Aurora™ Microplates

Application note

384-well neurite outgrowth assay using human iPSC-derived neurons

【Introduction】

Neuronal cells develop functional intercellular networks through neurite outgrowth, including the extension of axons and dendrites, as well as synapse formation. Regulation of these networks is a major focus of basic neurobiological research related to neurodevelopment and neural plasticity, and it is also of significant importance in drug discovery research for neurodegenerative diseases and neurotoxicity.

In this study, we employed a neurite outgrowth assay using human iPSC-derived neurons and a 384-well CELLAZIP™ F-coated. Compounds previously reported to inhibit or promote neurite outgrowth[1,2] were applied to the neurons, and we attempted to generate dose–response curves and determine IC_{50} and EC_{50} values.

【Materials and Methods】

Human iPSC-derived neurons (iCell® GABANeurons) suspended in culture medium were seeded onto 384-well CELLAZIP™ F-coated at 4,500 cells/well (4.5×10^4 cells/cm²) and incubated at 37℃ in a 5% CO_2 atmosphere. After 24 hours, compounds (dissolved in DMSO) known to inhibit or promote neurite outgrowth, as described in Fig.1, were added. The final DMSO concentration in each well was normalized to 0.1%. After 72 hours, cells were fixed by adding 4% paraformaldehyde in phosphate buffer and incubating at room temperature for 15 minutes. Following two washes with D-PBS(–), blocking solution was added and the cells were incubated at room temperature for 1 hour. A βIII-tubulin primary antibody, diluted to the appropriate concentration with antibody diluent, was dispensed into each well and incubated overnight at 4℃. After washing three times with D-PBS(–), secondary antibody, F-actin staining solution, and nuclear staining solution were added, followed by light-protected incubation at room temperature for 1 hour. After three additional washes with D-PBS(–), images of βIII-tubulin (green), F-actin (red), and nuclei (blue) were captured using the CellVoyager™ CQ1 confocal microscope (Yokogawa Electric Corp.) (10× and 40× objective lenses). Neurite outgrowth was analyzed using images acquired with the 10× objective lens for βIII-tubulin. Dose–response curves for neurite outgrowth relative to each compound concentration were generated using image analysis software (Table 1, Fig.1).

【Results and Discussion】

The results for staurosporine, which exhibited a particularly distinctive change in neurite outgrowth among the test compounds used in this study, are shown in Fig.2. Staurosporine functions as a broad-spectrum protein kinase inhibitor and is known to promote neurite outgrowth at low concentrations[3]. In the present study, neurite outgrowth was promoted at concentrations between 0.025 and 0.2 μM, whereas higher concentrations resulted in inhibition. These findings clearly demonstrate concentration-dependent changes in neurite outgrowth induced by the compound.

Images of neurons treated with various test compounds that promote or inhibit neurite outgrowth, along with the dose–response curves obtained from image analysis, are shown in Fig.3 and 4. Clear inhibition or promotion of neurite outgrowth by the compounds was observed, and the image-based analysis yielded IC_{50} and EC_{50} values for neurite outgrowth in response to the test compound concentrations.

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Table 1. Cells、reagents、imaging system

Cell culture	384-well microplate	・CELLAZIP™ F-coated		
	Human iPSC-derived neurons	・iCell® GABANeurons (#C1018, FUJIFILM Cellular Dynamics) : 4,500 cells/well ・Culture medium composition: iCell® Neural Basal Medium1 (#M1010, FUJIFILM Cellular Dynamics) iCell® Neural Supplement A (#M1032, FUJIFILM Cellular Dynamics) laminin (#L2020, Sigma-Aldrich) , 3.3μg/mL		
Test compounds	Inhibition of neurite outgrowth	Staurosporine (at high concentration)	Broad-spectrum kinase inhibitor	#T4000, TCI
		Colchicine	Microtubule polymerization inhibitor	#C0380, TCI
		Rotenone	Mitochondrial complex I inhibitor	#R0090, TCI
		BIO	GSK3B inhibitor	#S7198, Selleck
		Triptolide	Plant-derived natural diterpene	#T4000, TCI
	Promotion of neurite outgrowth	Staurosporine (at low concentration)	Broad-spectrum kinase inhibitor	#S3604, Selleck
		Alverine	Smooth muscle relaxant	#S5945, Selleck
		Blebbistatin	Myosin inhibitor	#E1249, Selleck
		Ifenprodil	NMDA receptor antagonist	#E48811, Selleck
		Perphenazine	Typical antipsychotics and D2R antagonists	#S4731, Selleck
		Trifluoperazine	Typical antipsychotics and D2R antagonists	#S5856, Selleck
Staining	Cell fixation	4% paraformaldehyde in phosphate-buffered solution (#09154-85, nacalai tesque)		
	Blocking	Blocking One Histo (#06349-64, nacalai tesque) (diluted 20-fold with PBS) +0.3% Triton X-100 (#35501-02, nacalai tesque), r.t., 1 hr		
	Primary antibody	Purified anti-Tubulin β3 (TUBB3) Antibody (#801202, BioLegend), 1:500 dilution, 4℃ O.N.		
	Secondary antibody	Goat anti-Mouse IgG, IgM (H+L) Secondary Antibody, Alexa Fluor™ 488 (#A32723, Invitrogen), 1:250 dilution, r.t., 1 hr in the dark		
	F-actin	Texas Red™-X Phalloidin (#T7471, Invitrogen), 1:400 dilution, r.t., 1 hr in the dark		
	Cell nucleus	Hoechst33342 (#3579, Invitrogen), 10mg/mL water solution 1:1000 dilution, r.t., 1 hr in the dark		
	Dilution buffer	Blocking One Histo(diluted 20-fold with PBS)+0.3% Triton X-100		
Imaging and analysis	Imaging system	CellVoyager CQ1 (Yokogawa Electric Corp.)		
	Data analysis software	CellPathfinder (Yokogawa Electric Corp.)		

Neurite Outgrowth	Test compounds
Inhibition	Staurosporine (at high concentration), Colchicine, Rotenone, BIO, Triptolide
Promotion	Staurosporine (at low concentration), Alverine, Blebbistatin, Ifenprodil, Perphenazine, Trifluoperazine

Fig.1 Workflow of the neurite outgrowth assay

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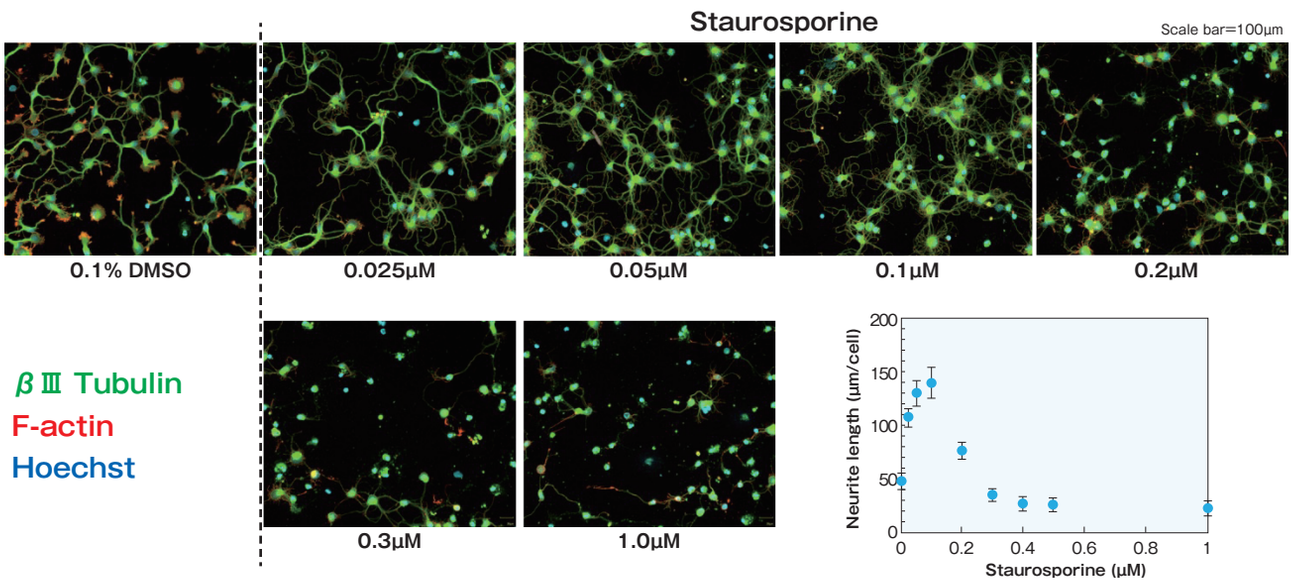

Fig.2 Effect of staurosporine on neurite outgrowth: The assay was performed at n = 9 for each concentration, and images captured with a 40× objective lens are presented.

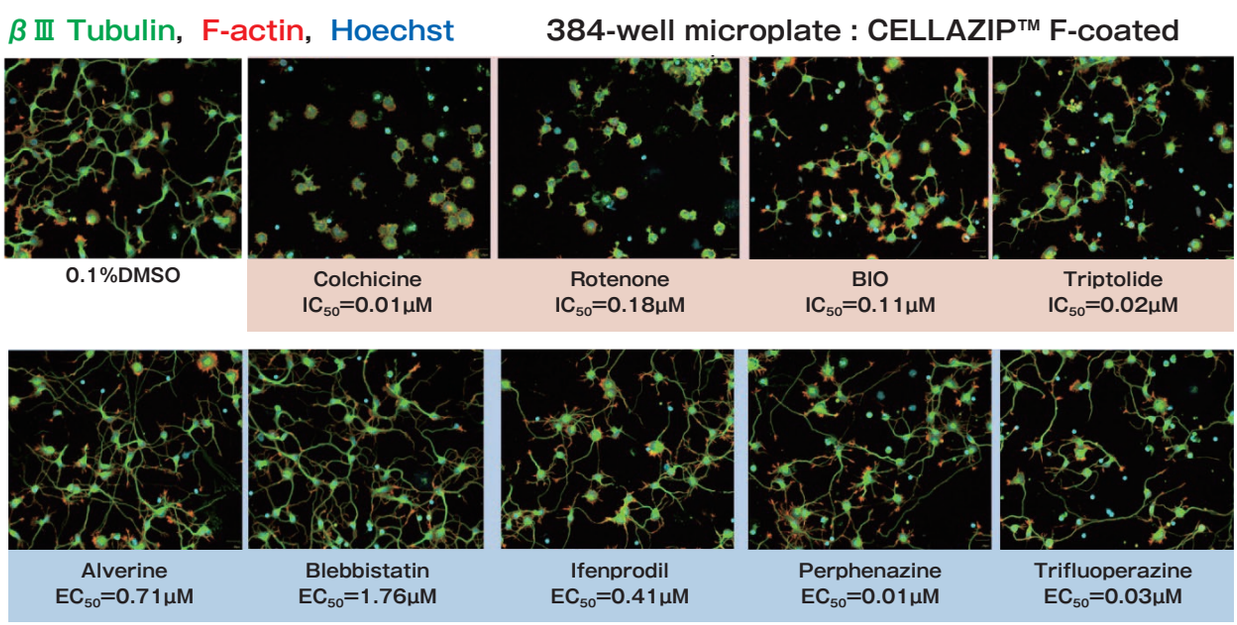

Fig.3 Staining images obtained after treatment with test compounds that inhibit or promote neurite outgrowth (images captured using a 40× objective lens).