CHAPTER IV

RESULTS

1. P19 cell differentiation

P19 embryonal carcinoma cells grew rapidly in culture as "packed colonies". Although cells with processes were observed occasionally, undifferentiated cells grew to be densely packed, displaying a characteristic cuboidal morphology evidence on phase contrast microscopy and showed predominantly homogeneous morphology characterized by scant cytoplasm and distinct nucleoli (Figure 1A). Neuronal differentiation was initiated by treating the cell with all-*trans* retinoic acid (RA). By the second day of the RA treatment, compact cell aggregates or embryoid bodies (EBs) were formed (Figure 1B). Aggregates of P19 cells were treated with 1 μM RA for 4 days, dissociated, replated, and grown as a monolayer in tissue culture dishes or 24-well plates which treatment with 20 μM Ara C eliminated nonneuronal cell growth and cell viability and morphological properties were monitored. At day 2 after plating, neuronal processes or neurites appeared in some cells (Figure 1C). At day 4, neuronal cells began to dominate the culture, during which time the cells developed numerous long processes indicative of the neural differentiation and the network of neuronal processes appeared (Figure 1D). At day 7, P19 neurons were uniformly spread and were interconnected by extensive network of neurites, remarkable increase in networks of neuronal processes was observed (Figure 1E).

Within 24 h of plating, a flat layer of fibroblast-like cells migrated out of the periphery of the aggregate. Between day1 and day 2 after plating, neuron-like cells appeared whose processes grew rapidly from the aggregates over the fibroblast-like cell layer. After days 4-5 of plating, a population of glial cells initially appeared at the junction of the fibroblast-like monolayer and increased rapidly. Thus, RA-treated cultures contained three distinct cell types, neuron-like cells, glial-like cells, and fibroblast-like cells. Undifferentiated cells were flat, round in shape and devoid of neurites. Following 4 days exposure to 1 μM RA, the majority of cells exhibited neuron-like morphology. P19 cells were induced to differentiate along the neuroectodermal

pathway by culturing free-floating EBs in RA. After 4 days, EBs were plated into tissue-culture dishes, whereupon extensive morphological differentiation occurred. Neurons were distinguished as phase-bright rounded cells with extended processes. The number of nonneuronal cells steadily declined upon treatment with 20 μM of AraC. This treatment was selective for retaining postmitotic neurons and resulted in highly enriched neuronal populations. Glial and other nonneuronal cells were often seen beneath neuron-like cells and appeared as flattened nonphase-bright cells which were mostly observed on NLCs without the treatment of Ara C (Figure 1F). Dense aggregate of neuronal cell bodies formed multifocal "ganglia" points" of the networks were specifically stained by cresyl violet, which clearly appeared a process of neuron (Figure 2).

2. Acetylcholineresterase as a marker enzyme in neuron-like cells

P19 cells did not exhibit AChE activity. However, after aggregation with RA and plating for morphological differentiation, neuronally induced cells showed increasing levels AChE activity in cell homogenates. The undifferentiated cells exhibited no or only a small amount of AChE, 0.311 ± 0.039 mU/mg of protein. At day 2 after the treatment of cells with RA at a concentration of 1 μM, levels of AChE activity were induced to 0.74 ± 0.047 mU/mg of protein. An eight-fold increase in AChE activity (5.86 ± 0.22 mU/mg of protein) occurred after day 4, and then became stable at day 6 of the treatment (Figure 3). After the induction of the morphological differentiation by plating onto the tissue culture dish, the dramatically 24-fold increase in AChE activity was observed at 17.78 ± 0.58 mU/mg of protein, which was paralleled to the appearance of morphologically identifiable neurons after plating (Figure 1D). The level of AChE activity at day 5 after plating increased continuously to 20.18 ± 0.83 mU/mg of protein, and reached the plateau at in day 7. In contrast, in the absence of RA treatment, the level of AChE activity in undifferentiated aggregated cells at day 4 was only 0.131 ± 0.011 mU/mg of protein and was maintained at that level even though at after plating. The differentiation of P19 cells into NLCs and the expression of the AChE specific activity were dependent on the concentration of RA present in the culture medium as shown in Figure 3, which was the response of EBs and NLCs to RA at a concentration of 0.1 μM.

The optimization of the extent of neuronal differentiation in the culture was also established using the RA treatment at the concentration of 1 μM (Figure 3). If EBs were maintained in culture, the free floating aggregates expressed AChE activity but at a lower level and appearing at later time than in the morphologicaiiy differentiating neuronal cells. Replating onto tissue-culture dishes was necessary to observe high levels of cell-associated AChE activity in RA-treated, neuronally induced cells. The temporal expression pattern of AChE in P19 cells was similar to that observed previously in regardless of the inducer utilized to induced neuronal differentiation. In cell line, there is a basal level of AChE activity that increases upon neurites initiation, accentuating AChE's role in assembling microtubules in neurites (Coleman and Taylor, 1996).

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

To verify the absence of intrinsic neuronal toxicity, VPA or VPU was tested in the absence of $A\beta_{1-42}$. The neuron-like cells were exposed to VPA or VPU at the concentration ranging from 1-1000 μM for 72 h. Activity in cell survival was determined using the XTT reduction assay and the trypan blue exclusion assay (Figure 4). The presence of VPA or VPU at a concentration of 100 μM for 72 h did not significantly decrease the mitochondrial function when measured with the XTT assay by 97% and 94%, respectively, as compared to control (no drug treatment). Trypan blue exclusion assay did not significantly decrease the cell viability caused by 100 μM VPA or VPU by 97% and 99%, respectively. Thus, the results indicated that both VPA and VPU were not toxic to NLCs under these conditions. It should be noted that VPA at a concentration of 1000 μM slightly increased the cell viability when measured with the XTT assay and the trypan blue exclusion assay.

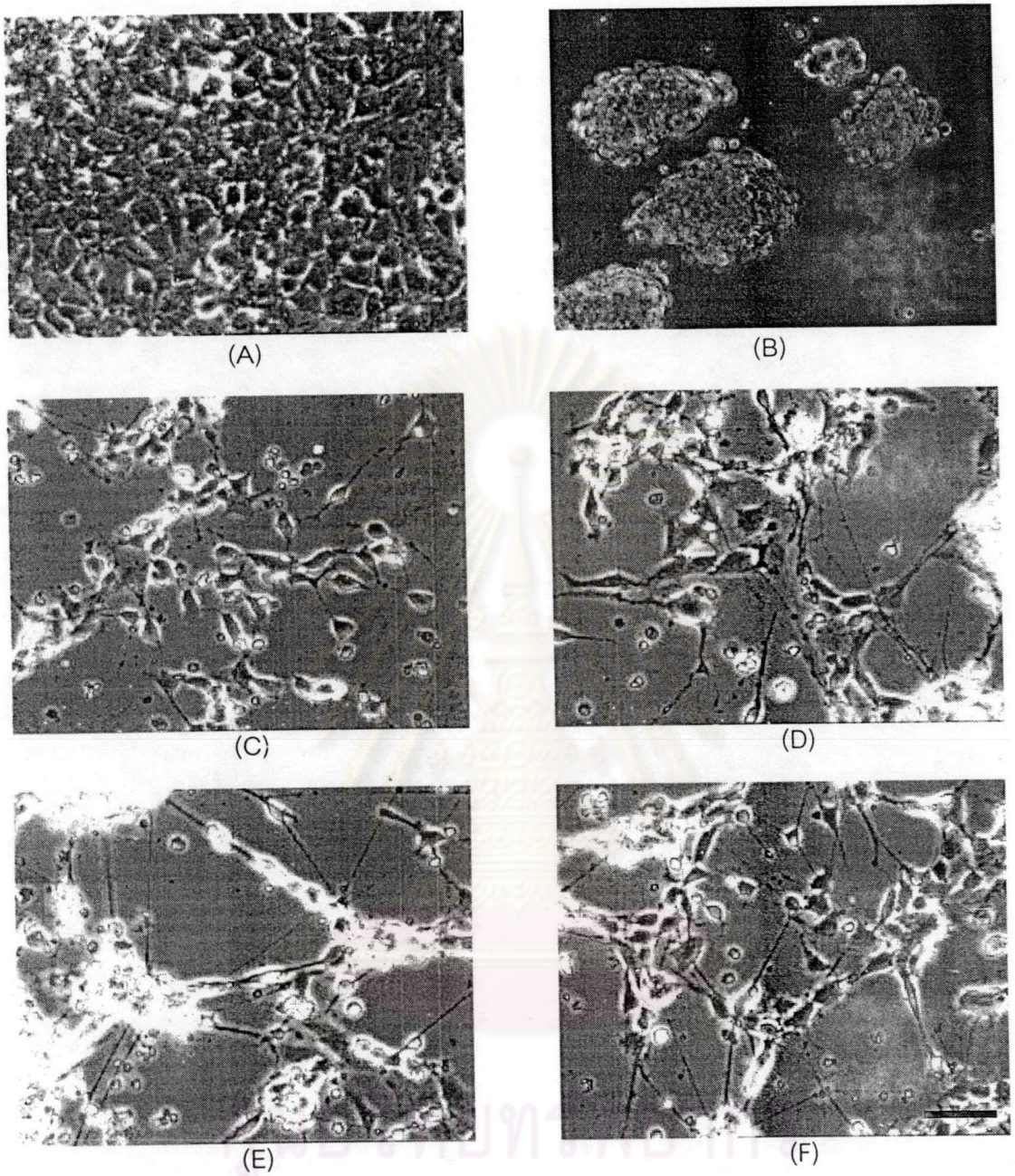


Figure 1. P19 embryonal carcinoma cells differentiated to ectodermal phenotype in response to retinoic acid. (A) P19 cells, (B) embryoid bodies, (C) 2 days of NLCs, (D) 4 days of NLCs, (E) 7 days of NLCs with the treatment of Ara C, and (F) 7 days of NLCs without the treatment of Ara C. Scale bar = 50 μm.

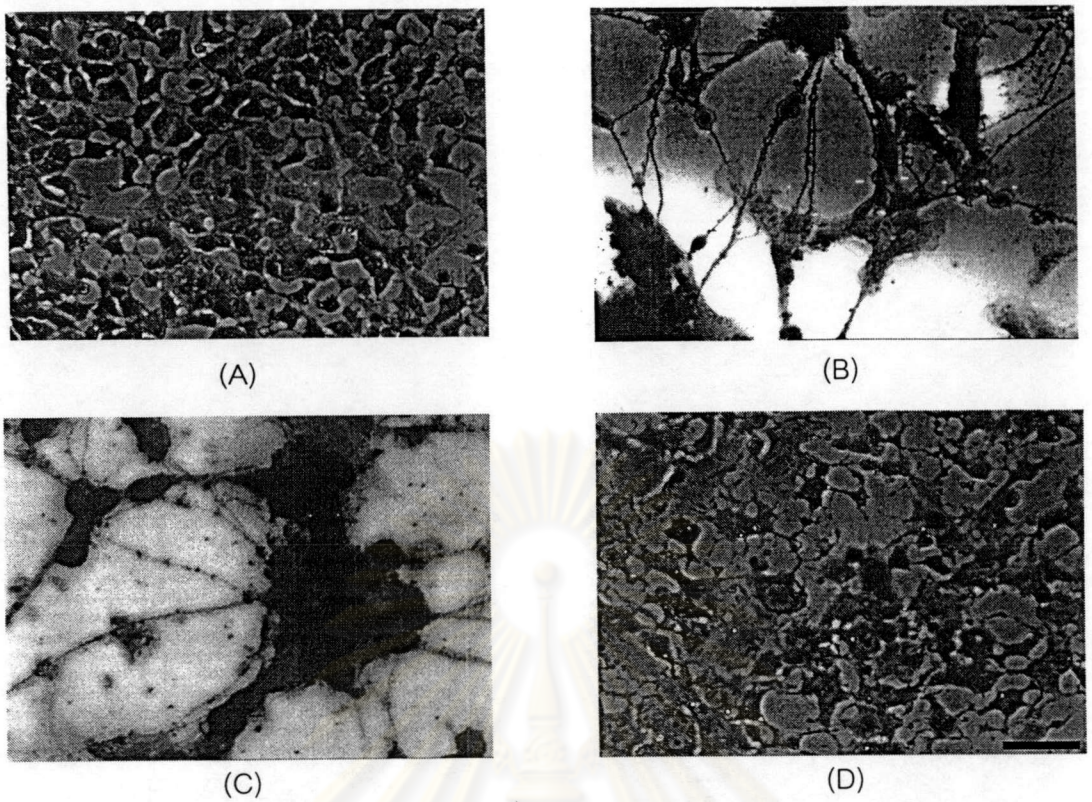


Figure 2. Cresyl violet staining of P19 embryonal carcinoma cells differentiated to ectodermal phenotype in response to retinoic acid. (A) P19 cells, (B) 4 days of NLCs, (C) 7 days of NLCs with the treatment of Ara C, and (D) 7 days of NLCs without the treatment of Ara C. Scale bar = 50 μm.

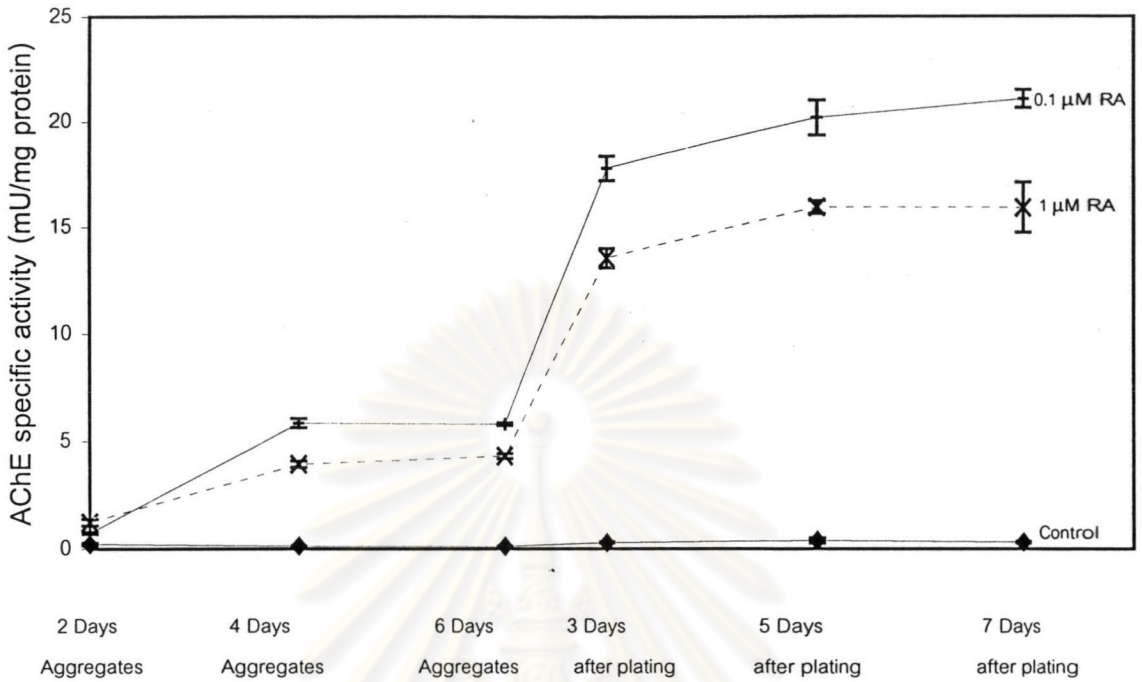


Figure 3. Specific AChE activity in P19 cells under different culture conditions. EBs were cultured with RA at the concentrations of 0.1 and 1.0 µM for 6 days and maintained as free-floating aggregates. After 6 days of culturing EBs, cells were plated onto tissue culture dishes for morphological differentiation. The specific AChE activity increased indicating the differentiation of neuronal cells. The results represented the mean ± S.E.M from triplicate experiments.

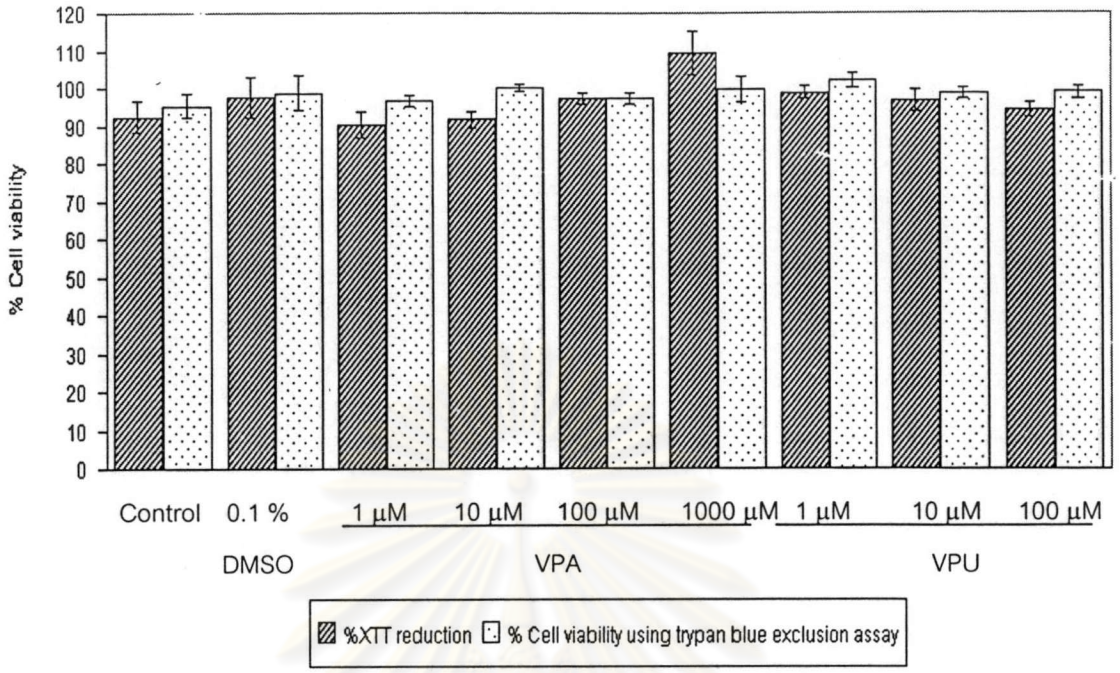


Figure 4. Effect of VPA and of VPU on metabolic activity of NLCs determined by XTT reduction assay and on neuronal survival determined by trypan blue exclusion assay. NLCs were cultured in medium in the absence and the presence of 1, 10, 100, and 1000 µM VPA or 1, 10, and 100 µM VPU for 72 h. Both VPA and VPU did not induce significant changes in the XTT values and trypan blue exclusion at the indicated concentrations $p<0.05$ as compared to control and vehicle control treatments. The results represented the mean ± S.E.M from triplicate experiments, and were illustrated by histogram representation.

4. Effect of $A\beta_{1-42}$ on neuron-like cells

4.1 $A\beta_{1-42}$ induced neuronal toxicity in a concentration-dependent manner

Among various forms of β–amyloid peptide, $A\beta_{1-42}$ was found to be a major cause in the pathogenesis of AD and was therefore used for the entired study. To test the toxicity following the $A\beta_{1-42}$ treatment to NLCs, the concentration dependence and the nature of the cytotoxic response elicited by $A\beta_{1-42}$ was established. The NLCs were cultured at 37°C for 7 days to stabilize and establish neurites before performing toxicity assays. The cells of the control group were healthy for the following 7 days. Cells were exposed to $A\beta_{1-42}$ at the concentrations ranging from 0.1 to 10 μM for 24, 48, 72, and 96 h. The measurement of the extent of cell survival was assayed by the XTT reduction and the trypan blue exclusion, as well as the extent of cell death was assayed by LDH release in the culture medium (Figures 5-7).

Using XTT reduction assay, $A\beta_{1-42}$ at a concentration of 0.1 μM resulted in no difference in cell viability from the control group (Figure 5). After 24 h incubation with $A\beta_{1-42}$ at the concentrations of 5 and 10 μM decreased the reduction of XTT to 66% and 50%, respectively. At 72 h incubation with $A\beta_{1-42}$ at the concentrations more than 0.1 μM decreased the XTT reduction in a concentration dependent manner presenting with an EC_{50} value of 2.82 μM (Figure 8). The results obtained from the trypan blue exclusion assay (Figure 6) showed a similar manner as the XTT reduction assay with an EC_{50} value approximated to be 5.01 μM (Figure 8). The cell viability decreased in a concentration dependent manner.

The LDH release was observed only when cells were exposed to $A\beta_{1-42}$ at the concentration greater than 1 μM for more than 48 h (Figure 7). We also observed a concentration-dependent increase in the LDH release into the culture medium in the $A\beta_{1-42}$-treated NLCs, giving an EC_{50} value of 5.62 μM, that was accompanied by a decrease of XTT reduction values (Figure 8). Exposure of neurons to 5 μM $A\beta_{1-42}$ for 72 h induced marked neuronal cell death associated with a considerable increase in the LDH release by 65%.

While the LDH release is an index of cell death, the XTT reduction is an index of the cell survival. The results suggested that Aβ-induced toxicity measured as a percentage of XTT reduction correlated with the percentage release of LDH. It represents an excellent marker of metabolic compromise that ultimately leads to neuronal degeneration and cell death. The EC_{50} values of each assay, suggested that there were two phase in $A\beta_{1-42}$ toxicity; an early phase inducible by low concentrations of $A\beta_{1-42}$, as detected by the XTT reduction assay, and the late phase, which required high concentrations of $A\beta_{1-42}$, as detected by the LDH release assay (Figure 8).

4.2 Time course study of $A\beta_{1-42}$ induced neuronal toxicity

To define the time course of Aβ toxicity, we quantified cell viability by the same three criteria described above. These assays were performed on neuronal cultures treated in parallel with Aβ or vehicle only. Metabolic integrity was assessed by the XTT reduction, which quantify cellular reducing potential; by this measure, neuronal viability began to decline at 24 h of treatment and the maximum inhibition lasted until the end of the measurement (Figure 5). The trypan blue exclusion assay showed the similar results, and reached the plateau at 96 h of the exposure time (Figure 6).

At a concentration of 0.1 μM of $A\beta_{1-42}$ applied, the LDH release was not detectable for up to 72 h. The $A\beta_{1-42}$ at the concentrations of 1-10 μM, a marked increase in the LDH in the medium started to rise after 48 h, with the most pronounced increase and reached the plateau level at 72 h of exposure time (Figure 7). Cellular morphological change was also observed, including somal shrinkage, plasma membrane rupture as viewed in light microscope. This effect after exposure to 5 μM $A\beta_{1-42}$ was obvious after 48 h, and remarkably reached the plateau at 72 h. The increase in the LDH release after $A\beta_{1-42}$ treatment was not due to DMSO, since vehicle treated cells received the equivalent amount of DMSO was not different from the control (without DMSO). The percentage of the basal LDH release of the control group and of the vehicle-treated cells (0.01% DMSO) at 72 h were 18.35 ± 3.124% and 17.99 ± 1.67%, respectively. An increase in the LDH release in the media was observed

following 48 h incubation with $A\beta_{1-42}$ at the concentration grater than 1 μM, indicating that $A\beta_{1-42}$ was exerting a toxic effect.

The Aβ-induced degeneration resulted in obvious changes in neuronal morphology, including swollen and beaded neurites, vacuolar inclusion, and cellular collapse. The light and phase-contrast microscopy observation demonstrated that addition of aggregated Aβ to differentiated cells resulted in decreased cell number and retraction of neurites as compared to neuronal cells alone (Figure 11D). These degenerative events exhibited a delayed onset, with observable morphological changes typically beginning within 48 h of Aβ treatment. The morphological changes were succeeded by a significant decrease in the metabolic activity as assayed by XTT, and eventually cell lysis as determined by the LDH release and the positive trypan blue staining.

The optimal conditions for obtaining a significant $A\beta_{1-42}$ toxicity in culture of NLCs derived from P19 were described above. Thus we concluded that the $A\beta_{1-42}$ treatment of NLCs was at a concentration of 5 μM with an exposure time for at least 72 h to induce neuronal toxicity and was subsequently used for the entired experiments.

5. Effects of VPA or VPU on $A\beta_{1-42}$–induced cytotoxicity

5.1 Cotreatment with VPA or VPU and $A\beta_{1-42}$ on NLCs

To evaluate whether VPA or VPU could attenuate $A\beta_{1-42}$-induced neurotoxicity, the various concentrations of valproates were coincubated with $A\beta_{1-42}$ in culture. The NLCs were incubated at 37°C for 7 days prior to a cotreatment experiment. In the cotreatment experiment, VPA or VPU at a concentration of 100 μM, added along with the peptide for the additional 3-treatment-days significantly protected from neurotoxicity and cell death induced by $A\beta_{1-42}$ at a concentration of 5 μM. Using the XTT reduction assay, VPA at the concentration of 100 μM, when treated with 5 μM $A\beta_{1-42}$, significantly enhanced cell survival to 63% of the control as shown in Figure 9A. Additionally, VPU at the same concentration also enhanced cell viability to 77% of the control. Trypan exclusion assay also showed the ability of VPA and VPU to increase cell

viability to 63% and 70%, respectively (Figure 9B). For the detection of membrane integrity by the LDH release assay, both VPA and VPU at the indicated concentration reduced the release of LDH into the medium to 65% and 59%, respectively, while NLCs treated with 5 μM $A\beta_{1-42}$ alone exhibited 71% (Figure 9C). The toxicity induced by 5 μM $A\beta_{1-42}$ in the presence of VPA was strongly reduced in a concentration-dependent manner and the protective effect of VPA was significantly observed at a concentration of 100 μM. VPU at the concentration higher than 100 μM of VPU was not tested due to its solubility limitation. However, VPU not VPA at a concentration of 50 μM significantly reduced the release of LDH into the medium (Figure 9C). The addition of VPU to cultures simultaneously to the addition of $A\beta_{1-42}$ promoted the survival of neurons, since both the release of LDH into the culture media was abrogated and cellular reduction of XTT was maintained in a concentration dependent manner when evaluated at 72 h post-peptide exposure. Figure 10 depicted the concentration response curve of VPA and VPU protection against neuronal death induced by 5 μM $A\beta_{1-42}$. VPA protected NLCs against the Aβ-induced neurotoxicity in a concentration dependent manner. Across assays, efficacy of VPU for promotion of neuronal survival following 72 h of exposure to $A\beta_{1-42}$ ranged from approximate 45-75% recovery as compared to vehicle controls and was achieved at a concentration of 100 μM. VPA at the higher concentration (500-1000 μM) must be used to achieve 40-75% recovery as compared to vehicle control. The results indicated that VPU was more potent than VPA in the prevention of the loss in metabolic function and membrane damage.

Phase-contrast microscopic examination revealed that a majority of cells when treated with 5 μM $A\beta_{1-42}$ were floating in the medium and even the attached cells appeared round and shrunken (Figure 11D). The light microscopic examination showed different in cell morphology and degree of cell attachment between $A\beta_{1-42}$ treatment and cotreatment . Even in attached cells in $A\beta_{1-42}$–treated cultures, sign of cell injury were evident. When the cotreatment with either VPA or VPU and $A\beta_{1-42}$ at the toxic concentration, the sign of cell injury was markedly decreased. Under a microscopic examination, most cells maintained normal shapes (Figures 11E and 11F). However, the toxic influence of $A\beta_{1-42}$ was not completely blocked by VPA and VPU.

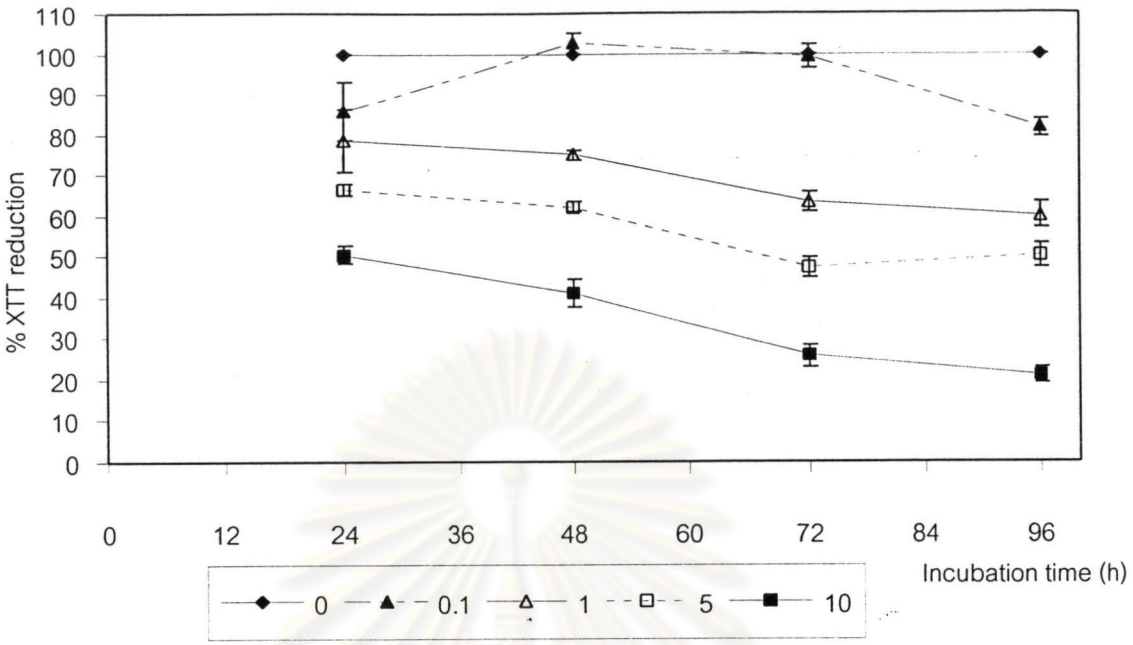


Figure 5. Changes in mitochondrial function, as measured by the XTT reduction assay, in 7-day-old cultured neurons treated with the various concentrations of $A\beta_{1-42}$. The XTT reduction assay was monitored at 24-h intervals during a 3-day exposure period. Neurons with impaired mitochondrial function were unable to reduce the XTT dye effectively relative to healthy cells. The $A\beta_{1-42}$ was preincubated for 72 h for "aging" before addition to the cell cultures. Mitochondrial function was evaluated 24-96 h after addition of the peptide. Data shown were the mean of three different experiments with each experimental value being the average of two trials. Error bars represented S.E.M values.

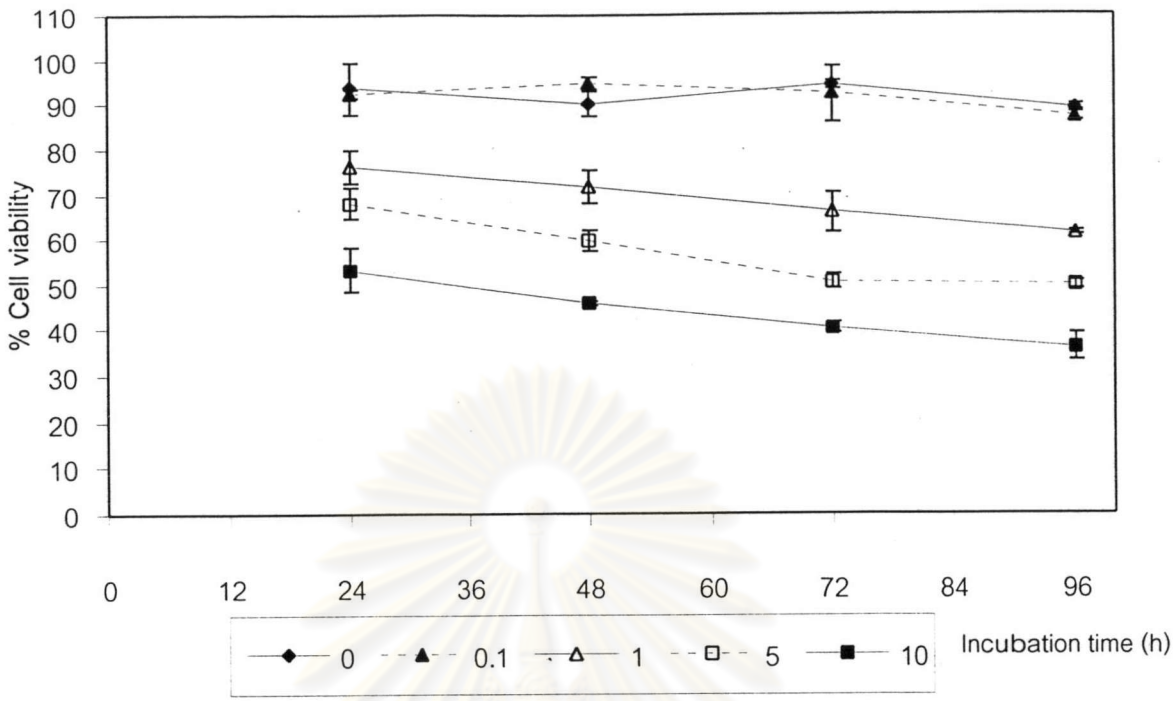


Figure 6. Neurotoxicity in 7-day-old neurons treated with the various concentrations of $A\beta_{1-42}$, as measured by the trypan blue exclusion assay. The neurotoxicity was monitored at 24-h intervals during a 3-day exposure period. Dead neurons internalize the dye. The $A\beta_{1-42}$ was preincubated for 72 h for "aging" before addition to the cell cultures. Neuronal toxicity was evaluated 24-96 h after addition of the peptide. Data shown were the mean of three different experiments with each experimental value being the average of two trials. Error bars represented S.E.M values.

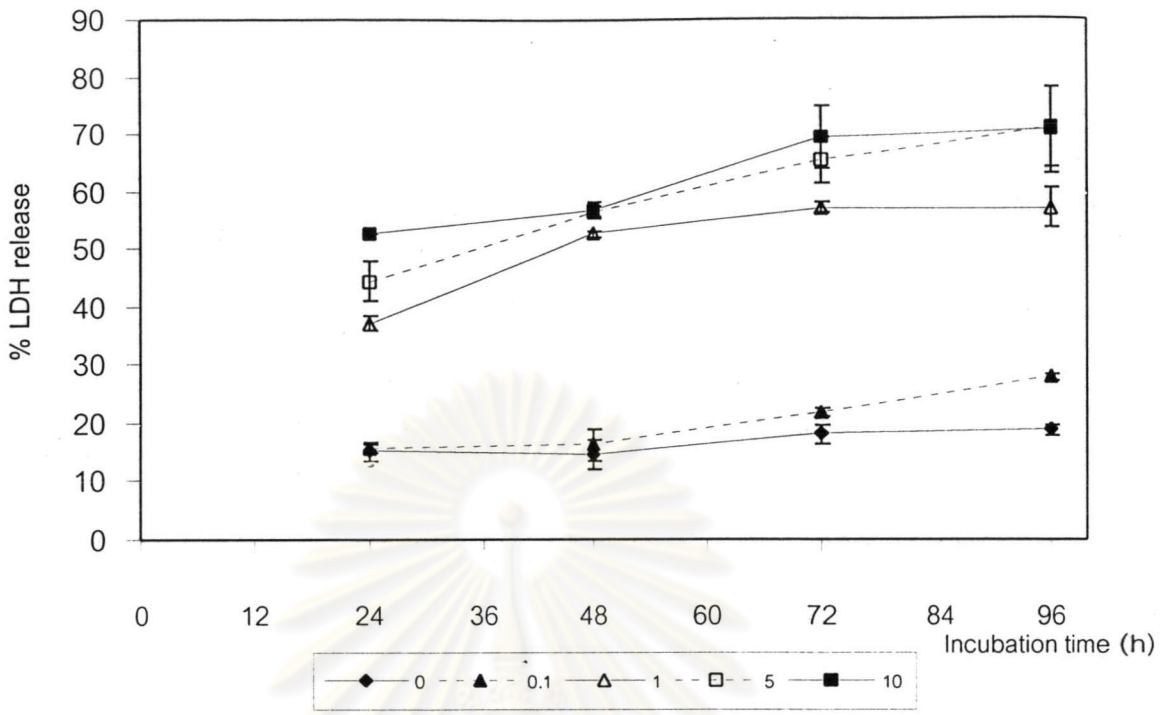


Figure 7. Neurotoxicity in 7-day-old neurons treated with the various concentrations of $A\beta_{1-42}$, as measured by LDH release into the supernatant. The neurotoxicity monitored at 24-h intervals during a 3-day exposure period. The $A\beta_{1-42}$ was preincubated for 72 h before addition to the cell cultures. Neuronal toxicity was evaluated 24-96 h after addition of the peptide. Data shown were the mean of three different experiments with each experimental value being the average of two trials. Error bars represented S.E.M. values.

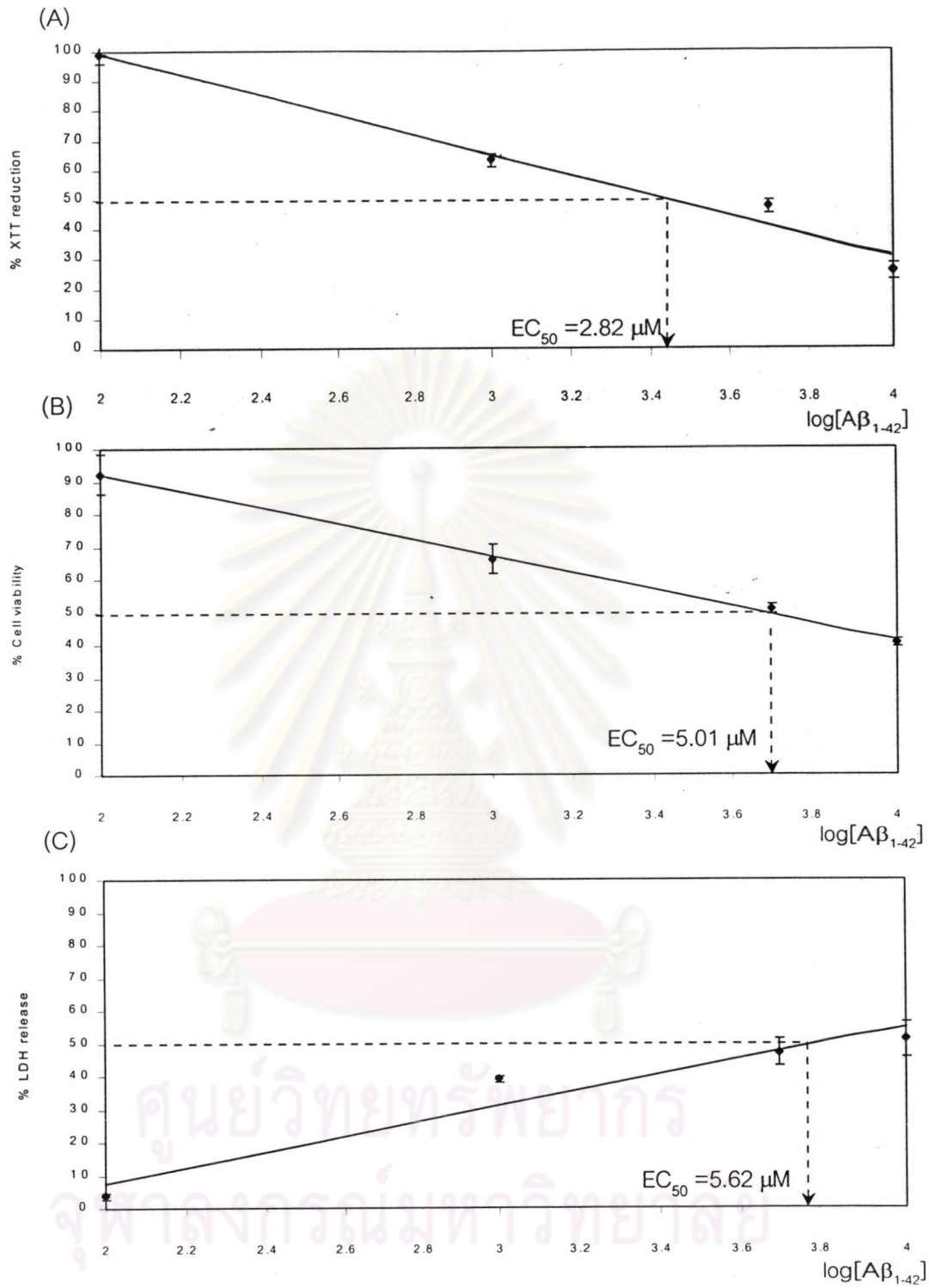
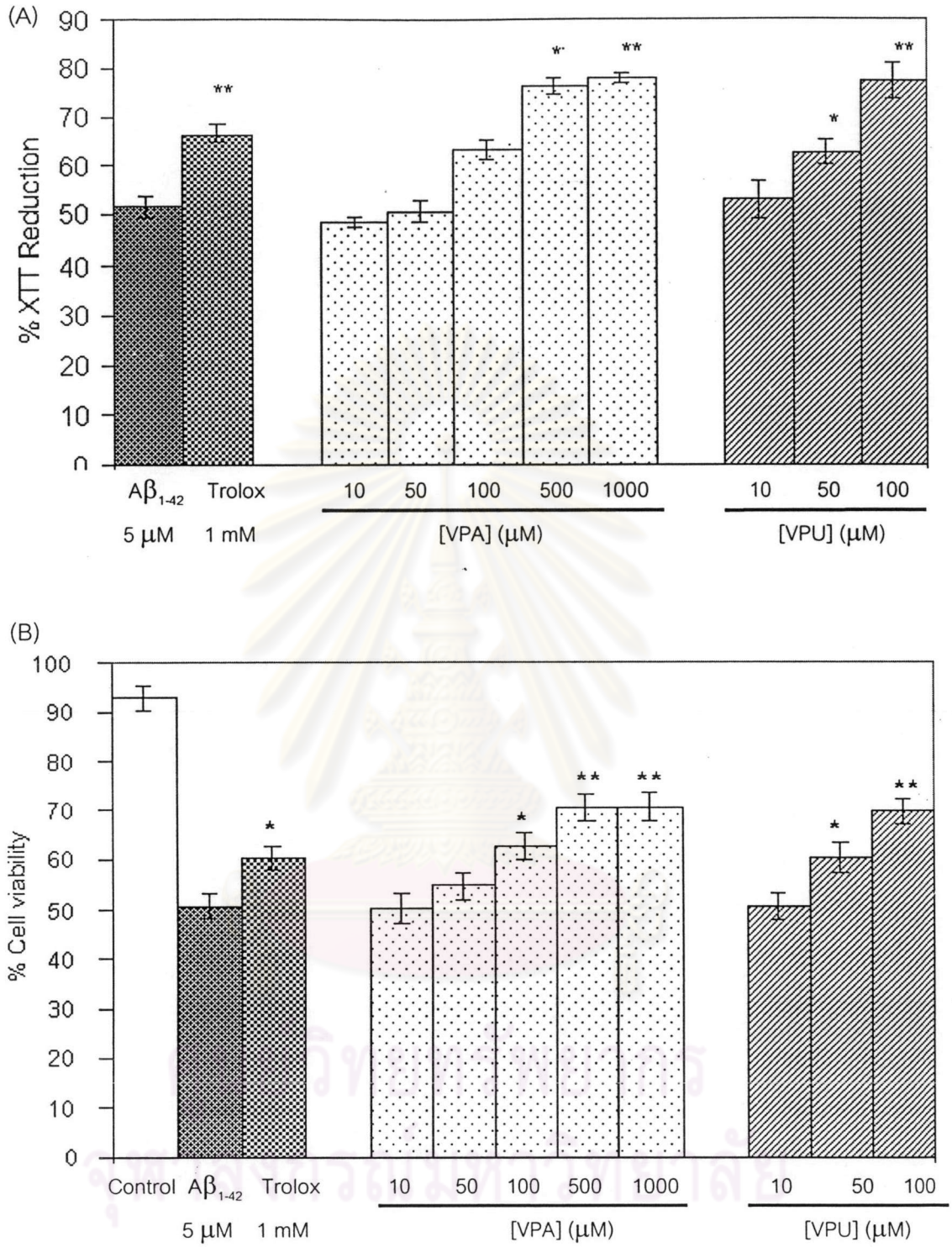


Figure 8. Concentration dependent $A\beta_{1-42}$-induced neuronal damage. Neuron-like cells were incubated in the presence of increasing concentration of $A\beta_{1-42}$ for 72 h, and neurotoxicity was measured by (A) cellular reduction of XTT (B) trypan blue exclusion, and (C) LDH activity release into the culture medium. Results were the mean ± S.E.M. from three independent experiments and expressed relative to the cells treated with vehicle alone.


(A)
% XTT Reduction
0
10
20
30
40
50
60
70
80
90
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*
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*
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Aβ1-42 Trolox
5 μM 1 mM
10 50 100 500 1000
[VPA] (μM)
10 50 100
[VPU] (μM)
(B)
% Cell viability
0
10
20
30
40
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Control Aβ1-42 Trolox
5 μM 1 mM
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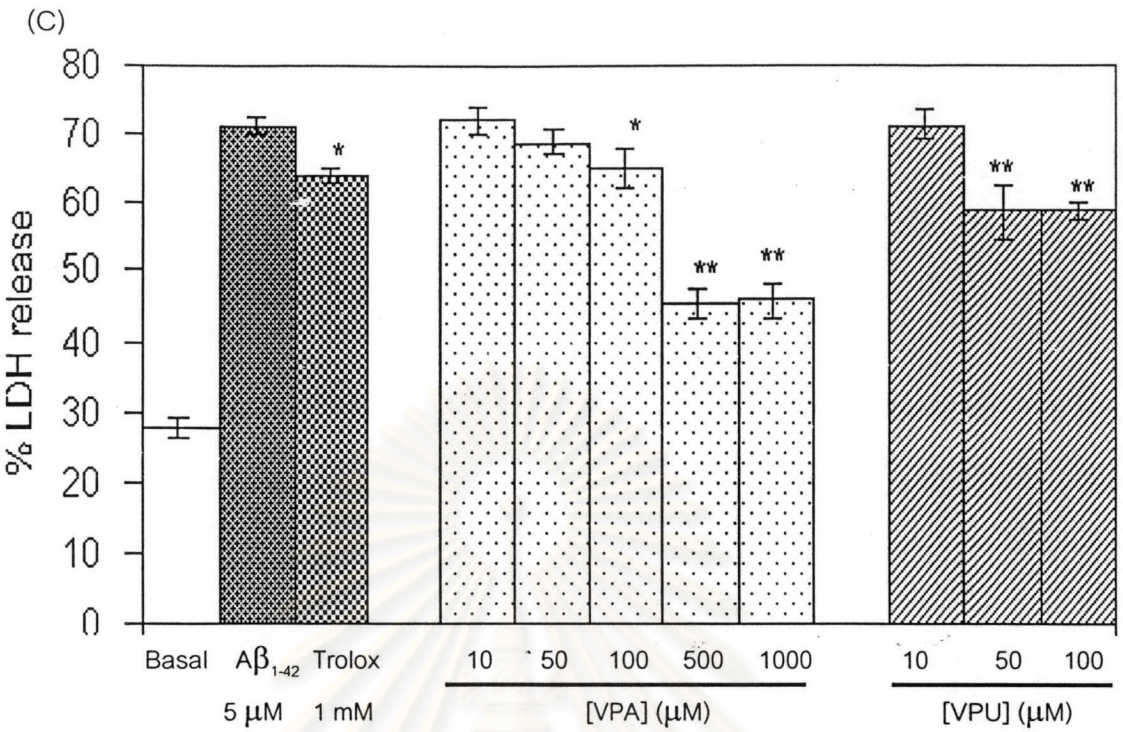


Figure 9. Evaluation of attenuation potential of VPU compared with VPA against $A\beta_{1-42}$-induced toxicity in cultured neuronal-like cells. At day 7, cells were incubated with 5 μM $A\beta_{1-42}$ in the absence or presence of increasing concentration of VPU or VPA at 72 h peptide exposure. (A) The effect of VPU and VPA on cellular reduction in XTT was determined and the data expressed as a percentage of the reduction of vehicle control cultures. (B) The effect of VPU and VPA on $A\beta_{1-42}$-induced cell damage assayed by trypan blue exclusion. (C) The effect of VPU and VPA on $A\beta_{1-42}$-induced LDH release. LDH release measurements were normalized to the total LDH release (assigned as 100%). Data represented the mean ± S.E.M. Statistical significance was determined by ANOVA (**p< 0.01; *p< 0.05) in the comparison to the Aβ treatment alone.

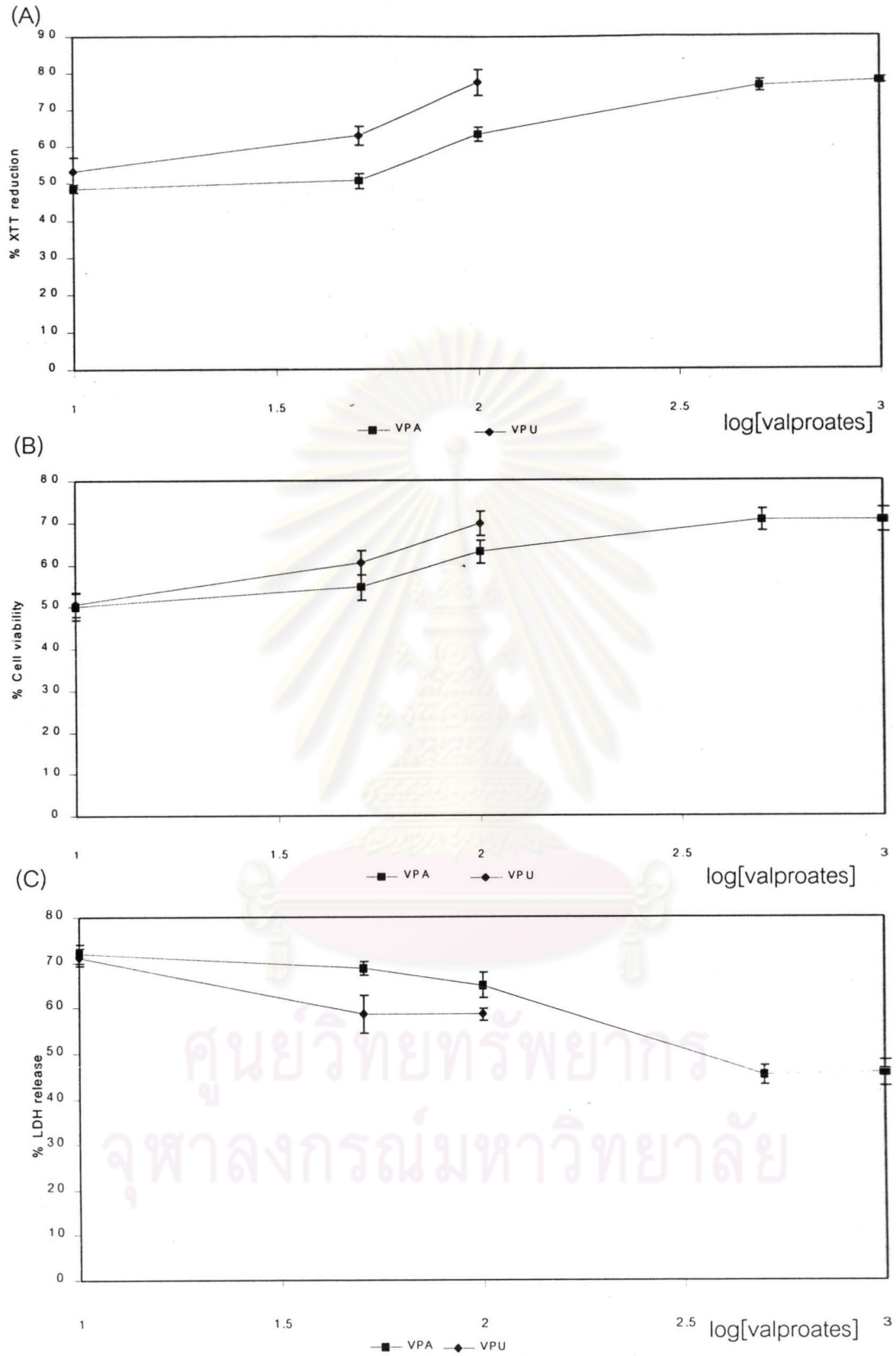


Figure 10. Concentration dependent manner of VPA and VPU on the protection of neurotoxicity-induced by 5 μM $A\beta_{1-42}$. The cell viability was determined by (A) XTT reduction assay (B) trypan blue exclusion assay (C) LDH release.

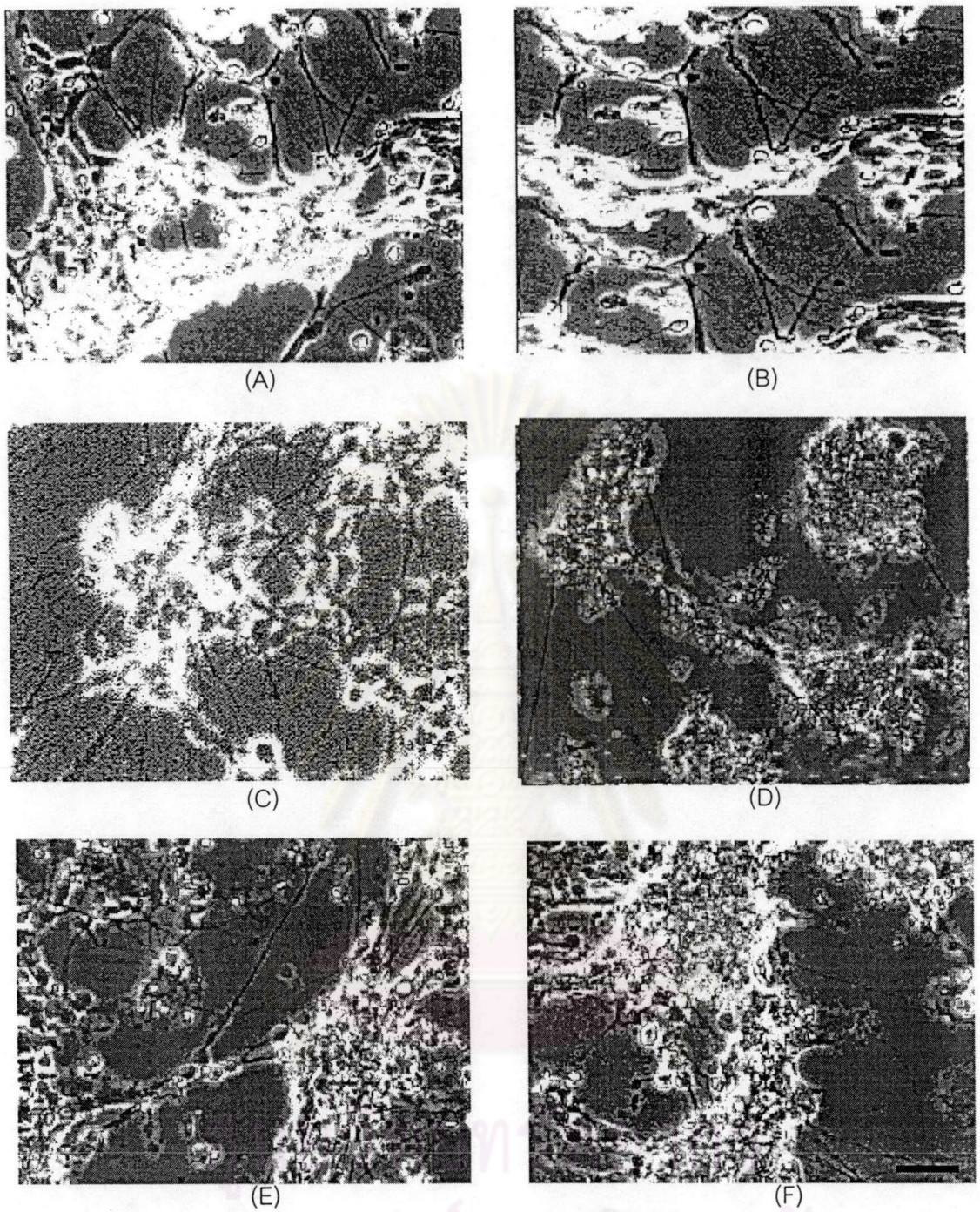


Figure 11. Photomicrographs showing $A\beta_{1-42}$-induced neurotoxicity and neuroprotective effects of VPA and VPU. Phase contrast micrographs of neurons exposed for 72 h with different treatments. (A) The control neurons incubated with vehicle only (0.01% DMSO). (B) The control cells + VPA (100 μM). (C) The control cells + VPU (100 μM). (D) The cells treated with $A\beta_{1-42}$ (5 μM) alone. (E) The cells treated with $A\beta_{1-42}$ (5 μM) in the presence of 100 μM VPA. (F) The cells treated with $A\beta_{1-42}$ (5 μM) in the presence of 100 μM VPU. Scale bar = 50 μm.

5.2 Pretreatment with VPA or VPU on $A\beta_{1-42}$–treated cells

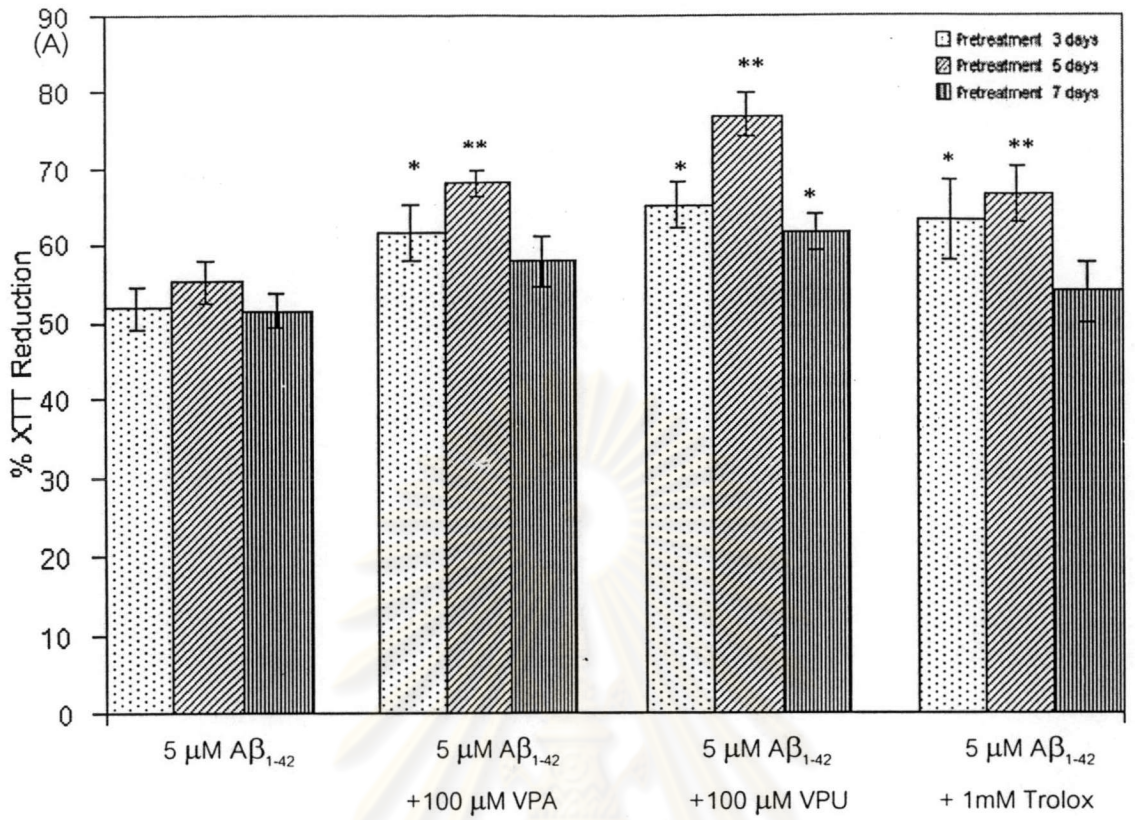
In order to investigate the neuroprotective potential of VPA and VPU, preincubation of the NLCs with each compound prior to $A\beta_{1-42}$ exposure were performed. The NLCs were pretreated with VPU or VPA at a concentration of 100 μM for 3, 5 and 7 days and were then exposed to aggregated $A\beta_{1-42}$ at a concentration of 5 μM for 72 h. Cell viability was excellent in cells exposed to either VPU, VPA alone or DMSO vehicle but greatly diminished in cells treated with $A\beta_{1-42}$. When the NLCs were exposed to $A\beta_{1-42}$, the cell viability was significantly decreased. Pretreatment of the cells with VPA or VPU for 3 days prior to $A\beta_{1-42}$ exposure significantly altered the degree of $A\beta_{1-42}$-induced loss of redox activity. In the presence of $A\beta_{1-42}$ alone, the XTT reduction decreased to 52% of the control, while the pretreatment of VPA or VPU for 3 days, the XTT reduction significantly increased to 62% or 65% of the control, respectively (Figure 12A). However, when measured by the trypan blue exclusion assay, both VPA and VPU did not affect the Aβ-induced cell death (Figure 12B). The $A\beta_{1-42}$-induced LDH release from NLCs (61%) was not changed after pretreatment with VPA (59%) or 1 mM Trolox (63%) (Figure 12C). Interestingly, only the pretreatment with VPU diminished the membrane damage-induced by Aβ, when measured the LDH release by 46%.

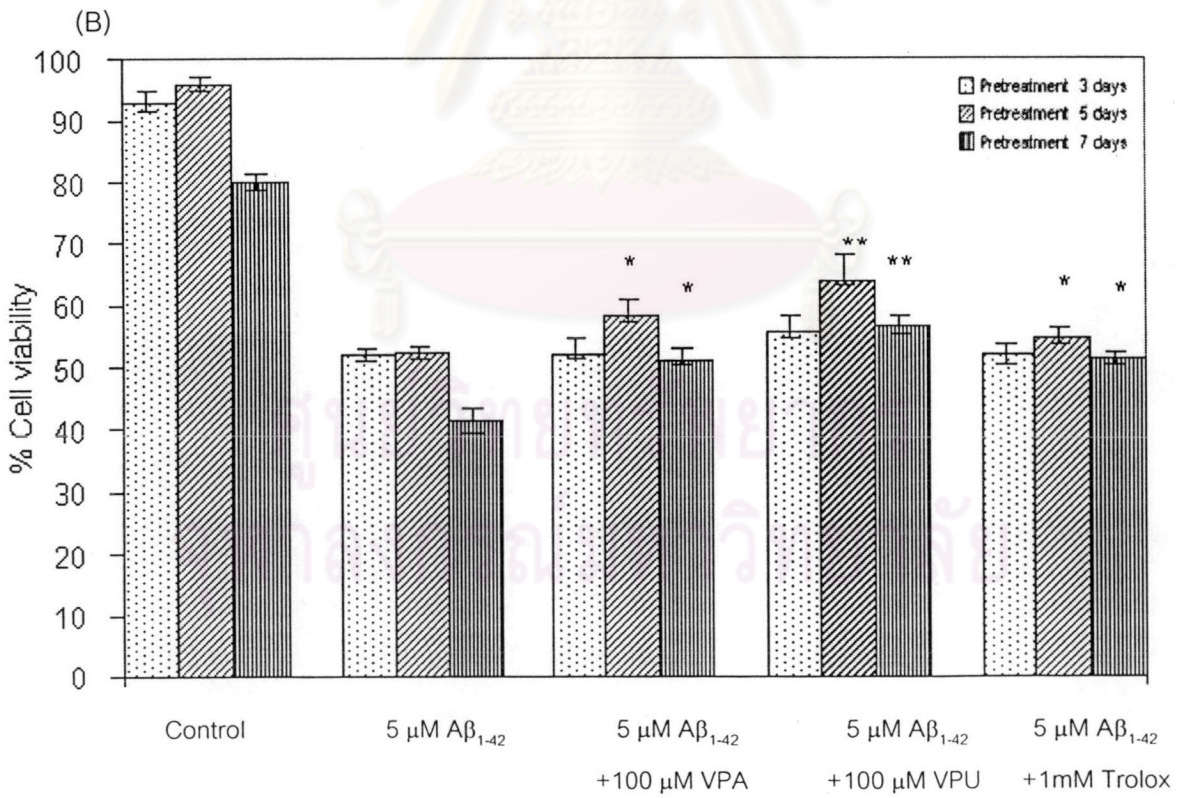
To determine the onset of VPA and VPU action, the extent of various incubation periods was examined. Pretreatment with the compounds for 5 days protected the cells from damage induced by $A\beta_{1-42}$ when compared with the control group. The XTT reduction for the treatment with $A\beta_{1-42}$ alone was 55% of the control, while 5 days-pretreatment with VPA or VPU were 68% or 77% of the control, respectively (Figure 12A). Similarly, the trypan blue exclusion assay detected the significant protection effect of both VPA (58%) and VPU (64%) on the Aβ-induced cell death (Figure 12B). Effect of both VPA and VPU pretreatment on the alteration of membrane integrity from $A\beta_{1-42}$ was observed. The LDH release induced by $A\beta_{1-42}$ was 67% while the pretreatment with VPA or VPU at the concentration of 100 μM, the LDH release was significantly reduced to 59% ($p<0.05$) or 46% ($p<0.01$) respectively.

The effect of pretreatment with VPA or VPU at a concentration of 100 μM for 7 days prior exposure to 5 μM $A\beta_{1-42}$ was determined. The XTT reduction of 7 days pretreatment with either VPA or VPU was 56% or 62% respectively. Trypan blue exclusion assay, cell viability was determined as 80% of control while pretreated with either VPA or VPU for 7 days were 51% or 57%, respectively. LDH release into the supernatant when treated with 5 μM of $A\beta_{1-42}$ alone was 73%. Pretreatment with VPA was not associated with a marked reduction in the LDH release in NLCs, however pretreatment with VPU could significantly minimize the release of LDH to 65% (Figure 12 C).

Preincubation of cells with VPA or VPU protected the loss of mitochondrial integrity, the significant protection was obvious in cultures obtained from 3-days and 5-days pretreatment. In contrast, 7-days pretreatment with VPA did not prevent the mitochondrial damage induced by $A\beta_{1-42}$. In addition, short-term pretreatment of cultured neurons with 100 μM VPA for 3 days before $A\beta_{1-42}$ application did not result in a significant increasing in cell viability examined by the assays of the trypan blue exclusion and the LDH release. On the other hand, when pretreatment with VPU for 3 days could protect cells from neurotoxic insults measured by assays of the XTT reduction and the LDH release. Under phase contrast microscopy, most cells were attached to the culture plate and no sign of abnormal morphology was detectable (Figures 13C and 13D).

Representative phase-contrast micrographs of neuronal cultures from differentiated P19cells under control condition and after $A\beta_{1-42}$ exposure without and with the long-term pretreatment with VPU were summarized in Figure 13. $A\beta_{1-42}$ triggered morphological signs of apoptosis including degeneration of neurites, shrinkage of cell bodies, and fragmentation into condensed particles, which were at least partly prevented by pretreatment with VPU.


(A)
% XTT Reduction
Pretreatment 3 days
Pretreatment 5 days
Pretreatment 7 days
5 μM Aβ1-42
5 μM Aβ1-42 +100 μM VPA
5 μM Aβ1-42 +100 μM VPU
5 μM Aβ1-42 + 1mM Trolox


(B)
% Cell viability
Pretreatment 3 days
Pretreatment 5 days
Pretreatment 7 days
Control
5 μM Aβ1-42
5 μM Aβ1-42 +100 μM VPA
5 μM Aβ1-42 +100 μM VPU
5 μM Aβ1-42 +1mM Trolox

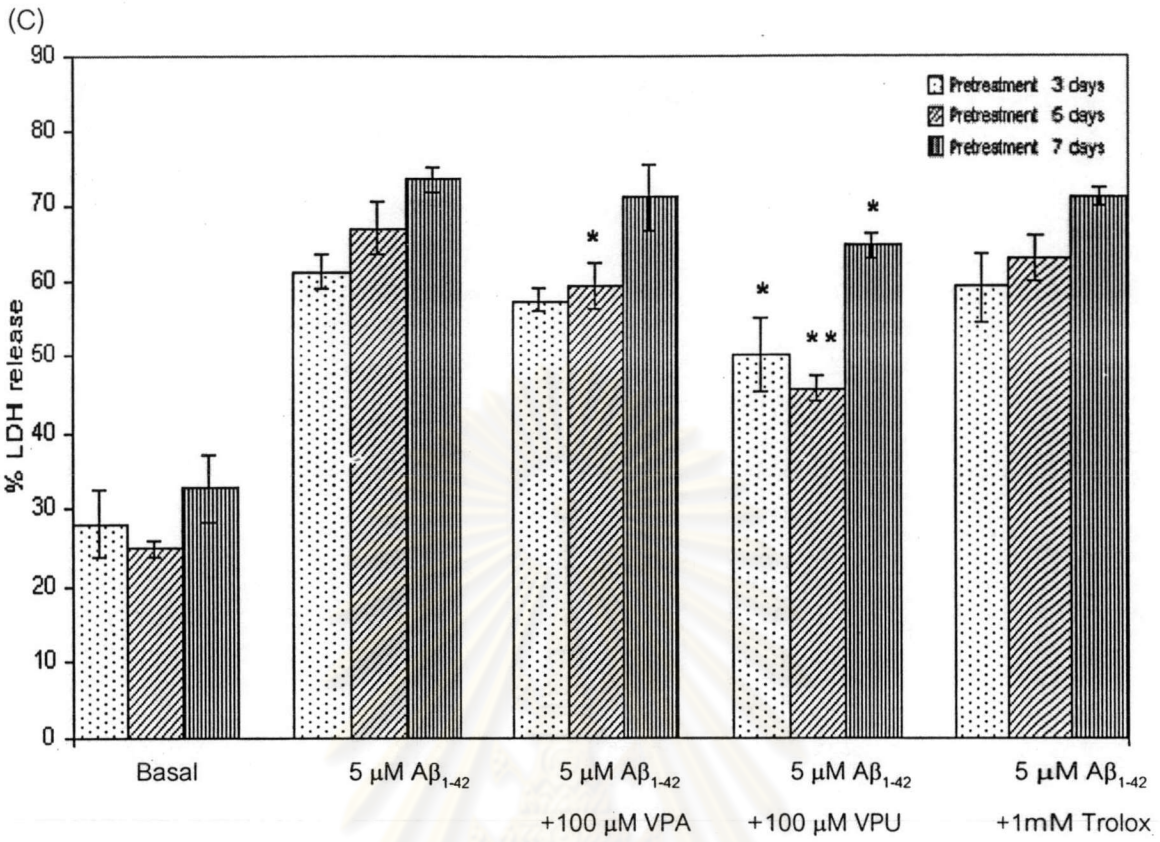
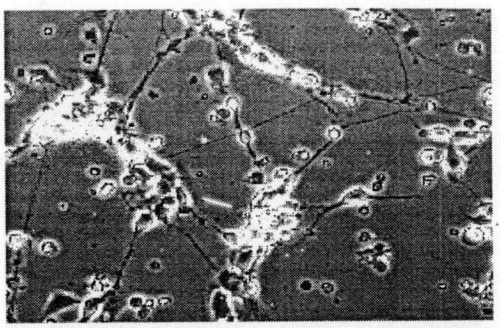
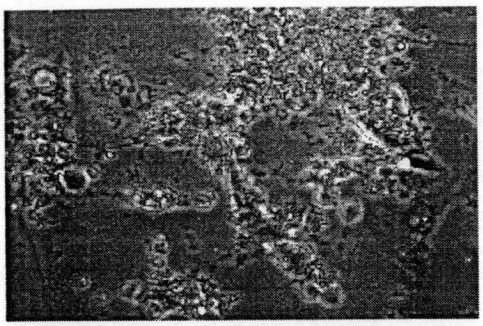


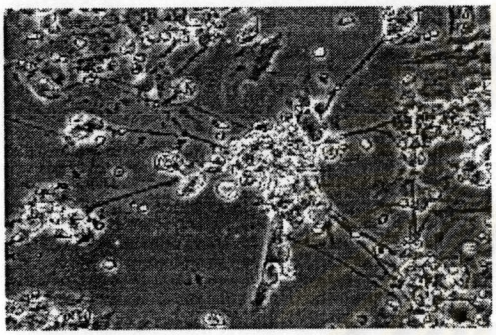
Figure 12. Neuroprotective effect of VPA compared with VPU against $A\beta_{1-42}$-induced toxicity in cultured neuron-like cells in the pretreatment experiments. At 7 days NLCs were pretreated for 3, 5, and 7 days with VPU or VPA at a concentration of 100 μM. Cultures were then exposed $A\beta_{1-42}$ at a concentration of 5 μM for 72 h, and neuronal survival was quantified. (A) The effect of VPU and VPA on cellular reduction in XTT was determined and the data expressed as a percentage of the reduction of vehicle control cultures. (B) The effect of VPU and VPA on $A\beta_{1-42}$-induced cell damage assayed by trypan blue exclusion. (C) The effect of VPU and VPA on $A\beta_{1-42}$-induced LDH release. The measurements of LDH release were normalized to the total LDH release (assigned as 100%). Data represented the mean ± S.E.M from at least triplicate experiments. Statistical significance was determined by ANOVA (**p< 0.01; *p< 0.05) in the comparison to the Aβ treatment alone.



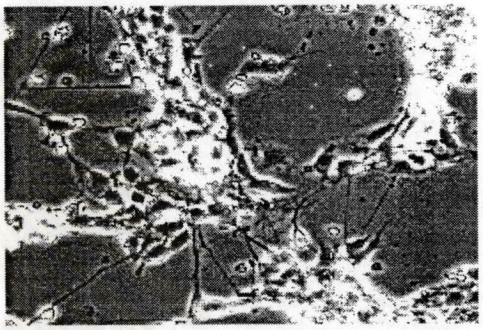
(A)



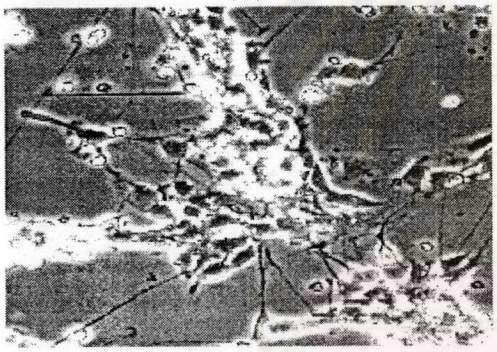
(B)



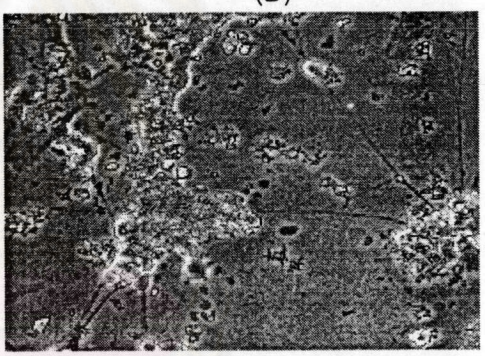
(C)



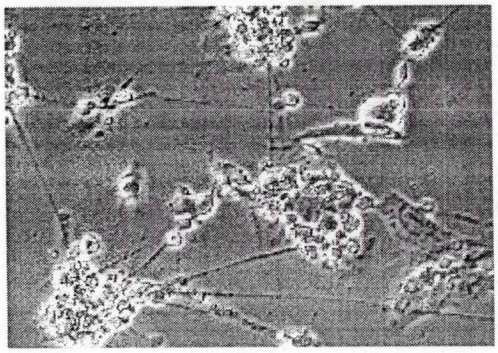
(D)



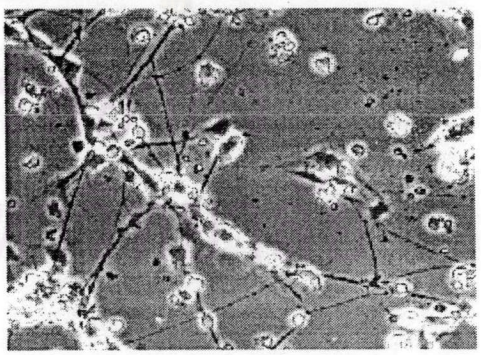
(E)



(F)



(G)



(H)

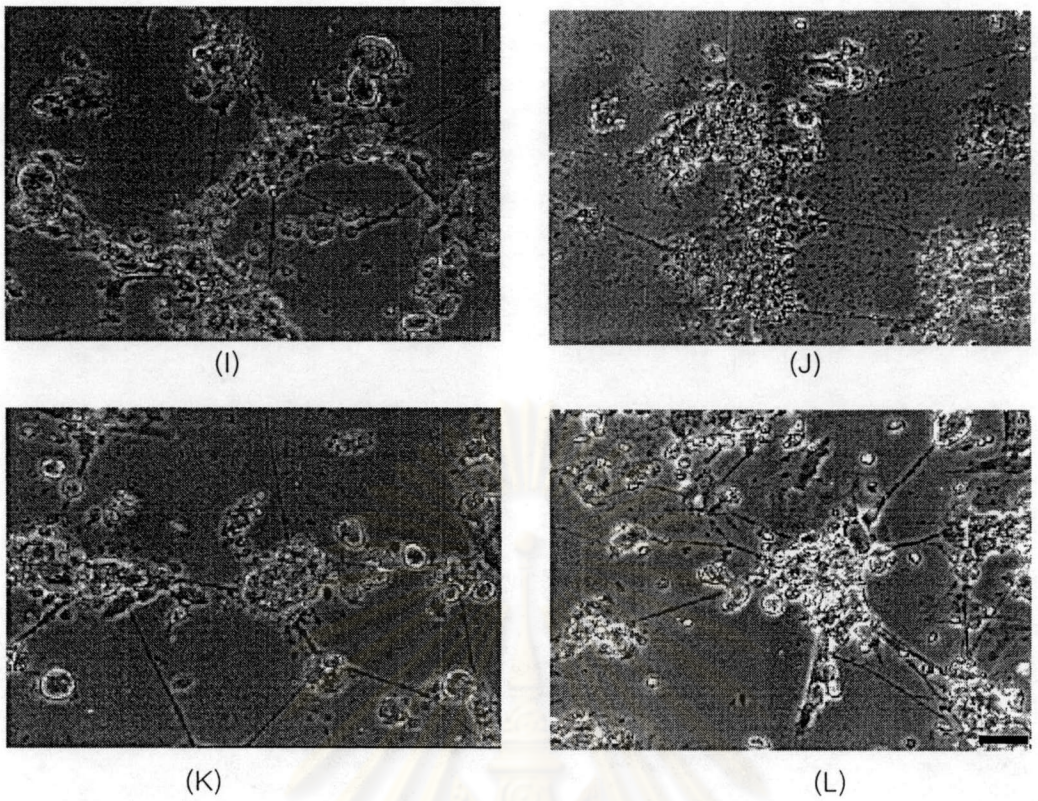


Figure 13. Phase-contrast microscopy showing effect of VPA and VPU on survival promotion in neuron-like cell cultures. NLCs were pretreated either VPA or VPU at a concentration of 100 μM for 3, 5 and 7 days, and then incubated with $A\beta_{1-42}$ at a concentration of 5 μM. The photographs were taken 72 h after application of $A\beta_{1-42}$. Scale bar = 50 μm.

A–D The effect of short –term (3 days) pretreatment with VPU or VPA. (A) Vehicle-treated control cultures; (B) $A\beta_{1-42}$ -treated cultures; (C) $A\beta_{1-42}$-treated cultures after pretreatment with VPA; (D) $A\beta_{1-42}$ -treated cultures after pretreatment with VPU.

E–H The effect of 5 days pretreatment with VPU or VPA. (E) Vehicle-treated control cultures; (F) cultures exposed to $A\beta_{1-42}$ (G) $A\beta_{1-42}$ -treated cultures after pretreatment with VPA; (H) $A\beta_{1-42}$ -treated cultures after pretreatment with VPU.

I-L The effect of 7 days pretreatment with VPU or VPA. (I) Vehicle-treated control cultures; (J) cultures exposed to $A\beta_{1-42}$ (K), $A\beta_{1-42}$ -treated cultures after pretreatment with VPA; (L) $A\beta_{1-42}$-treated cultures after pretreatment with VPU.

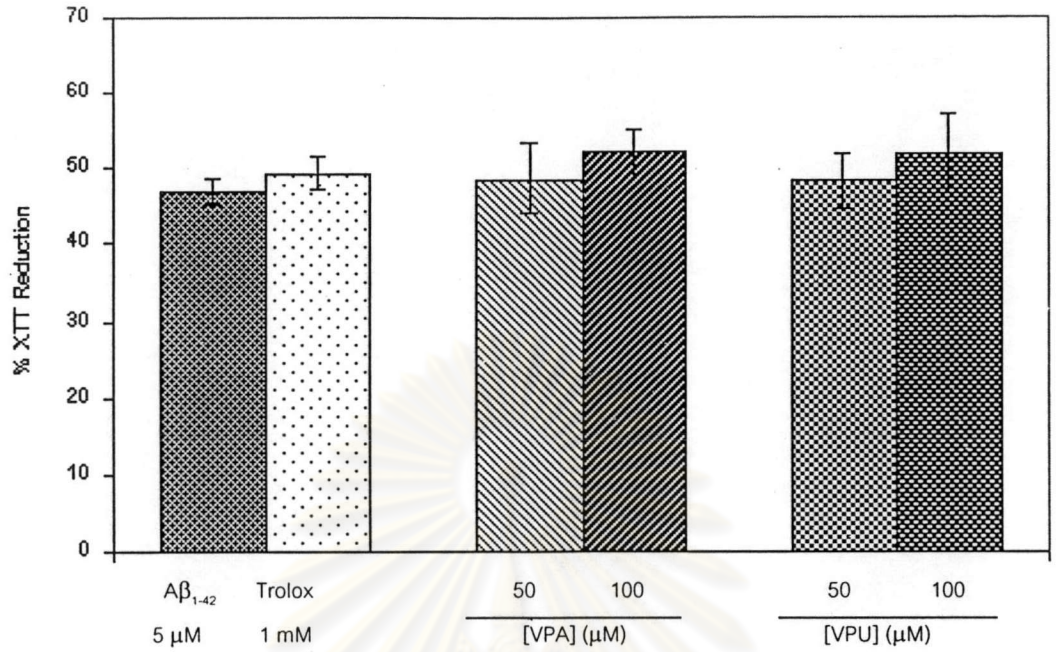
5.3 Post-treatment with VPA or VPU on $A\beta_{1-42}$-treated cells

To define the neurorescuing effect, cell viability indicators were measured after the addition of valproates to Aβ–pretreated cultures. While the replacement of medium was unlikely to deplete any $A\beta_{1-42}$ associated with cell membranes, the degree of reduction in total $A\beta_{1-42}$ within the culture achieved by replacement of medium was sufficient to allow the experimental. As the analysis, the addition of valproates to neuronal cultures was delayed for 72 h post-peptide addition because measurable toxicity of $A\beta_{1-42}$ could be achieved within 3 days (Figures 5-7). The results showed that both VPU and VPA at the concentrations of 50 and 100 μM were not able to significantly rescue neurons against toxicity induced by 5 μM $A\beta_{1-42}$ even if added up to 3 days post-Aβ treatments (Figure 14).

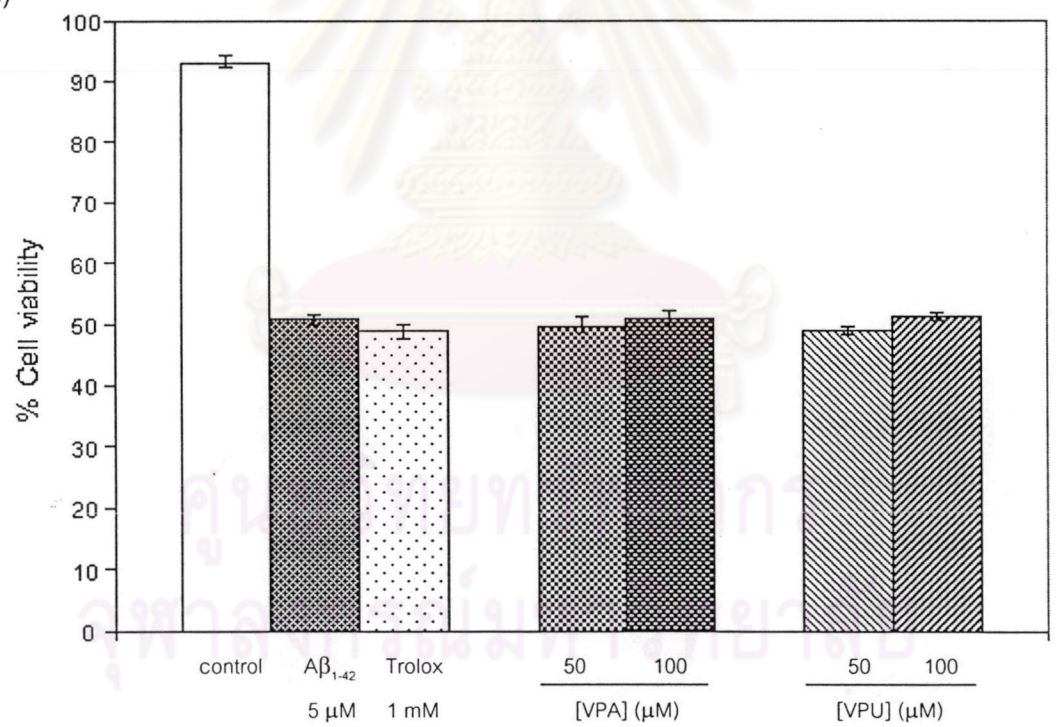
The morphology of NLCs after exposure to $A\beta_{1-42}$ for 72 h was examined under a phase-contrast microscope. Cells had round, shrunken morphology and a number of cells were floating in the culture medium, indicating that NLCs were degenerating. In post-treatment case, the addition of VPA or VPU to the culture at this time point did not change cell morphology (Figure 15).

In summary, protection against $A\beta_{1-42}$ toxicity was not observed following post-treatment of cultures with either VPA or VPU at the concentration of 100 μM.

(A)


% XTT Reduction
70
60
50
40
30
20
10
0
Aβ1-42
5 μM
Trolox
1 mM
50
100
[VPA] (μM)
50
100
[VPU] (μM)

(B)


% Cell viability
100
90
80
70
60
50
40
30
20
10
0
control
Aβ1-42
5 μM
Trolox
1 mM
50
100
[VPA] (μM)
50
100
[VPU] (μM)

(C)

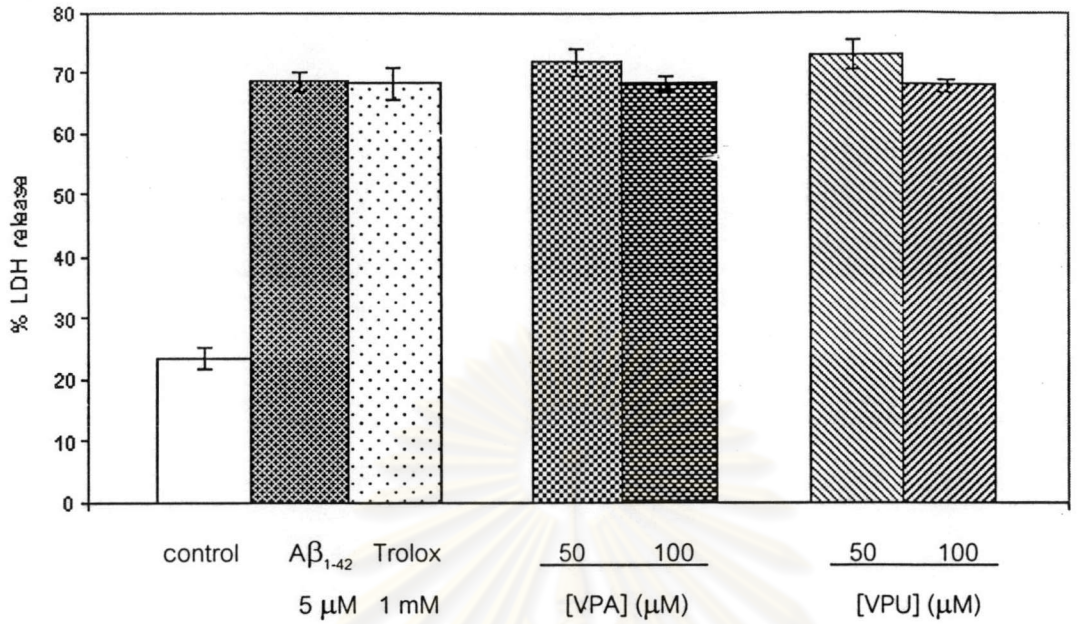


Figure 14. The post-treatment effect of VPA compared with VPU against $A\beta_{1-42}$-induced toxicity in cultured neuron-like cells. At the 7 days of NLCs were pretreated with 5 μM $A\beta_{1-42}$ for 72 h before the addition of VPU or VPA at the concentration of 50 and 100 μM or the corresponding vehicle and was then measured 72 h after the addition the compounds. (A) The effect of VPU and VPA on cellular reduction in XTT was determined and the data expressed as a percentage of the reduction of vehicle control cultures. (B) The effect of VPU and VPA on $A\beta_{1-42}$-induced cell damage assayed by trypan blue exclusion. (C) The effect of VPU and VPA on $A\beta_{1-42}$-induced LDH release. The measurements of LDH release were normalized to the total LDH release (assigned as 100%). Data represented the mean ± S.E.M. Statiscal significance was determined by ANOVA (**$p< 0.01$; *$p< 0.05$) in the comparison to the Aβ treatment alone.

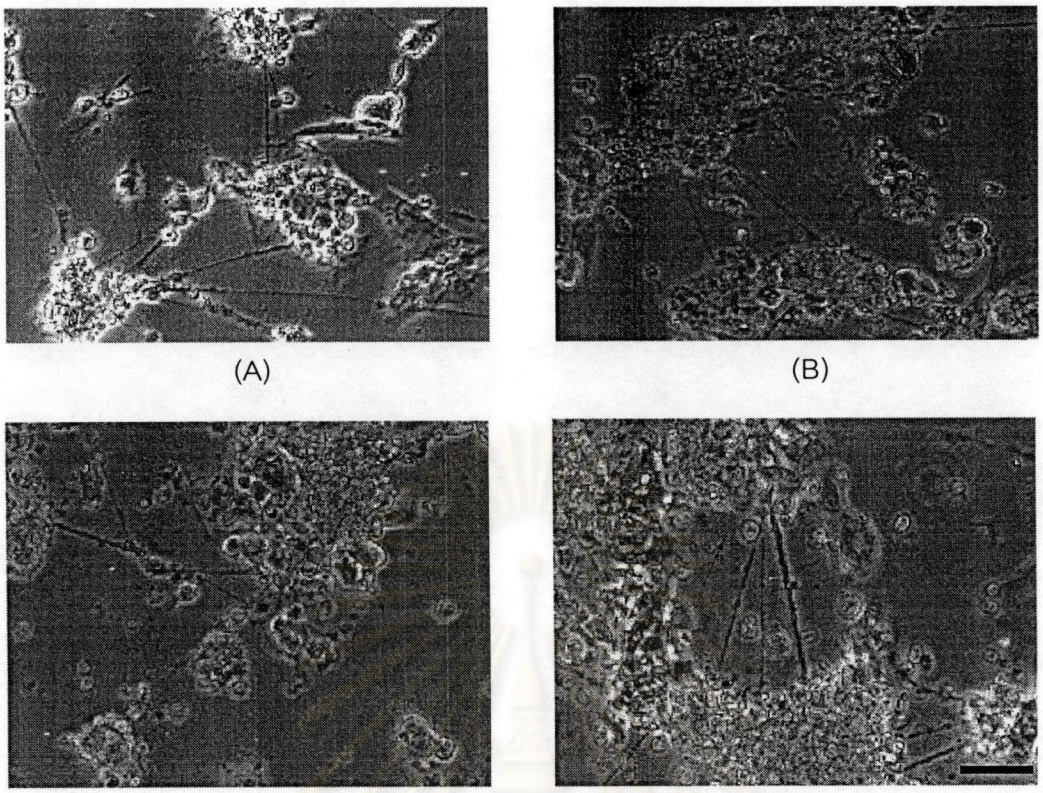


Figure 15. Photomicrographs showing the post-treatment effect of VPA or VPU on $A\beta_{1-42}$ -induced cytotoxicity. Phase contrast micrographs of neurons exposed for 72 h with different treatments. (A) The control neurons incubated with vehicle only (0.01% DMSO) (B) The cells treated with $A\beta_{1-42}$ (5 μM) alone. (C) The cells treated with $A\beta_{1-42}$ (5 μM) for 72 h and then treated with 100 μM VPA. (D) The cells treated with $A\beta_{1-42}$ (5 μM) for 72 h and then treated with 100 μM VPU. Scale bar = 50 μm.