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TMC-95A, a reversible proteasome inhibitor, induces neurite outgrowth in PC12 cells

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Abstract—TMC-95A has been characterized as a potent proteasome inhibitor that binds to enzymes non-covalently at low nanomolar concentrations. Herein, the neuritogenic activity of TMC-95A in PC12 rat pheochromocytoma cells is reported for the first time. TMC-95A induced a positive neurite initiation of PC12 cells at concentration ranging from 1 to 20 μM.

The ubiquitin/proteasome system is the major pathway of proteolysis in eukaryotic cells.[1] The proteasome is a large multicatalytic complex that catalyzes the degradation of ubiquitinated cellular proteins. Substrates of the ubiquitin/proteasome pathway include several cell regulatory molecules, and the regulated degradation of these substrates is critical for the control of cell proliferation, differentiation, and cell cycle progression. Therefore, it is not surprising that the inhibition of proteasome activity could have profound biological consequences.

Several cell-permeable proteasome inhibitors[2] were found to induce neurite outgrowth when added to neuronal cell lines. The inhibitors with neuritogenesis activity include synthetic *N*-benzyloxycarbonyl-Leu-Leu-leucinal (ZLLLal),[3] a natural product lactacyctin,[4,5] and its analogue *clasto*-lactacystin β-lactone (Fig. 1).[6,7] These molecules exert their activities through a covalent attachment to the N-terminal catalytic threonine residue of the proteasome.

In contrast to these covalent inhibitors, the natural product from *Apiospora montagnei*, TMC-95A (Fig. 1), has been determined to display non-covalent, reversible inhibition of the proteasome in the low nanomolar range by forming the characteristic hydrogen bonds.[8,9] It is important to note that TMC-95A is specific to the proteasome, and does not inhibit other proteases such

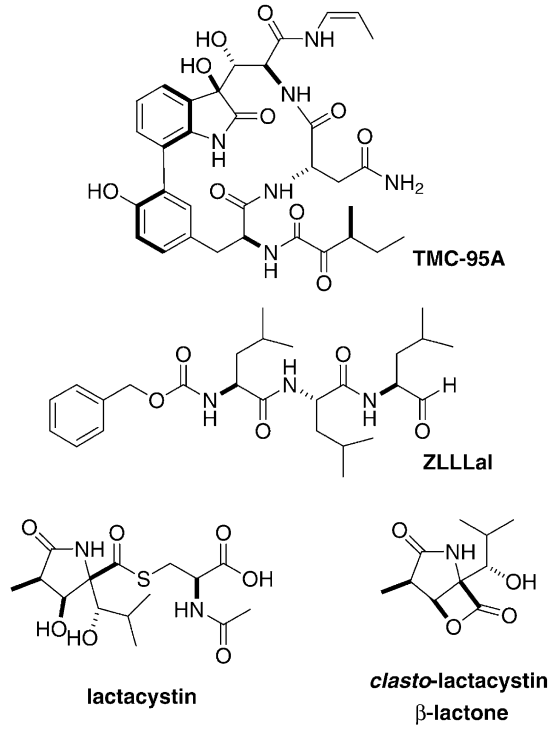

Figure 1. Structures of various proteasome inhibitors.

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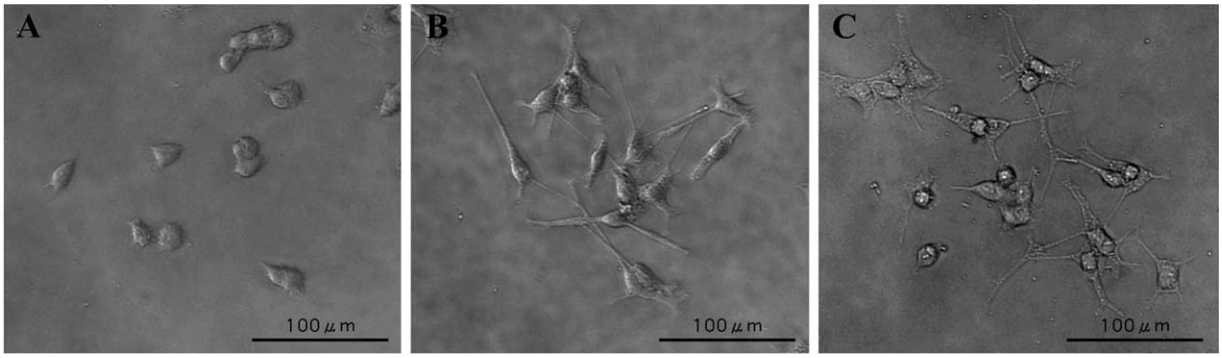

Figure 2. Neuritogenic effect of TMC-95A on PC12 cells. PC12 cells were cultured in DMEM medium supplemented with 10% horse serum and 5% fetal bovine serum, and treated with control vehicle solution (0.5% ethanol) and TMC-95A for 16 h. In comparison to the control (A), TMC-95A induced neurite outgrowth from cell body. (B), TMC-95A 5 μM; (C), TMC-95A 20 μM. Phase-contrast photographs were taken by a reverse microscope equipped with CCD digital camera and LuminaVision/MacSCOPE imaging system (Mitani Co., Japan).

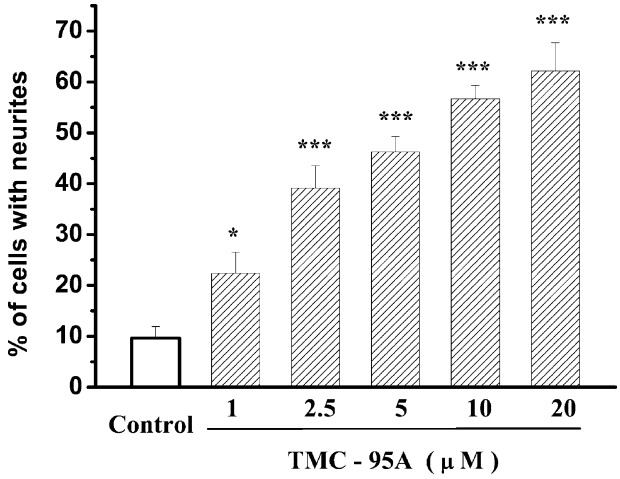

Figure 3. TMC-95A initiated neurite outgrowth from PC12 cells dose-dependently. PC12 cells were cultured in 24-well plate, and treated with control vehicle solution (0.5% ethanol) and TMC-95A for 16 h. The percentage of cells with neurites was calculated from 5 random fields of one well. Data were expressed as mean percentage ± SE of 4 wells in one representative experiment. Statistical analysis was performed with software Origin 7.0 (Origin Lab, USA). *, $p < 0.05$; ***, $p < 0.001$ versus control in one-way ANOVA followed by Bonferroni post hoc means comparison.

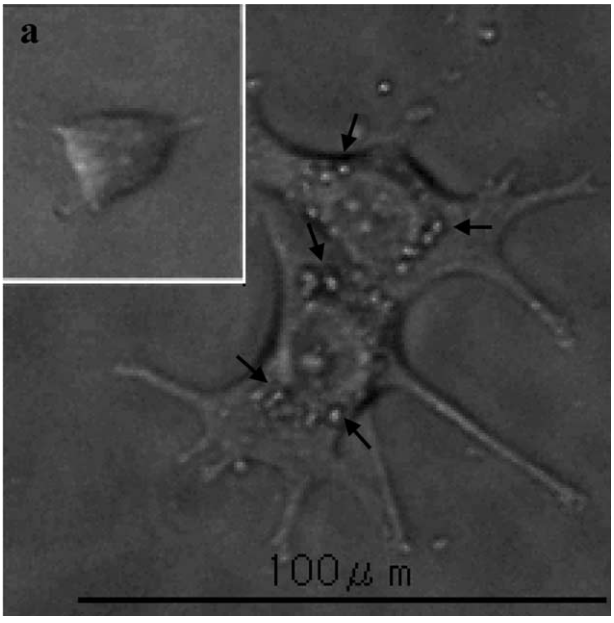

Figure 4. TMC-95A induced inclusion bodies in PC12 cells. When PC12 cells cultured in DMEM medium were treated with TMC-95A above 10 μM, inclusions appeared around cell nuclei paralleling neurite outgrowth. Demonstration here shows two flattened PC12 cells containing inclusion body (indicated by dark arrows). Inset (a) is a PC12 cell from control group as comparison.

as calpain, cathepsin, and trypsin. The TMC-95A molecule consists of modified amino acid residues which form a strained heterocyclic ring system.[8] The combination of the structural novelty, inhibitory potency, and the unique inhibition mechanism of TMC-95A prompted us to undertake a program of total synthesis, which was achieved in 2003.[10–12] TMC-95A is not related to any previously reported proteasome inhibitors, and the only biological effect published on this molecule is the cytotoxicity against human cancer cells (HCT-116, HL-60).[8] In this context, we came to be interested in the effects of TMC-95A in neuronal cells. PC 12 rat pheochromocytoma cells, which have been widely used for studying neuronal differentiation, were selected as the neuronal cells to be used for the preliminary experiment. Herein, the neurite-initiating effect of TMC-95A in PC12 cells is demonstrated.

PC12 cells (JCRB0733) were grown in DMEM media containing 10% donor horse serum (HS), 5% fetal bovine serum (FBS), 50 IU/mL penicillin and 50 μg/mL streptomycin. To prepare for the neurite outgrowth assay, the PC12 cells were trypsinized to a single cell suspension, and seeded into poly-L-lysine coated plates at a cell density of 1×10^4 cm^{-2}. After one day, the culture medium was replaced with DMEM supplemented with 10% HS, 5% FBS, and TMC-95A at various concentrations. The cells were further incubated for 16 h, and then fixed with 10% paraformaldehyde in order to determine the percentage of cells with neuritic processes longer than the diameter of the cell.

As shown in Figure 2, TMC-95A induced apparent morphological changes in PC12 cells. In the control

assay (Fig. 2A), only 10% of the PC12 cells had neurite growth. When TMC-95A was added to the culture medium, some of the PC12 cells became flattened, polarized and differentiated with apparent neurite outgrowth (Fig. 2B,C). As shown in Figure 3, neuritogenesis was enhanced in a dose-dependent manner from 1 to 20 μM of TMC-95A, and after the 16 h incubation, over 60% of the cells bore neurites at a concentration of 20 μM. In addition to the initiation of neurite outgrowth, other morphological changes were observed in the cells. One such change was the formation of inclusion bodies around the nuclei (Fig. 4).[13] After the PC12 cells had fully developed the neurites (more than 24 h), partial apoptosis-like cell death was observed (data not shown).

The neuronal differentiation by a proteasome inhibitor was not unprecedented. ZLLLal is a proteasome inhibitor that exibits neurite-initiating effect in PC12 cells, however, is not specific to proteasome, and inhibits other proteases such as calpains.[3] *Clasto*-lactacystin β-lactone was reported to induce neurite outgrowth in PC12 cells at a narrow dose range,[14] whereas lactacystin exhibited dose-dependent toxic effects in PC12.[7] Interestingly, lactacystin is known to induce neurite outgrowth in Neuro 2A mouse neuroblastoma cells.[5] TMC-95A is distinct from these inhibitors in its novel mechanism of specific binding to proteasome, and its potent neuritogenesis activity in PC12 cells over a broad range of concentrations.

In summary, TMC-95A was found to differentiate the neurite in PC12 cells for the first time. Because of its unique biological properties, TMC-95A will be useful in regulating the neuronal differentiation as well as understanding the role of the ubiquitin/proteasome system in the development and maintenance of the neurons. Furthermore, the established synthetic route to TMC-95A will enable the design of fully synthetic neuritogenic compounds with less cytotoxicity, which may be applicable to repair damaged neurons by cell or tissue transplantation, such as in the treatment of Parkinson's disease and retinal degenerations. Active studies along this line are currently underway in our laboratory.

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