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APPENDICES

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APPENDIX I

CHEMICALS, SUPPLIES, EQUIPMENT

Chemicals

1. P19 embryonal carcinoma cells (ATCC, USA)
2. A β ₁₋₄₂ (Sigma Chemical Co., USA, and Biosource, German).
3. Valproic acid (Aldrich, German)
4. α -Minimal essential medium (α -MEM) (Life Technologies, USA)
5. Minimal essential medium (MEM) (Life Technologies, USA)
6. Fetal bovine serum (FBS) (Biochrom AG, German)
7. Newborn calf serum (NCS) (Life Technologies, USA)
8. All-*trans* retinoic acid (Sigma Chemical Co., USA)
9. Trypsin (Sigma Chemical Co., USA)
10. DNase I (Pharmacia Biotech, USA)
11. Cytosine arabinoside (Sigma Chemical Co., USA)
12. Cresyl violet (Sigma Chemical Co., USA)
13. Trypan blue (Sigma Chemical Co., USA)
14. Trypsin (Sigma Chemical Co., USA)
15. Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., USA)
16. Phosphate buffered saline (PBS)
17. CytoTox 96 Non-radioactive Cytotoxicity Assay Kit (Promega Co., U.S.A.)
18. 3,3-[(Phenylamino)carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) (Sigma Chemical Co., USA)
19. Phenazine methosulfate (PMS) (Sigma Chemical Co., USA)
20. Acetylthiocholine (Sigma Chemical Co., USA)
21. 5,5'-Dithiobis(nitrobenzoic acid) (DTNB) (Sigma Chemical Co., USA)
22. Tetra-isopropylpyrophosphoramidate (iso-OMPA) (Sigma Chemical Co., USA)
23. Sodium bicarbonate (Sigma Chemical Co., USA)
24. Triton X-100 (Sigma Chemical Co., USA)
25. Trolox (Sigma Chemical Co., USA)

Supplies

1. 24-Well flat bottom sterile tissue culture (Nunclon, Denmark and TPP, Switzerland)
2. 6-Well flat bottom sterile tissue culture (Nunclon, Denmark and TPP, Switzerland)
3. 96-Well flat bottom sterile tissue culture microtiter plates (Nunclon, Denmark and TPP, Switzerland)
4. T75 tissue culture flask (Nunclon, Denmark and TPP, Switzerland)
5. 15-ml and 50-ml polypropylene conical tubes
6. Eppendorf vials
7. Acrocap filter units (0.2 μm)
8. Pipette man
9. Sterile pipettes (1 ml, 5 ml, 10 ml)
10. Pasteur pipettes, sterilized
11. Sterilize pipette tips, (1-10 μl , 1-20 μl , 20-100 μl and 200 -1000 μl)

Equipment

1. Centrifuge (EBA12R Hettich Centrifuge, USA)
2. Spectrophotometer (Spectronic Spectrophotometer, USA)
3. CO₂ humidified tissue culture incubator (CO₂ Water Jacketed Incubator, Forma Scientific, USA)
4. Laminar flow (Issco Laminar Flow model BV 225, Dwyer Instruments, USA)
5. Inverted microscope (Olympus[®] CK30/CK40 Culture Microscope, Olympus Optical Co., Japan; Zeiss Axiovert 100 microscope, USA)
6. Spectrophotometer microplate reader (Anthos htII, Anthos labtech instruments, USA; Bio-Rad, Bio-Rad laboratories, USA)
7. Deep freezer (-80°C)
8. Freezer (-20°C)
9. Water bath
10. Hemacytometer

APPENDIX II

PREPARATION OF REAGENTS

all trans RA (1 mM stock solution)

Dissolve 50 mg of *all trans* RA in 156 ml 100% ethanol and 8 ml of distilled water. Stored in dark bottle in refrigerator.

Growth medium of NLCS

Combine the following components in the corresponding proportions, 90% MEM medium, 10% heat inactivated FBS, 25 μ M of L-glutamine and 20 μ M of AraC. Filter through a 0.2 μ m filter (if necessary) and store at 2-8° C in a sterile container.

Growth medium of P19 cells

Combine the following components in the corresponding proportions, 90% α -MEM medium, 7.5% heat inactivated NCS, and 2.5% heat inactivated FBS. Filter through a 0.2 μ m filter (if necessary) and store at 2-8° C in a sterile container.

Heat-inactivated FBS and NCS

Thaw the desired amount of FBS or NCS at ambient temperature or 2°C–8°C. Adjust the waterbath to a temperature of 56°C \pm 2°C. Place the bottle of FBS or NCS into the waterbath so that the entire contents of the bottle are immersed in water. Heat the bottle for 45 minutes, swirling periodically. Remove the bottle from the waterbath, allow to cool. Aliquot 50 ml of the FBS or NCS in sterile 50 ml sterile bottles. Label each container with name, lot number, expiration date, and the heat inactivation date. Store at -20°C \pm 10° C or 2-8°C.

Induction medium of aggregates

Combine the following components in the corresponding proportions, 95% α -MEM medium, 5% heat inactivated FBS and 1 μ M of *all trans* RA. Filter through a 0.2 μ m filter (if necessary) and store at 2-8° C in a sterile container.

α -MEM medium

To a mixing container that is as close to final volume as possible, add distilled water 5% less than the desired total volume of medium. Dissolve α -MEM powder to room temperature water with gentle stirring. Add the 29.3 ml of 7.5% sodium bicarbonate solution and stir until dissolved. Adjust pH to 7.4 \pm 0.10 with 1N HCl or 1 N NaOH and bring solution to 1000 ml with purified water. Filter through a 0.2 μ m filter into sterile bottles. Store at 2-8° C, which will expire in 8 weeks.

MEM medium

Aseptically dilute 100 ml of 10X concentrate with approximately 850 ml of sterile water. Aseptically add the 29.3 ml of 7.5 % sodium bicarbonate solution. Adjust pH to 7.4 \pm 0.10 with 1N HCl or 1 N NaOH and bring solution to 1000 ml with purified water. Dispense the solution into sterile container. Store at 2-8° C, which will expire in 8 weeks.

Phosphate buffered saline (PBS)

Per liter of PBS to be made, add 950 ml deionized water to the container. Dissolve ingredients including, 7.650 g NaCl, 0.724 g anhydrous Na₂HPO₄, and 0.210 g KH₂PO₄. Stir for approximately four hours at room temperature until all solids are dissolved and adjust pH to 7.4 (with 1 N NaOH). Bring solution to 1000 ml with purified water. Solution is autoclaved for 45 min at 121°C.

Plating of P19 cells

After 2-3 days, aspirate the medium from the flasks. Add 5 ml PBS to each flask; swirl and lay the flasks flat to cover the monolayer. Aspirate the PBS. Add 2-3 ml trypsin to each flask; swirl and lay the flasks flat making sure the monolayer is

coated. Allow the flasks to sit for approximately 1-2 minutes to detach the cells from the surface. Add 15 ml of growth medium to each flask. Mix by swirling the flasks and triturating.

Thawing cells

The cells (P19EC, stored in liquid nitrogen or -80°C freezer) are quick thawed in a $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ waterbath. The contents of the vials are combined in a 15 ml conical tube and mixed thoroughly. The total volume is recorded. An aliquot of the undiluted cell suspension is taken and used to make 1 in 2 dilution in trypan blue (i.e. 50 μl cells + 50 μl trypan blue). Add 10 ml growth medium to the remaining cells. Live cells (i.e. cells not stained by trypan blue) are counted in all four outer squares on both sides of the hemacytometer. To calculate the number of cells per ml of cell suspension. A final concentration of 2×10^5 cells per flask is required. The concentration is achieved by appropriate dilutions of the cell suspension using growth medium. To calculate the number of flasks and the appropriate volume of cells to be used. Place flasks in a $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 5% $\text{CO}_2 \pm 0.5\%$ CO_2 humidified incubator for 2-3 days.

0.25 % Trypsin solution

Dissolve 0.25 g of trypsin powder in 100 ml PBS, swirling periodically. Filter through a 0.2 μm filter into sterile bottles, store at $2-8^{\circ}\text{C}$.

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APPENDIX III

TABLES OF EXPERIMENTAL RESULTS

1. Acetylcholinesterase as a marker enzyme in neuron-like cells

	Specific AChE activity*		
	(mU/mg protein)		
	Control	0.1 μ M RA	1 μ M RA
1. Aggregates (2 days)	0.225 \pm 0.06	1.25 \pm 0.143	0.736 \pm 0.047
2. Aggregates (4 days)	0.131 \pm 0.01	3.94 \pm 0.150	5.86 \pm 0.215
3. Aggregates (6 days)	0.122 \pm 0.002	4.31 \pm 0.113	5.79 \pm 0.067
4. 3 days after plating	0.298 \pm 0.03	13.53 \pm 0.458	17.78 \pm 0.577
5. 5 days after plating	0.392 \pm 0.13	15.95 \pm 0.309	20.18 \pm 0.827
6. 7 days after plating	0.311 \pm 0.04	15.94 \pm 1.190	21.06 \pm 0.425

* Each value represents the mean \pm S.E.M of (three) independent experiment.

2. Effect of VPA and of VPU alone on neuron-like cells

	%XTT reduction*	% Cell viability*
1. Control	92.34 \pm 4.16	95.26 \pm 3.17
2. 0.1 % DMSO	97.53 \pm 5.42	98.65 \pm 4.67
3. 1 μ M VPA	90.23 \pm 3.23	96.64 \pm 1.56
4. 10 μ M VPA	91.50 \pm 2.40	100.11 \pm 1.01
5. 100 μ M VPA	97.06 \pm 1.61	97.16 \pm 1.46
6. 1000 μ M VPA	109.08 \pm 5.81	99.65 \pm 3.42
7. 1 μ M VPU	98.69 \pm 1.82	102.02 \pm 1.99
8. 10 μ M VPU	96.59 \pm 2.92	98.50 \pm 1.59
9. 100 μ M VPU	94.00 \pm 2.04	98.89 \pm 1.70

* Each value represents the mean \pm S.E.M of (three) independent experiment.

3. Effect of A β_{1-42} on neuron-like cells

3.1 XTT reduction assay*

	Incubation time			
	24 h	48 h	72 h	96 h
0.1 μM A β_{1-42}	85.51 \pm 7.37	102.51 \pm 2.87	99.16 \pm 3.03	81.59 \pm 2.34
1 μM A β_{1-42}	78.24 \pm 7.72	74.76 \pm 1.17	63.23 \pm 2.33	60.06 \pm 3.02
5 μM A β_{1-42}	66.17 \pm 1.31	61.98 \pm 1.33	47.35 \pm 2.33	50.28 \pm 3.06
10 μM A β_{1-42}	50.34 \pm 2.03	40.75 \pm 3.33	25.63 \pm 2.67	20.82 \pm 2.02

* Each value represents the mean \pm S.E.M of (three) independent experiment.

3.2 % cell viability by trypan blue exclusion assay*

	Incubation time			
	24 h	48 h	72 h	96 h
Control	93.71 \pm 5.76	90.17 \pm 2.82	94.21 \pm 0.91	89.28 \pm 0.64
0.1 μM A β_{1-42}	92.63 \pm 0.89	94.80 \pm 1.23	92.29 \pm 6.18	87.39 \pm 0.74
1 μM A β_{1-42}	76.42 \pm 3.66	71.66 \pm 3.59	65.95 \pm 4.15	61.55 \pm 0.47
5 μM A β_{1-42}	68.02 \pm 3.47	59.63 \pm 2.10	50.50 \pm 1.59	49.94 \pm 0.88
10 μM A β_{1-42}	53.37 \pm 4.70	45.84 \pm 0.58	40.36 \pm 1.16	36.12 \pm 3.05

* Each value represents the mean \pm S.E.M of (three) independent experiment.

3.3 LDH release*

	Incubation time			
	24 h	48 h	72 h	96 h
Control	15.09 \pm 1.50	14.62 \pm 2.60	17.99 \pm 1.67	18.72 \pm 1.02
0.1 μM A β_{1-42}	15.56 \pm 0.67	16.15 \pm 2.72	21.79 \pm 0.88	27.88 \pm 0.58
1 μM A β_{1-42}	37.13 \pm 1.20	52.53 \pm 0.58	57.07 \pm 0.88	57.10 \pm 3.51
5 μM A β_{1-42}	44.37 \pm 3.51	56.43 \pm 0.95	65.24 \pm 3.97	71.14 \pm 7.02
10 μM A β_{1-42}	52.46 \pm 0.99	56.59 \pm 1.33	69.31 \pm 5.33	70.62 \pm 7.33

* Each value represents the mean \pm S.E.M of (three) independent experiment.

4. Cotreatment with VPA or VPU and A β_{1-42} on NLCs

4.1 XTT reduction assay

Treatments	% XTT reduction*
1. 5 μ M A β_{1-42}	51.77 \pm 1.98
2. 10 μ M VPU + 5 μ M A β_{1-42}	53.20 \pm 3.96
3. 50 μ M VPU + 5 μ M A β_{1-42}	63.00 \pm 2.59
4. 100 μ M VPU + 5 μ M A β_{1-42}	77.40 \pm 3.48
5. 10 μ M VPA + 5 μ M A β_{1-42}	48.65 \pm 1.19
6. 50 μ M VPA + 5 μ M A β_{1-42}	50.70 \pm 2.17
7. 100 μ M VPA + 5 μ M A β_{1-42}	63.20 \pm 1.95
8. 500 μ M VPA + 5 μ M A β_{1-42}	76.50 \pm 1.47
9. 1000 μ M VPA + 5 μ M A β_{1-42}	77.94 \pm 0.88
10. 1 mM Trolox + 5 μ M A β_{1-42}	66.58 \pm 1.81

* Each value represents the mean \pm S.E.M of (three) independent experiment.

4.2 % Cell viability by trypan blue exclusion assay

Treatments	% Cell viability*
1. Control	92.93 \pm 2.48
2. 5 μ M A β_{1-42}	50.68 \pm 2.55
3. 10 μ M VPU + 5 μ M A β_{1-42}	50.66 \pm 2.75
4. 50 μ M VPU + 5 μ M A β_{1-42}	60.45 \pm 3.00
5. 100 μ M VPU + 5 μ M A β_{1-42}	69.66 \pm 2.77
6. 10 μ M VPA + 5 μ M A β_{1-42}	50.10 \pm 3.07
7. 50 μ M VPA + 5 μ M A β_{1-42}	54.60 \pm 3.05
8. 100 μ M VPA + 5 μ M A β_{1-42}	62.90 \pm 2.75
9. 500 μ M VPA + 5 μ M A β_{1-42}	70.52 \pm 2.68
10. 1000 μ M VPA + 5 μ M A β_{1-42}	70.58 \pm 2.87
11. 1 mM Trolox + 5 μ M A β_{1-42}	60.44 \pm 2.56

* Each value represents the mean \pm S.E.M of (three) independent experiment.

4.3 LDH release

Treatments	% LDH release*
1. Control	27.53 ± 1.37
2. 5 μM Aβ ₁₋₄₂	71.21 ± 1.19
3. 10 μM VPU + 5 μM Aβ ₁₋₄₂	71.19 ± 2.01
4. 50 μM VPU + 5 μM Aβ ₁₋₄₂	58.50 ± 4.11
5. 100 μM VPU + 5 μM Aβ ₁₋₄₂	58.50 ± 1.40
6. 10 μM VPA + 5 μM Aβ ₁₋₄₂	71.96 ± 2.07
7. 50 μM VPA + 5 μM Aβ ₁₋₄₂	68.82 ± 1.51
8. 100 μM VPA + 5 μM Aβ ₁₋₄₂	64.92 ± 2.88
9. 500 μM VPA + 5 μM Aβ ₁₋₄₂	45.27 ± 2.08
10. 1000 μM VPA + 5 μM Aβ ₁₋₄₂	45.69 ± 2.73
11. 1 mM Trolox + 5 μM Aβ ₁₋₄₂	63.82 ± 1.03

* Each value represents the mean ± S.E.M of (three) independent experiment.

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5. Pretreatment with VPA or VPU on A β_{1-42} -treated cells

5.1 XTT reduction assay

Treatments	% XTT reduction*
1. 5 μ M A β_{1-42} (3 days)	51.90 \pm 2.76
2. 5 μ M A β_{1-42} (5 days)	55.20 \pm 2.66
3. 5 μ M A β_{1-42} (7 days)	51.50 \pm 2.14
4. 100 μ M VPA + 5 μ M A β_{1-42} (3 days)	61.64 \pm 3.73
5. 100 μ M VPA + 5 μ M A β_{1-42} (5 days)	68.00 \pm 1.69
6. 100 μ M VPA + 5 μ M A β_{1-42} (7 days)	57.80 \pm 3.19
7. 100 μ M VPU + 5 μ M A β_{1-42} (3 days)	65.00 \pm 2.99
8. 100 μ M VPU + 5 μ M A β_{1-42} (5 days)	76.85 \pm 2.99
9. 100 μ M VPU + 5 μ M A β_{1-42} (7 days)	61.50 \pm 2.31
10. 1 mM Trolox + 5 μ M A β_{1-42} (3 days)	63.20 \pm 5.15
11. 1 mM Trolox + 5 μ M A β_{1-42} (5 days)	66.60 \pm 3.73
12. 1 mM Trolox + 5 μ M A β_{1-42} (7 days)	53.80 \pm 3.87

* Each value represents the mean \pm S.E.M of (three) independent experiment.

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5.2 % Cell viability by trypan blue exclusion assay

Treatment	% Cell viability*
1. Control (3 days)	92.92 ± 1.62
2. Control (5 days)	95.91 ± 1.16
3. Control (7 days)	79.83 ± 1.64
4. 5 μM Aβ ₁₋₄₂ (3 days)	51.80 ± 0.96
5. 5 μM Aβ ₁₋₄₂ (5 days)	52.40 ± 0.92
6. 5 μM Aβ ₁₋₄₂ (7 days)	41.60 ± 1.89
7. 100 μM VPA + 5 μM Aβ ₁₋₄₂ (3 days)	52.00 ± 2.59
8. 100 μM VPA + 5 μM Aβ ₁₋₄₂ (5 days)	58.00 ± 2.87
9. 100 μM VPA + 5 μM Aβ ₁₋₄₂ (7 days)	51.10 ± 1.73
10. 100 μM VPU + 5 μM Aβ ₁₋₄₂ (3 days)	55.56 ± 2.77
11. 100 μM VPU + 5 μM Aβ ₁₋₄₂ (5 days)	64.06 ± 3.88
12. 100 μM VPU + 5 μM Aβ ₁₋₄₂ (7 days)	56.50 ± 1.44
13. 1 mM Trolox + 5 μM Aβ ₁₋₄₂ (3 days)	52.05 ± 1.58
14. 1 mM Trolox + 5 μM Aβ ₁₋₄₂ (5 days)	55.08 ± 1.38
15. 1 mM Trolox + 5 μM Aβ ₁₋₄₂ (7 days)	51.43 ± 1.11

* Each value represents the mean ± S.E.M of (three) independent experiment.

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5.3 LDH release

Treatment	% LDH release*
1. Control (3 days)	28.23 ± 4.40
2. Control (5 days)	24.98 ± 1.13
3. Control (7 days)	32.77 ± 6.56
4. 5 µM Aβ ₁₋₄₂ (3 days)	61.21 ± 2.28
5. 5 µM Aβ ₁₋₄₂ (5 days)	66.99 ± 3.25
6. 5 µM Aβ ₁₋₄₂ (7 days)	73.44 ± 1.79
7. 100 µM VPA + 5 µM Aβ ₁₋₄₂ (3 days)	57.27 ± 1.54
8. 100 µM VPA + 5 µM Aβ ₁₋₄₂ (5 days)	59.24 ± 2.99
9. 100 µM VPA + 5 µM Aβ ₁₋₄₂ (7 days)	71.19 ± 4.38
10. 100 µM VPU + 5 µM Aβ ₁₋₄₂ (3 days)	50.24 ± 5.0
11. 100 µM VPU + 5 µM Aβ ₁₋₄₂ (5 days)	45.78 ± 1.71
12. 100 µM VPU + 5 µM Aβ ₁₋₄₂ (7 days)	64.57 ± 1.75
13. 1 mM Trolox + 5 µM Aβ ₁₋₄₂ (3 days)	59.12 ± 4.61
14. 1 mM Trolox + 5 µM Aβ ₁₋₄₂ (5 days)	62.96 ± 3.09
15. 1 mM Trolox + 5 µM Aβ ₁₋₄₂ (7 days)	71.12 ± 1.17

* Each value represents the mean ± S.E.M of (three) independent experiment.

6. Post-treatment with VPA or VPU on Aβ₁₋₄₂-treated cells

6.1 XTT reduction assay

Treatment	% XTT reduction*
1. 5 µM Aβ ₁₋₄₂	47.00 ± 1.84
2. 5 µM Aβ ₁₋₄₂ + 50 µM VPA	48.38 ± 3.76
3. 5 µM Aβ ₁₋₄₂ + 100 µM VPA	51.90 ± 5.41
4. 5 µM Aβ ₁₋₄₂ + 50 µM VPU	48.62 ± 4.59
5. 5 µM Aβ ₁₋₄₂ + 100 µM VPU	52.30 ± 3.01
6. 5 µM Aβ ₁₋₄₂ + 1 mM Trolox	49.50 ± 2.34

* Each value represents the mean ± S.E.M of (three) independent experiment.

6.2 % Cell viability by trypan blue exclusion assay

Treatments	% Cell viability*
1. Control	93.23 \pm 0.91
2. 5 μ M A β ₁₋₄₂	50.87 \pm 0.73
3. 5 μ M A β ₁₋₄₂ + 50 μ M VPA	49.69 \pm 1.28
4. 5 μ M A β ₁₋₄₂ + 100 μ M VPA	50.82 \pm 1.39
5. 5 μ M A β ₁₋₄₂ + 50 μ M VPU	48.72 \pm 0.79
6. 5 μ M A β ₁₋₄₂ + 100 μ M VPU	51.32 \pm 0.76
7. 5 μ M A β ₁₋₄₂ + 1 mM Trolox	48.90 \pm 1.18

* Each value represents the mean \pm S.E.M of (three) independent experiment.

6.3 LDH release

Treatments	% LDH release*
1. Control	23.36 \pm 1.52
2. 5 μ M A β ₁₋₄₂	68.56 \pm 1.71
3. 5 μ M A β ₁₋₄₂ + 50 μ M VPA	71.73 \pm 2.01
4. 5 μ M A β ₁₋₄₂ + 100 μ M VPA	68.50 \pm 1.33
5. 5 μ M A β ₁₋₄₂ + 50 μ M VPU	73.04 \pm 2.38
6. 5 μ M A β ₁₋₄₂ + 100 μ M VPU	67.70 \pm 1.17
7. 5 μ M A β ₁₋₄₂ + 1 mM Trolox	68.24 \pm 2.64

* Each value represents the mean \pm S.E.M of (three) independent experiment.

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Miss Boonrat Chantong was born on February 20th, 1976 in Samutsakorn province, Thailand. She was graduated in Bachelor of Science in Pharmacy in 1998 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Since graduation, she has been appointed as a lecturer in the Department of Pharmacy, Sirinthon College of Public Health.



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