Final Report

Project Title Effect of Asiatic acid on a reduction of hippocampal cell proliferation and survival and cognitive deficits caused by 5 fluorouracil chemotherapy in rats

By Assistant Professor Dr Jariya Umka Welbat

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Abstract

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Project Period :

Abstract:

Valproic acid (VPA) is commonly prescribed as an anticonvulsant and mood stabilizer used in the treatment of epilepsy and bipolar disorder. A recent study has demonstrated that VPA reduces histone deacetylase (HDAC) activity. This action is believed to contribute to the effects of VPA on neural stem cell proliferation and differentiation which may explain the cognitive impairments produced in rodents and patients. The hippocampus has a crucial role in cognition which is associated with cell proliferation in the SGZ of the hippocampal dentate gyrus. Asiatic acid is a triterpenoid derivative from medicinal plant, Centella asiatica. Asiatic acid has demonstrated biological effects such as antioxidant, anti-inflammatory and neuroprotective properties both in vitro and in vivo. Previous work in rodents has shown that asiatic acid stimulates learning and memory. An analytical study has shown that asiatic acid increases cell proliferation in the SGZ of the dentate gyrus (unpublished data). Furthermore, asiatic acid has been shown to improve memory and reduce cell death in primary cultured cortical neuronal cells. Asiatic acid has shown the ability to have neuroprotective effect and induce learning and memory. It also stimulates cell proliferation the SGZ of the hippocampal dentate gyrus which is associated to cognition. Therefore, the aim of this study is to explore the effect of asiatic acid on cognitive deficit and reduction of cell proliferation caused by VPA. Male Sprague Dawley rats were orally administered asiatic acid (30 mg/kg/day) for 28 days, while VPA-treated rats received VPA injections (300 mg/kg) twice a day from Day 15 to Day 28 for 14 days. Spatial working memory was determined using the novel object location (NOL) test. Hippocampal cell proliferation and survival were quantified by immunostaining for Ki-67 and Bromodeoxyuridine (BrdU), respectively. The results demonstrated that VPA-treated animals were unable to discriminate between objects in familiar and novel locations. Moreover, VPA significantly reduced numbers of Ki-67 and BrdU positive cells. These results indicate that VPA treatment caused

impairments of spatial working memory, cell proliferation and survival in the SGZ of the hippocampal DG. However, these abnormalities were restored to control levels by co-treatment with asiatic acid.

Keywords : 3-5 words

Asiatic acid, valproic acid, spatial memory, neurogenesis

Final report content:

1. Abstract

Valproic acid (VPA) is commonly prescribed as an anticonvulsant and mood stabilizer used in the treatment of epilepsy and bipolar disorder. A recent study has demonstrated that VPA reduces histone deacetylase (HDAC) activity. This action is believed to contribute to the effects of VPA on neural stem cell proliferation and differentiation which may explain the cognitive impairments produced in rodents and patients. The hippocampus has a crucial role in cognition which is associated with cell proliferation in the SGZ of the hippocampal dentate gyrus. Asiatic acid is a triterpenoid derivative from medicinal plant, Centella asiatica. Asiatic acid has demonstrated biological effects such as antioxidant, anti-inflammatory and neuroprotective properties both in vitro and in vivo. Previous work in rodents has shown that asiatic acid stimulates learning and memory. An analytical study has shown that asiatic acid increases cell proliferation in the SGZ of the dentate gyrus (unpublished data). Furthermore, asiatic acid has been shown to improve memory and reduce cell death in primary cultured cortical neuronal cells. Asiatic acid has shown the ability to have neuroprotective effect and induce learning and memory. It also stimulates cell proliferation the SGZ of the hippocampal dentate gyrus which is associated to cognition. Therefore, the aim of this study is to explore the effect of asiatic acid on cognitive deficit and reduction of cell proliferation caused by VPA. Male Sprague Dawley rats were orally administered asiatic acid (30 mg/kg/day) for 28 days, while VPA-treated rats received VPA injections (300 mg/kg) twice a day from Day 15 to Day 28 for 14 days. Spatial working memory was determined using the novel object location (NOL) test. Hippocampal cell proliferation and survival were quantified by immunostaining for Ki-67 and Bromodeoxyuridine (BrdU), respectively. The results demonstrated that VPA-treated animals were unable to discriminate between objects in familiar and novel locations. Moreover, VPA significantly reduced numbers of Ki-67 and BrdU positive cells. These results indicate that VPA treatment caused impairments of spatial working memory, cell proliferation and survival in the SGZ of the hippocampal DG. However, these abnormalities were restored to control levels by co-treatment with asiatic acid. These data demonstrate that asiatic acid may be a potent cognitive enhancer which improves hippocampal-dependent spatial memory, likely by increasing hippocampal neurogenesis. Moreover, asiatic acid could prevent the spatial working memory and neurogenesis impairments caused by VPA.

2. Executive summary

Asiatic acid may be a potent cognitive enhancer which improves hippocampal-dependent spatial memory, likely by increasing hippocampal neurogenesis. Moreover, asiatic acid could prevent the spatial working memory and neurogenesis impairments caused by VPA.

3. Objective

To examine protective effect of asiatic acid on a reduction of cell proliferation and survival in the rat hippocampus and cognitive deficit induced by VPA.

4. Research methodology

Animals

Male Wistar rats (the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom) weighing 180-220 g were housed in cages of 3 under standard conditions (12 h light/dark cycle) and food and water were provided ad libitum. The animal study was approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethics of the Animal Experiment of National Research Council of Thailand.

Drug treatment protocols

Rats were weighed daily from arrival and allowed to habituate for 1 week prior to drug administration. To investigate the preventive effects of asiatic acid on reduction of spatial working memory and hippocampal cell proliferation caused by valproic acid in the rats. After 7 days of habituation, the animals were randomly divided into 4 groups with 10 animals in each group (figure 1).

Group 1: Control animals were orally administered propylene glycol via gavage tube for 28 days and intraperitoneally injected with 0.9% normal saline twice a day (10.00 a.m. and 3.00 p.m.) on the day 15 to day 28 for 14 days.

Group 2: Asiatic acid-treated animals were received asiatic acid (30 mg/kg/day) via gavage tube in a volume of 1 ml/kg for 28 days.

Group 3: VPA-treated animals were intraperitoneally injected with VPA (300 mg/kg, dissolved in 0.9% normal saline at a volume 1 ml/kg), twice a day, on the day 15 to day 28 for 14 days.

Group 4: Asiatic acid plus VPA animal were orally administered asiatic acid via gavage tube for 28 days and intraperitoneally injected with VPA twice a day, on the day 15 to day 28 for 14 days.

All animal groups were be administered 3 BrdU (100 mg/kg at a volume of 4 ml/kg, Sigma Aldrich, UK) intraperitoneal (i.p.) injections, 24 hours apart starting 2 days prior to their first 5-FU/saline injection.

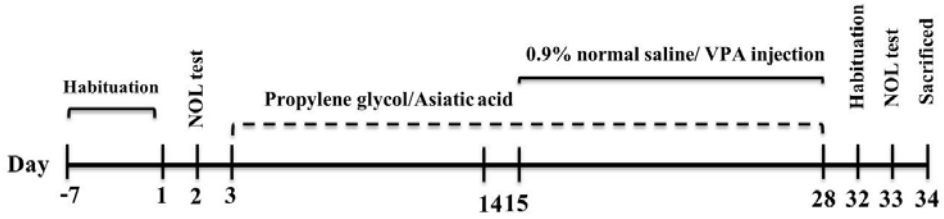

Figure 1 Shows timeline of animal drug administrations.

Behavioural testing

Novel object location (NOL) spatial memory task

Behavioural testing was carried out 3 days after terminal administration to have it washed out of the system. Rats were tested using the NOL test, adapted from Dix and Aggleton (Dix and Aggleton, 1999). This test were used and carried out as described previously by Umka et al. (2010) and Lyons et al. (2012). The experiments were conducted at an illumination of 100 lx between 09.00 and 16.00 hours. The test apparatus were consisted of an arena (open square opaque Perspex box, dimensions; 36-cm wide x 50-cm long x 30-cm high) and two identical weighted water bottles (19-cm high, 6.5-cm diameter). The procedure was described as follows.

1. Rats were habituated to an arena for 30 minutes, 24 hours prior to testing.

2. During habituation phase, the locomotor activity was measured by analyzing mean velocity using Foxit Reader 5.1 (Umka et al., 2010, Lyons et al., 2012).

3. The next day, rats were habituated again for 3 minutes, 5 minutes before the familiarization trial.

4. During the familiarization trial, rats were placed to an arena to explore two identical objects in two corners.

5. Rats were returned to their home cage for 15 minutes.

6. After that, were returned to the arena for 3 minutes choice trial to explore the two objects which one object were placed in the same location and the other one were moved to a new location.

7. The starting location of objects was randomized. Exploration was defined as the rat directing its nose in the direction of the object less than 1 cm from the object, and actively exploring it (Dix and Aggleton, 1999).

8. Time spent exploring both objects and trials were scored blind twice and averaged using stopwatch from digitized recording. During the trials, there was no observer in the room.

9. The preference index (PI) is defined as a percentage of exploration time spent on object in the novel location over the total exploration times of novel and familiar locations in the choice trial

as described by Lyons et al. (Lyons et al., 2011). A PI which is significantly different from chance shows that the animals are spending more time on the object in the novel location and are able to recall the previous object locations.

10. Total exploration time for both familiarization and choice trials combined were determined to analyze locomotor activity between groups.

Brain tissue analysis for immunohistochemistry staining

Brain tissue preparation

After behavioural analysis, animals were killed by rapid stunning and cervical dislocation and brains were removed. For both Ki67 and BrdU staining, brains were cryoprotected in 30% sucrose for 3 h at 4° C before embedding in OCT (Thermo Fisher Scientific, Germany). Then, they were snap-frozen in liquid nitrogen cooled isopentane and stored at -80°C prior sectioning and examination. Frozen brains were serially sectioned through the entire dentate gyrus at a thickness of 20 μm in the coronal plane using a cryostat. Sections were thaw mounted on APES coated slides and stored at -20°C until used for Ki67 and BrdU immunohistochemistry.

Ki67 staining

1. Selected slices were defrosted for 10 minutes.

2. A PAP marker pen (Vector Labs, UK), which is a hydrophobic barrier, were used to circle around each individual section.

3. After the PAP markings were dry, the sections were washed three times to remove the OCT compound.

4. The sections were fixed with 2.5% paraformaldehyde (PFA) for three minutes and washed three times with phosphate buffer saline (PBS).

5. The sections were then treated with the primary antibody monoclonal Mouse Ki67 for 60 minutes.

6. Following this, the sections were washed gently three times and then treated with the secondary antibody Alexa 488 Rabbit Anti-mouse IgG for 40 minutes.

7. Then, the sections were once again washed three times.

8. Before investigation, all the sections were counter stained with Propidium iodide, a nuclear stain, for 30 seconds.

9. After staining is complete, the sections were washed three times and mounted in glycerol and cover-slipped for observation by fluorescence microscopy.

BrdU staining

1. 20 µm frozen sections were defrosted for 20 minutes at room temperature (RT).

2. Sections were washed off OCT with 3 x PBS for 3 minutes and then fixed with PFA for 3 minutes at RT.

3. After that, sections were incubated with 2 M HCl and triton X-100 for 20 minutes.

4. Sections were incubated with 5 M HCl for 10 minutes, followed by 0.1 M borate buffer for 2 minutes.

5. Sections were incubated with primary antibody for 60 minutes, followed by washing with 3 x borate buffer.

6. Then, sections were incubated in secondary antibody for 60 minutes.

7. Sections were washed with 3 x borate buffer, followed by mounting with DAPI.

Microscopic quantification

A systemic random sampling technique (Mayhew and Burton, 1988) was used to select every 20th section from the length of dentate gyrus in a total of 9 sections per brain (Umka et al., 2010). Sections were viewed at X40 on Nikon ECLIPSE 80i fluorescence microscope. The Ki67 and BrdU positive cells were counted within the SGZ. The sum of cell counts per section for the whole dentate gyrus was multiplied 22 to produce estimates of proliferating cell number (Huang and Herbert, 2006) and statistical analysis were performed to compare the number of the cell counts. All counting were performed blind.

Statistical analysis

All statistical parameters were calculated using GraphPad Prism 5.0 software and significance was regarded as $P < 0.05$.

5. Result

Asiatic acid prevents the spatial working memory deficits caused by VPA

The NOL test was used to assess spatial working memory 3 days after the end of drug administration. This period was used to avoid confounding issues associated with the acute effects of the drugs. In the familiarization trial, animals in all groups showed no significant difference in exploratory time between the objects in the arena ($P < 0.05$, paired Student t-test, Fig. 1A) indicating no preference for either object in the two locations prior to the choice trial. Fifteen minutes later, one object was moved to a new location and animals were tested for their ability to discriminate between the object in the familiar location (FL) and the object moved to a novel location (NL). During the choice trial, as expected, animals in the vehicle and asiatic acid groups spent significantly more time

attending the object in the novel location (mean±SEM; FL, vehicle: 6.270 ± 1.172 sec., asiatic acid: 9.181 ± 2.284 sec.; NL, vehicle: 12.250 ± 2.262 sec., asiatic acid: 15.450 ± 1.381 sec., $P < 0.05$, paired Student t-test, Fig. 1B). This indicated that these animals had no impairments in spatial working memory. A one-way ANOVA analysis with LSD post hoc test revealed that animals receiving asiatic acid spent significantly more time on the object in the novel locations in comparison to each other group [$F(3,37) = 4.074$; $P = 0.014$]. In contrast, the VPA treated group was impaired in their ability to discriminate between objects in familiar and novel locations resulting in no significant difference in the time spent on these two objects (mean±SEM; FL: 9.494 ± 1.190 sec., NL: 8.712 ± 1.171 sec., $P > 0.05$). This result indicates that the VPA treatment has caused a deficit in spatial working memory. Animals administered both VPA and asiatic acid however spent significantly more time on the object in the novel location compared with that in the familiar location (mean±SEM; FL: 3.718 ± 0.857 sec., NL: 9.399 ± 1.694 sec., $P < 0.05$). This group behaved similarly to control and asiatic acid alone treated animals and did not show spatial memory deficits exhibited by the VPA group.

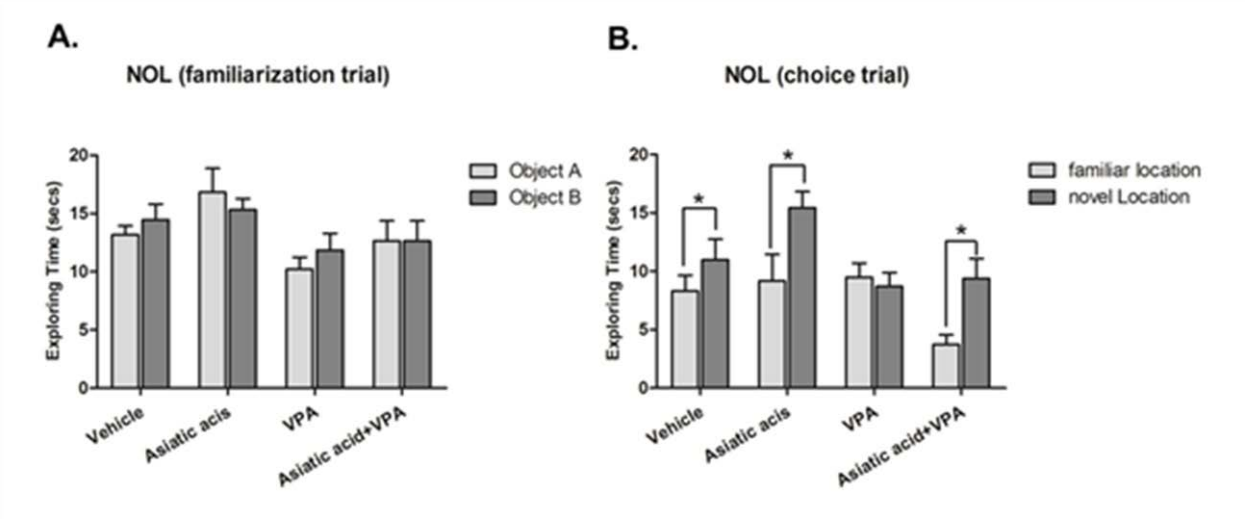

Figure 1 Mean exploration times of the animals exploring each object during the familiarization (A) and choice (B) trials of the novel object location test after treatment. There were no significant differences in the exploration times of either object for any group in the familiarization ($P > 0.05$). In the choice trial, vehicle, asiatic and VPA plus asiatic acid groups spent a significantly longer time exploring the object in the novel location compared with the familiar location ($P < 0.05$), whereas the VPA group failed to discriminate between objects ($P > 0.05$).

The exploration times of familiar and novel locations in the choice trial were converted into a preference index (PI). PI was calculated by expressing time spent exploring the object in the novel location as a percentage compared to 50% chance. The data showed that the animals in vehicle, asiatic acid and VPA plus asiatic acid treated groups were significantly different from 50% chance ($P < 0.05$; one-sample t test, Fig. 2A), demonstrating a normal ability in remembering the location of objects and expressing greater interest in objects in novel locations. In contrast, the PI of the VPA group was not different from 50%, indicating spatial working memory deficits. One-way ANOVA with LSD post-hoc test confirmed that the PI of vehicle, asiatic acid and VPA plus asiatic acid animals were significantly higher than the VPA-treated group [$F(3,29) = 8.851$; $P = 0.003$, Fig. 2A]. The total exploration times showed no significant difference among groups, indicating that animals did not have impaired locomotor ability during the performance of the task [$F(3,32) = 1.500$; $P > 0.05$, one-way ANOVA, LSD post-hoc test, Fig. 2B].

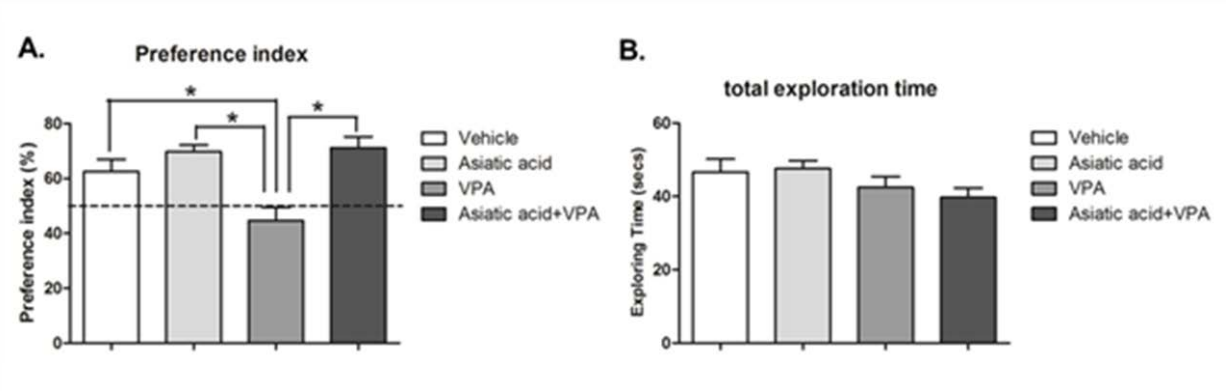

Figure 2 The preference index (PI) showed a significant difference from 50% chance in vehicle, asiatic acid and VPA plus asiatic acid groups (A; $P < 0.05$) while the VPA group showed no significant difference from chance. Additionally, PI in animals in vehicle, asiatic acid and VPA plus asiatic acid groups were significantly higher than VPA group ($P > 0.05$). The total exploration time of familiarization and choice trial combined was not significantly different among groups (B; $P > 0.05$)

Asiatic acid prevents the reduction in cell proliferation in the SGZ caused by VPA

Ki-67 immunostaining was used to quantify the numbers of proliferating cells in the SGZ of the hippocampal dentate gyrus at the end of the experiment. A one-way ANOVA analysis with a LSD post hoc test showed that animals treated with VPA alone had a significantly decreased number of Ki-67 positive cells ($P < 0.05$). Treatment with asiatic acid alone significantly increased the number of Ki-67 positive cells compared with controls [mean±SEM; vehicle: 2918 + 64.31 cells, asiatic acid: 3270 + 64.58 cells, VPA: 2513 + 100.60 cells and VPA plus asiatic acid treated groups: 3420 + 102.10 cells $F(3,20) = 22.65$, $P < 0.001$, Fig. 3]. Interestingly, animals treated with both VPA and asiatic acid

showed no significant difference in Ki-67 positive cell numbers from the vehicle treated control group ($P > 0.05$). This demonstrates that asiatic acid had prevented the reduction in cell proliferation in the SGZ caused by VPA on its own.

Asiatic acid prevents the reduction in cell survival in SGZ caused by VPA

At the end of the experiment, BrdU-positive cells were counted in the dentate gyrus and SGZ to quantify cell survival. There were significantly fewer BrdU-positive cells in animals treated with VPA when compared with vehicle, asiatic and VPA plus asiatic acid [mean±SEM; vehicle: 549.30 + 38.85 cells, asiatic acid: 625.30 + 34.40 cells, VPA: 270.70 + 100.24.04 cells and VPA plus asiatic acid treated groups: 582.70 + 78.23 cells $F(3,20) = 9.197$, $P < 0.001$, one-way ANOVA, LSD post-hoc test, Fig. 4]. These results indicate that VPA treatment reduced cell survival in the SGZ over the course of the experiment. In contrast, animals treated with both VPA and asiatic acid showed no significant difference in the BrdU-positive cell numbers from vehicle-treated animals ($P > 0.05$). These results suggeste that asiatic acid can protect against the decrease in the survival of proliferating cells found after VPA treatment.

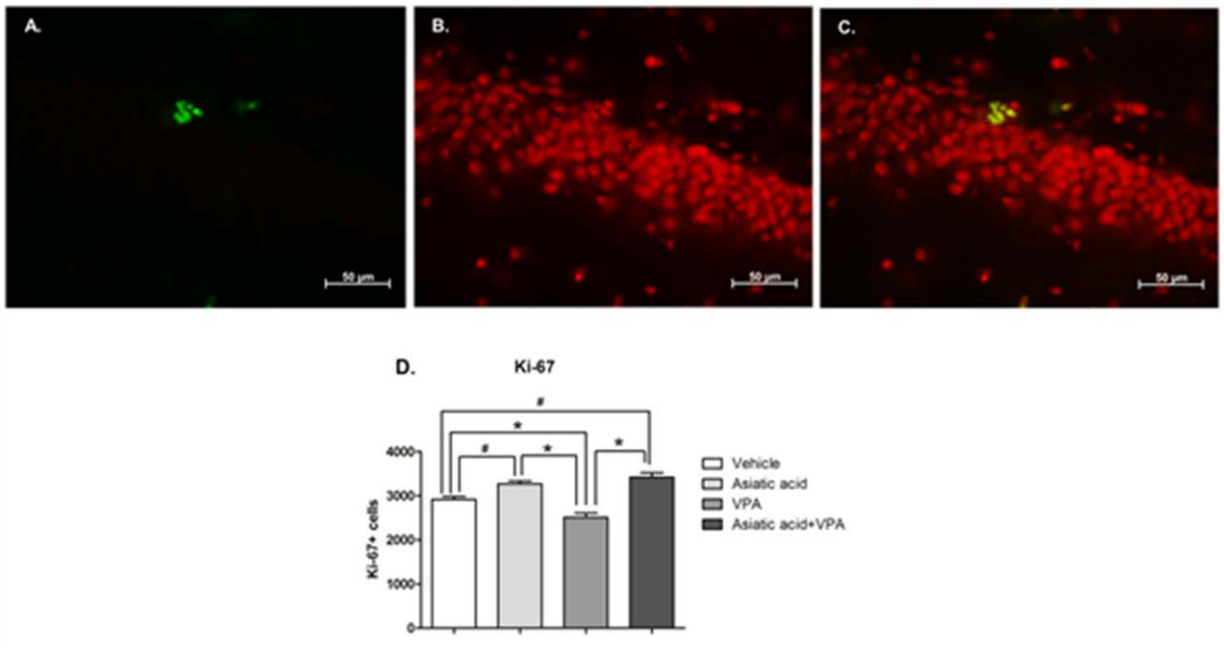

Figure 3 The number of Ki-67 positive cells in the SGZ of the hippocampal dentate gyrus. Ki-67 positive cells were stained in the SGZ of the dentate gyrus (green; A). All nuclei were counterstained with propidium iodide (red; B) and figure merged (C). The number of proliferating cells in animals receiving only VPA was significantly lower (* $P < 0.05$) than vehicle, asiatic acid and asiatic acid plus VPA groups. While, asiatic acid and asiatic acid plus VPA groups were significantly higher

when compared to the vehicle group (# P < 0.05). One-way ANOVA with LSD post hoc test was used to compare between all groups (D).

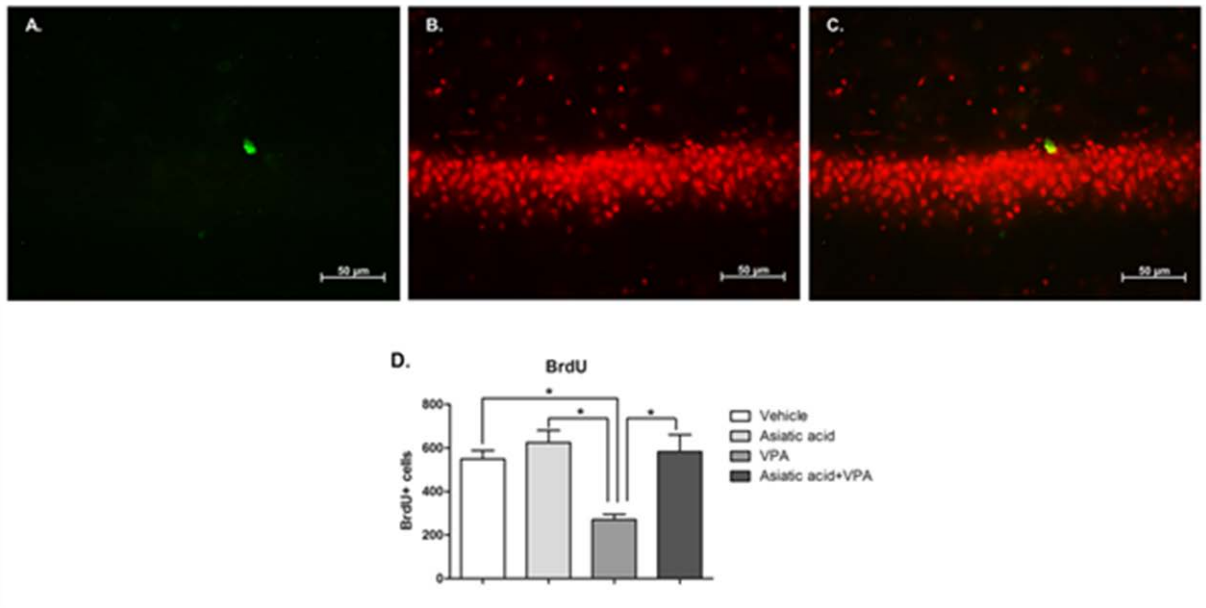

Figure 4 The number of BrdU positive cells in the SGZ of the hippocampal dentate gyrus. BrdU positive cells were stained in the SGZ of the dentate gyrus (green; A). Nuclei were counterstained with propidium iodide (red; B) and figure merged (C). BrdU positive cell number in VPA-treated group was significantly lower than vehicle, asiatic acid and asiatic acid plus VPA groups (* P < 0.05). One-way ANOVA with LSD post hoc test was used to compare between all groups (D).

6. Conclusion and Discussion

The goal of this study was to understand the impact of VPA and asiatic acid on spatial memory and hippocampal neurogenesis. Specifically, we wanted to determine whether co treatment with asiatic acid would be beneficial to VPA exposed animals which can suffer from reduced hippocampal neurogenesis and spatial memory. VPA is an anticonvulsant medication that has been used to effectively control various types of seizure disorders (Henry, 2003; Perucca, 2002). VPA exposure alone has previously been shown to produce cognitive impairments (Umka et al., 2010). In the present study, spatial memory was tested using the NOL test which is hippocampal dependent (Umka et al., 2010; Weible et al., 2009). It relies on the spontaneous preference animals have for objects in novel locations and does not require positive or negative reinforcements (Dix and Aggleton, 1999; Ennaceur et al., 2005). Animals with hippocampal abnormalities or damage have been shown to have a poorer performance on this test (Lyons et al., 2011; Mustafa et al., 2008; Umka et al., 2010). Our results showed that the animals receiving only asiatic acid were able to discriminate between objects placed in the familiar and novel locations significantly better than

vehicle treated controls. This confirms an earlier report that showed that asiatic acid treatment can improve spatial memory (Sirichoat et al., 2015). In contrast, VPA treated animals were impaired in their discrimination between objects in familiar and novel locations, indicating that VPA treatment caused a deficit in spatial working memory (Umka et al., 2010). Animals co-administered with both VPA and asiatic acid showed a significant preference for the object in the novel location compared with that in the familiar location. This result demonstrates that asiatic acid has a protective effect and prevents spatial memory impairments found in animals exposed to VPA.

The proliferation and survival of dividing cells can be quantified by immuno staining for Ki-67 and BrdU, respectively. Ki-67 is differentially and endogenously expressed in different phases of the cell cycle (Endl and Gerdes, 2000). Ki-67 is expressed in dividing cells at all stages of mitotis but is not found in G0 (Kee et al., 2002; Wojtowicz and Kee, 2006). BrdU is incorporated into newly formed DNA during S phase of the cell cycle and remains in the nucleus after the completion of cell division (Kuhn and Cooper-Kuhn, 2007). The results of the present study show that VPA significantly reduced the numbers of Ki-67 and BrdU positive cells showing that VPA treatment reduced cell proliferation and survival of dividing cells in the SGZ of the hippocampal dentate gyrus. Recent studies have demonstrated that the VPA inhibits the action HDAC enzymes resulting in expression of growth arrest genes including p21 and apoptosis (Clayton et al., 2006; Phiel et al., 2001). These effects have been demonstrated on embryonic neural stem cells where they produce brain malformations and abnormal cell migration into the dentate gyrus (Kuwagata et al., 2009). VPA exposure has been demonstrated to produce mild cognitive impairments in patients taking this drug for epilepsy or other psychiatric conditions. Moreover, patients experienced long term VPA therapy show slow thinking, cognitive difficulties and problems with motor behavior and display impairments of memory (Arif et al., 2009; Park and Kwon, 2008; Senturk et al., 2007b). VPA has been demonstrated, in the present study and previous publications, to produce a hippocampal specific spatial memory decline which is correlated with a decrease in hippocampal neurogenesis. This makes the present animal model suitable for use in testing substances for protection from VPA induced cognitive decline.

Asiatic acid is a triterpenoid derived from the medicinal plant *Centella asiatica* (*C. asiatica*) (Krishnamurthy et al., 2009; Zheng and Qin, 2007). It can cross the blood brain barrier and also has antioxidant activities which have shown it to be protective against neuronal damage in cell culture (Xu et al., 2012). Additionally previous studies have found that asiatic acid can improve learning and memory in an animal model via an increase of hippocampal neurogenesis (Nasir et al., 2011; Sirichoat et al., 2015). The present study demonstrated that co-administration of asiatic acid with VPA maintained levels of cell proliferation and the survival of dividing cells at control levels. Furthermore, animals treated only with asiatic acid had significantly higher levels of proliferation than

the control group, a result in agreement with our previous study (Sirichoat et al., 2015). These findings confirm the neuroprotective effect of asiatic acid previously reported in cortical cell culture where asiatic acid was able to rescue primary cortical neuronal cells from C2-caramides induced cell death and against beta-amyloid neurotoxicity when tested on B103 cell cultures and hippocampal slices (Lee et al., 2014; Mook-Jung et al., 1999; Zhang et al., 2012).

In summary, we have shown that administration of asiatic acid is beneficial in preventing the spatial working memory deficit and cell proliferation and survival stimulated reduction produced by VPA. Therefore, asiatic acid might be useful in preventing memory deficit in patients taking VPA.

7. Appendix

Arif, H.; Buchsbaum, R.; Weintraub, D.; Pierro, J.; Resor, S.R., Jr.; Hirsch, L.J. Patient-reported cognitive side effects of antiepileptic drugs: Predictors and comparison of all commonly used antiepileptic drugs. Epilepsy & behavior : E&B 2009, 14, 202-209.

Clayton, A.L.; Hazzalin, C.A.; Mahadevan, L.C. Enhanced histone acetylation and transcription: A dynamic perspective. Mol Cell 2006, 23, 289-296.

Dix, S.L., Aggleton, J.P., 1999. Extending the spontaneous preference test of recognition: evidence of object-location and object-context recognition. Behavioral Brain Research 99, 191-200

Endl, E.; Gerdes, J. The ki-67 protein: Fascinating forms and an unknown function. Experimental cell research 2000, 257, 231-237.

Ennaceur, A.; Michalikova, S.; Bradford, A.; Ahmed, S. Detailed analysis of the behavior of lister and wistar rats in anxiety, object recognition and object location tasks. Behav Brain Res 2005, 159, 247-266.

Henry, T.R. The history of valproate in clinical neuroscience. Psychopharmacology Bulletin 2003, 37 Suppl 2, 5-16.

Huang, S.S., Chiu, C.S., Chen, H.J., Hou, W.C., Sheu, M.J., Lin, Y.C., Shie, P.H., Huang, G.J., 2011. Antinociceptive Activities and the Mechanisms of Anti-Inflammation of Asiatic Acid in Mice. Evid-Based Compl Alt, 1-10.

Kee, N.; Sivalingam, S.; Boonstra, R.; Wojtowicz, J.M. The utility of ki-67 and brdu as proliferative markers of adult neurogenesis. Journal of neuroscience methods 2002, 115, 97-105.

Krishnamurthy, R.G.; Senut, M.C.; Zemke, D.; Min, J.; Frenkel, M.B.; Greenberg, E.J.; Yu, S.W.; Ahn, N.; Goudreau, J.; Kassab, M., et al. Asiatic acid, a pentacyclic triterpene from centella asiatica, is neuroprotective in a mouse model of focal cerebral ischemia. J Neurosci Res 2009, 87, 2541-2550.

Kuwagata, M.; Ogawa, T.; Shioda, S.; Nagata, T. Observation of fetal brain in a rat valproate-induced autism model: A developmental neurotoxicity study. Int J Dev Neurosci 2009, 27, 399-405.

Lyons, L.; ElBeltagy, M.; Umka, J.; Markwick, R.; Startin, C.; Bennett, G.; Wigmore, P. Fluoxetine reverses the memory impairment and reduction in proliferation and survival of hippocampal cells caused by methotrexate chemotherapy. Psychopharmacology 2011, 215, 105-115.

Lyons, L., et al. Fluoxetine counteracts the cognitive and cellular effects of 5-fluorouracil in the rat hippocampus by a mechanism of prevention rather than recovery. PLoS ONE 2012, 7(1): e30010.

Mayhew, T.M., Burton, G.J., 1988. Methodological problems in placental morphometry: apologia for the use of stereology based on sound sampling practice. Placenta 9, 565-581.

Mustafa, S.; Walker, A.; Bennett, G.; Wigmore, P.M. 5-fluorouracil chemotherapy affects spatial working memory and newborn neurons in the adult rat hippocampus. European Journal of Neuroscience 2008, 28, 323-330.

Lee, K.Y.; Bae, O.N.; Weinstock, S.; Kassab, M.; Majid, A. Neuroprotective effect of asiatic acid in rat model of focal embolic stroke. Biol Pharm Bull 2014, 37, 1397-1401.

Mook-Jung, I.; Shin, J.E.; Yun, S.H.; Huh, K.; Koh, J.Y.; Park, H.K.; Jew, S.S.; Jung, M.W. Protective effects of asiaticoside derivatives against beta-amyloid neurotoxicity. J Neurosci Res 1999, 58, 417-425.

Nasir, M.N.; Habsah, M.; Zamzuri, I.; Rammes, G.; Hasnan, J.; Abdullah, J. Effects of asiatic acid on passive and active avoidance task in male spraque-dawley rats. J Ethnopharmacol 2011, 134, 203-209.

Park, S.P.; Kwon, S.H. Cognitive effects of antiepileptic drugs. Journal of clinical neurology 2008, 4, 99-106.

Perucca, E. Pharmacological and therapeutic properties of valproate: A summary after 35 years of clinical experience. CNS Drugs 2002, 16, 695-714.

Phiel, C.J.; Zhang, F.; Huang, E.Y.; Guenther, M.G.; Lazar, M.A.; Klein, P.S. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 2001, 276, 36734-36741.

Senturk, V.; Goker, C.; Bilgic, A.; Olmez, S.; Tugcu, H.; Oncu, B.; Atbasoglu, E.C. Impaired verbal memory and otherwise spared cognition in remitted bipolar patients on monotherapy with lithium or valproate. Bipolar Disorder 2007, 9 Suppl 1, 136-144.

Sirichoat, A.; Chaijaroonkhanarak, W.; Prachaney, P.; Pannangrong, W.; Leksomboon, R.; Chaichun, A.; Wigmore, P.; Welbat, J.U. Effects of asiatic acid on spatial working memory and cell proliferation in the adult rat hippocampus. Nutrients 2015, 7, 8413-8423.

Umka, J.; Mustafa, S.; ElBeltagy, M.; Thorpe, A.; Latif, L.; Bennett, G.; Wigmore, P.M. Valproic acid reduces spatial working memory and cell proliferation in the hippocampus. Neuroscience 2010, 166, 15-22.

Weible, A.P.; Rowland, D.C.; Pang, R.; Kentros, C. Neural correlates of novel object and novel location recognition behavior in the mouse anterior cingulate cortex. J Neurophysiol 2009, 102, 2055-2068.

Wojtowicz, J.M.; Kee, N. Brdu assay for neurogenesis in rodents. Nature protocols 2006, 1, 1399-1405.

Zhang, X.; Wu, J.; Dou, Y.; Xia, B.; Rong, W.; Rimbach, G.; Lou, Y. Asiatic acid protects primary neurons against c2-ceramide-induced apoptosis. Eur J Pharmacol 2012, 679, 51-59.

Zheng, C.J.; Qin, L.P. Chemical components of centella asiatica and their bioactivities. Zhong Xi Yi Jie He Xue Bao 2007, 5, 348-351.

8. **Output (Acknowledge the Thailand Research Fund)**

8.1 International Journal Publication

Jariya Umka Welbat, Apiwat Sirichoat, Wunnee Chaijaroonkhanarak, Parichat Prachaney, Wanassanun Pannangrong, Poungrat Pakdeechote, Bungorn Sripanidkulchai, Peter Wigmore. Asiatic Acid Prevents the Deleterious Effects of Valproic Acid on Cognition and Hippocampal Cell Proliferation and Survival. Nutrients. 2016;8(5). pii: E303. doi: 10.3390/nu8050303.

8.2 Application

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8.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent

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