show neuritogenic activity independent from the receptor will be useful.

Isolation of neurotrophic compounds from microbial metabolites

Neurotrophins are expected to have therapeutic and protective effects for neuron degenerative diseases; however, the permeability of neurotrophins across the blood-brain barrier is a difficult problem to resolve. If nonpeptide neurotrophic compounds could be isolated, they would be useful for the therapy of neuron degenerative diseases. Organic compounds isolated from microbial metabolites that enhance neurotrophin production, such as erinacin (10) and NG-011 (11), and those that show neuritogenic activity, including lactacystin (12), K-252a (13), PS-990 (14), nerfilin I (15) and stachybotrin C (16), have been reported (Fig. 2).

It is known that retinoic acid and dibutyryl cAMP induce neuronal differentiation in human neuroblastoma SH-SY5Y cells which lack the NGF receptor, TrkA. On the contrary, NGF did not induce neurite outgrowth in SH-SY5Y cells but did in rat pheochromocytoma PC12 cells which express TrkA.

In our laboratory, the screening for neuritogenic compounds from microbial products was carried out using both Trk-negative human neuroblastoma SH-SY5Y cells and Trk-positive rat PC12 cells (Fig. 3). The screening was carried out with the aim of isolating new nonpeptide compounds which permeate the blood-brain barrier and manifest their activity without using the Trk receptor.

Epolactaene

During the screening, we isolated a novel nonpeptide neurotrophic compound, epolactaene (Fig. 4), which is produced by *Penicillium* sp. 1689-P and is found at the sea bottom in the mouth of the Oi river, Shizuoka Prefecture, Japan (17). The compound has an

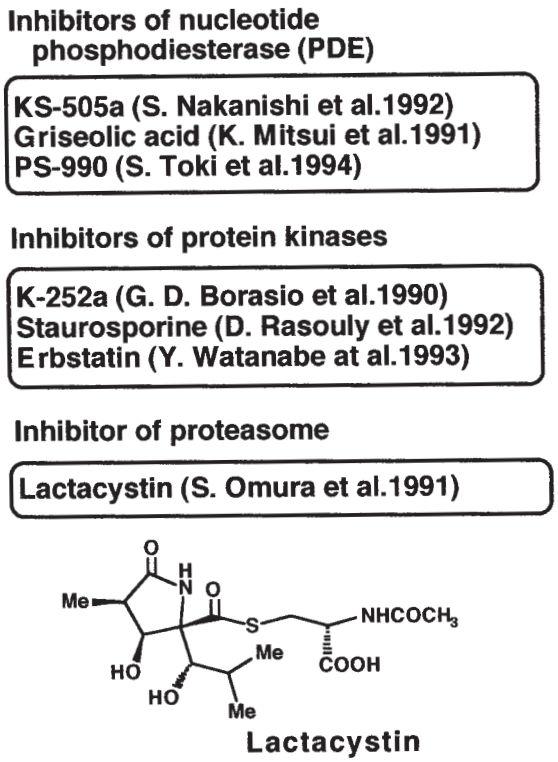


Fig. 2. Microbial metabolites which have neuritogenic activity.

epoxide-containing γ-lactam ring and a triene moiety in the molecule, and thus we named it epolactaene.

Epolactaene induced the neurite outgrowth of SH-SY5Y cells in the concentration range of 2.5-10 μg/ml. Epolactaene-treated cells exhibited bipolar

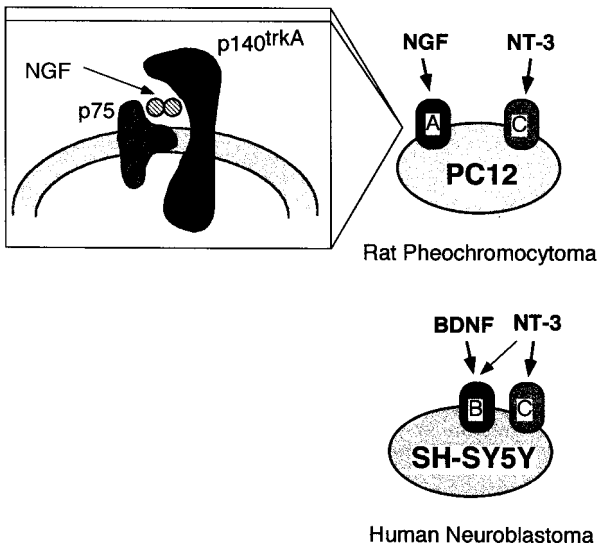


Fig. 3. Expression of neurotrophin receptors on PC12 and SH-SY5Y cells. PC12 cells have TrkA and TrkC on the cell surface but SH-SY5Y cells lack TrkA. Therefore, NGF induces neurite outgrowth of PC12 cells but not SH-SY5Y cells.

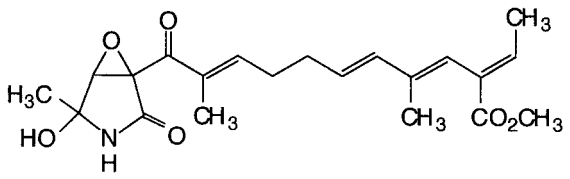


Fig. 4. Structure of epolactaene.

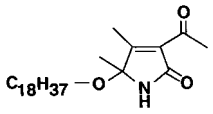
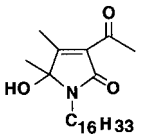
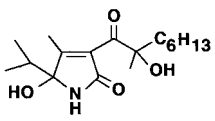
morphology, in which two neurites extended from opposite sides of the cell body. The cell cycle was arrested at the G_0/G_1 phase. Differentiation markers, phosphorylated neurofilaments and acetylcholine esterase activity were enhanced 2 days after treatment with epolactaene.

Only small quantities of epolactaene could be isolated from the fungus, and furthermore, the compound was unstable under light because of a labile triene group in the side chain and an epoxide fused to a γ-lactam ring. Therefore, we prepared a number of epolactaene derivatives, such as 3-substituted 3-pyrrolin-2-one derivatives possessing a double bond in the γ-lactam ring, in order to find compounds with more stable and effective neuritogenic activity (18). We then discovered that MT-5, a 3-acetyl-4,5-dimethyl-5-octadecyloxy-3-pyrrolin-2-one derivative, had the most effective neuritogenic activity on SH-SY5Y cells and also arrested the cell cycle progression at G_0/G_1, which is the same as epolactaene.

After synthesis of many derivatives, the following relationships between the structures and neuritogenic activity in SH-SY5Y cells were noted: an epoxide ring fused to the γ-lactam ring in epolactaene is not always necessary for the biological activity, but at least one straight long-chain alkyl group and a carbonyl group at the 3-position are required (Table II). The observed structure-activity relationships suggested that epolactaene and the related 3-pyrrolin-2-one derivatives may act via acylation of one or more relevant target molecules(s) in the cell.

During the synthesis study, we obtained other compounds, MT-19 and MT-20, which showed neuritogenic activity on PC12 but not on SH-SY5Y cells (Fig. 5).

Table II: Epolactaene derivatives which have different cell specificity.

Compounds		SH-SY5Y	PC12
	(MT-005)	+++	+
	(MT-019)	+	+++
	(PI-091)	++	–

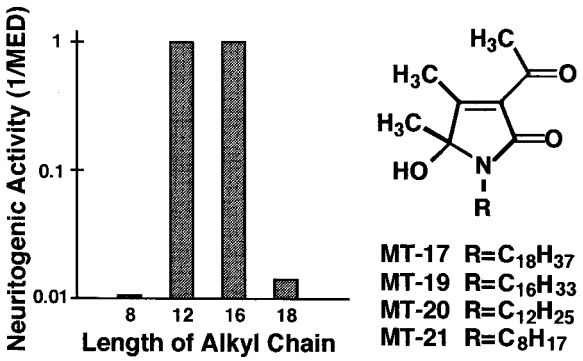


Fig. 5. Structures of MT-19 and MT-20. Among the many derivatives synthesized, MT-19 and MT-20 showed the strongest neuritogenic activity on PC12 cells.

MT-19 and MT-20

Since there is knowledge about the signal transduction of NGF in PC12 cells, we analyzed the signal transduction of MT-19 and MT-20 in PC12 cells. NGF binds to the Trk receptor and activates its tyrosine kinase activity, which leads to the activation of a guanine nucleotide binding protein, Ras. Expression of oncogenic Ras has been shown to induce neuronal differentiation, and NGF-induced differentiation of PC12 cells is blocked by microinjection of an anti-Ras monoclonal antibody or expression of a dominant negative Ras mutant (N17Ras/PC12) or membrane targeted-GAP, indicating that activation of Ras is necessary and sufficient for the induction of differentiation in PC12 cells. Furthermore, Ras activates the Raf-MEK-MAP kinase signaling pathway, which has been shown to play an important role in the induction of neurite outgrowth.

MT-19 and MT-20 induced neurite outgrowth not only of normal cells but also of mutant PC12 cells which lack Ras function. Furthermore, MT-19 and MT-20 did not induce MAP kinase activation, suggesting that they do not require the Ras-MAP kinase signaling pathway which is necessary and sufficient for NGF-induced neurite outgrowth in PC-12 cells. Consistent with this, none of the early- or late-response genes tested, which included *fos, zif268, Nur77, vgf* and *transin*, were induced. However, the protein level of three neurofilaments was increased after incubation with the compounds. Since the protein level of other cytoskeletons including actin and tubulin remained constant, MT-19 and MT-20 specifically affected neurofilament synthesis and/or turnover. Taken together, these findings indicate that MT-19 and MT-20 induce neurite outgrowth by activating the downstream target of MAP kinase or by a novel mechanism which is distinct from the NGF-activated pathway (19).

Recently, bone morphologenic protein (BMP)-2 was shown to induce differentiation in PC12 cells. Interestingly, BMP-2 did not induce MAP kinase activation or *fos* transcription but did increase the protein level of neurofilaments (20), suggesting that MT-19, MT-20 and BMP-2 have a common target (Fig. 6). Defining the target

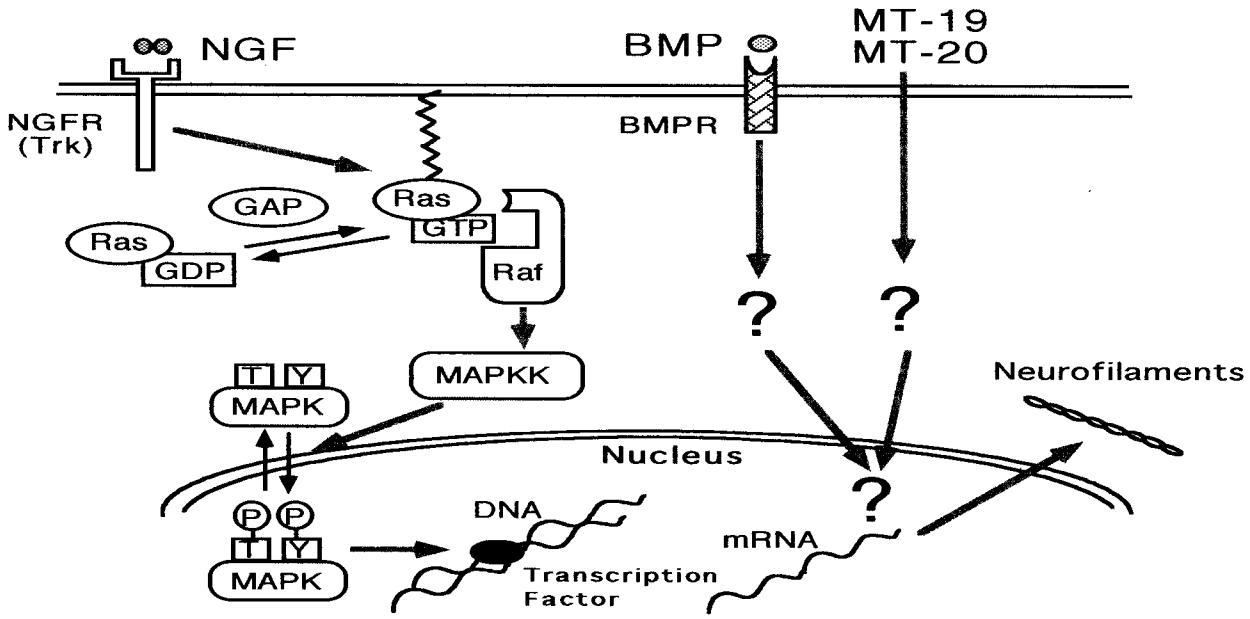


Fig. 6. Signal transduction pathway of the neuritogenic compounds in PC12 cells. The neuritogenic activity of MT-19 and MT-20 is mediated by a Ras-independent pathway.

of these compounds may provide a new approach for studying the mechanism of neuronal differentiation.

Staurosporine

Staurosporine (Fig. 7), a potent protein kinase inhibitor isolated from microbial metabolites, is known to mimic the effect of NGF in promoting neurite outgrowth (21). To elucidate the mechanism by which staurosporine induces neurite outgrowth in PC-12 cells, we performed an in-gel kinase assay using myelin basic protein (MBP) as a substrate and found that staurosporine induced the activation of a kinase with an apparent molecular mass of 57 kD. The dose of staurosporine required to activate this kinase was consistent with that required to induce neurite outgrowth. Interestingly, the staurosporine-activated kinase was immunoprecipitated by an anti-JNK isoform

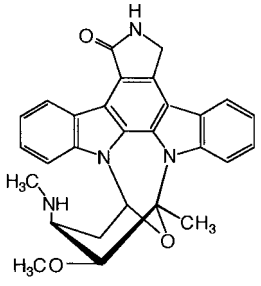


Fig. 7. Structure of staurosporine.

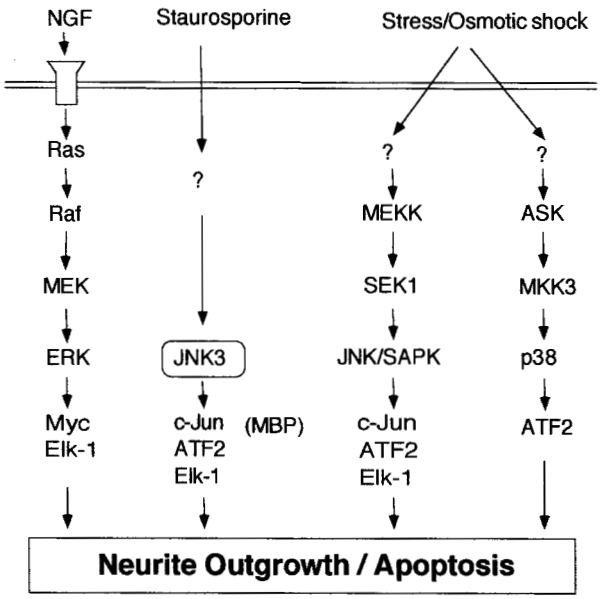


Fig. 8. Staurosporine activates a new member of the JNK family, which is different from JNK/SAPK.

antibody, but not by an anti-JNK1 specific antibody or anti-ERK1 antibody, raising the possibility that this kinase is a novel JNK isoform. The substrate specificity of the kinase was distinct from those of osmotic shock-activated JNKs and NGF-activated ERK1 (22). The kinase phosphorylated transcription factors including c-Jun, Elk-1 and ATF2, as well as MBP, suggesting that it plays a role in gene induction. Furthermore, staurosporine induced immediate-early genes, including *Nur77* and *fos*, but not

jun. The activation of the staurosporine-activated kinase, as well as the induction of neurite outgrowth, did not require Ras function, while Ras was required for the activation of ERKs and neurite outgrowth induced by NGF.

Taken together, these results indicate that staurosporine specifically activates a JNK isoform which may contribute to biological activities including neurite outgrowth, although the biological role and the activation mechanism of JNK3 remains uncertain (23) (Fig. 8).

Conclusions

Lactacystin was discovered as a chemical compound which induced the neurite of a neuroblastoma Neuro 2A, and it is also known now as an inhibitor of proteasomes. This review has described the discovery and mechanism of action of neuritogenic compounds different from lactacystin. Such nonpeptide organic compounds are expected to be chemotherapeutic agents as well as biochemical tools. Key work lies ahead in the discovery of the molecular target(s) for the neuritogenic compounds.

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