

Twenty-four hours after the transfection, measurable levels of both luciferase and  $\beta$ -galactosidase were observed. In the presence of DAPT, a commercially available  $\gamma$ -secretase inhibitor, luciferase but not  $\beta$ -galactosidase activity was decreased, indicating that this system can monitor the inhibition of  $\gamma$ -secretase (**Fig. 1B**). By ELISA, we confirmed the decrease of A $\beta$  production in the presence of DAPT (**Fig. 1C**).

Using this luciferase assay system, we screened ethanol-extracts of more than 1,600 plants, most of which are currently used in Chinese medicine, and found that among them Hop flower extracts had the most potent inhibitory activity. The inhibitory activity was dose-dependent (**Fig. 1B, C**). We observed very little inhibitory activity in water-extracts of Hop flowers. These results indicated that the responsible molecule(s) is lipid-soluble. Using ELISA, we confirmed that ethanol-extracts of Hop decreased production of both A $\beta$ 40 and A $\beta$ 42. These results indicated that ethanol-extracts of Hop flowers (referred to as “Hop extracts” hereafter) contain molecule(s) that inhibit  $\gamma$ -secretase.

#### *Isolation and identification of a major $\gamma$ -secretase-inhibitory component from Hop extracts*

In order to isolate major components of Hop extracts that inhibit  $\gamma$ -secretase, we first performed a Bligh-Dyer separation protocol (**Fig. S1**). As expected, the inhibitory activities were detected in the lipid-soluble fraction (Fraction 2) (**Fig. S2**). Next, we separated Fraction 2 by solid-phase extraction with a Sep-Pak Vac Silica cartridge: firstly eluted by with 100% chloroform (Fraction 2-1), followed by 100% methanol (Fraction 2-2). We found that the inhibitory activities were eluted in Fraction 2-2, but not Fraction 2-1 (**Fig. 2A**). We then applied Fraction 2-2 to normal-phase HPLC analysis with a 5CN-MS column, and observed that the inhibitory activities were eluted in Fractions 4-7 (**Fig. S3**). We next applied Fractions 4-7 to reverse-phase HPLC analysis with a COSMOSIL 5C18-AR-II column, and observed that the inhibitory activities were eluted in Fractions 9-12 (**Fig. 2B**). We then applied Fractions 9-12 to additional reverse phase HPLC analysis with a Symmetry Shield C18 column, and found that the inhibitory activities were eluted in Fractions 15-16 (**Fig. 2C**).

Lastly, we applied Fractions 15-16 to a COSMOSIL  $\pi$ -NAP reverse-phase HPLC column, and observed a single peak that eluted in Fraction 15 (**Fig. 3A**). We

confirmed that the purified compound indeed retained the ability to inhibit A $\beta$  production, as evidenced by the luciferase assay (**Fig. 3B**). It is notable, however, that the specific activity relative to the initial Hop extracts did not appreciably increase over the course of the purification (see discussion). We also measured the optical absorbance profile of the purified compound (**Fig. 3C**). Two absorbance peaks at 247 and 332 nm were assigned to  $\alpha$ ,  $\beta$ - and  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ -unsaturated carbonyl bonds, respectively (**Fig. 3D**). We then determined its molecular mass by LS/MS to be 416 g/mole (**Fig. 3D**). Repeated purifications yielded a total of approximately 2 mg of the purified compound. We then analyzed its chemical structure by NMR and found that its NMR values perfectly fit with those of a previously reported compound, Garcinielliptone HC [33], and its chemical formula was determined to be C<sub>25</sub>H<sub>36</sub>O<sub>5</sub> (**Fig. 3D**).

#### *Amelioration of AD phenotypes in AD model mice by continuous oral administration of Hop extracts*

We next examined the *in vivo* effects of Hop extracts on AD model mice, in which the FLAG-tagged  $\beta$ -C-terminal fragment of APP ( $\beta$ CTF) with Indiana mutation (V717F) was expressed under the control of the NSE (neuron-specific enolase) promoter. These transgenic mice were mated to obtain homozygous mice, and then the homozygous mice were bred to achieve sufficient numbers (referred to as AD mice hereafter). For the experiments, AD mice were separated into two groups. In one group, mice were allowed to drink water freely. In the other group, mice were allowed to freely drink Hop extract-containing water (2 g extract per liter of water) after weaning. In both groups and age-matched wild-type mice, total amounts of water consumption did not differ, approximately 4~6 ml/day/mouse (**Fig. 4A**). Body weights also did not differ between all 3 groups throughout the examination periods, up to 18 months of age (**Fig. 4B**). In both of the AD groups, expression of the transgene mRNAs in the brain were not statistically different (**Fig. 4C**), indicating that the Hop extract did not affect the NES promoter activity or stability of the of the transgene mRNA.

At 2 months of age, both AD groups did not display any impairment of spatial memory in the Morris water maze test, as compared with age-matched wild-type mice (**Fig. 4D**). At 9 months of age, water-drinking AD mice showed significant impairment in spatial memory in the Morris water maze test. These mice needed more time to reach the hidden platform, as compared with the age-matched wild-type mice. In contrast,

Hop extract-drinking AD mice were able to reach the hidden platform significantly faster than the water-drinking AD mice. The ability of the Hop extract-drinking AD mice to reach the platform appeared equal to or even slightly better than the age-matched wild-type mice (**Fig. 4D**). By 12 months, Hop extract-drinking AD mice were still able to reach the hidden platform significantly faster than the water-drinking AD mice, but apparently slower than the age-matched wild-type mice (**Fig. 4D**). In the follow-up probe test, water-drinking AD mice spent significantly less time around the area where the hidden platform had been placed than did the age-matched wild-type mice or Hop extract-drinking AD mice, at ages of 9 as well as 12 months (**Fig. 4E**).

We noted that no significant difference in basal activities were observed in the 3 groups of mice, up to ages of 18 months (**Fig. 4F, left panel**), and thus the observed differences in time to reach the hidden platform were not due to differences in physical activities. At ages of 15 and 18 months, however, a significant difference in time to reach the hidden platform was no longer observed between water-drinking AD mice and Hop extract-drinking AD mice (data not shown). These results indicate that Hop extracts have the ability to delay the AD phenotypes but not to completely prevent their manifestation. In the open field test, by which not only basal activities but also emotional states were able to be examined, all 3 groups of mice behaved indistinguishably at ages of 6, 12, and 15 months (**Fig. 4F, right panel**). At ages of 18 months, only water-drinking AD mice displayed significantly longer times away from the walls, indicating that the water-drinking AD mice were impaired in feeling anxiety, a type of emotional disturbance, and that Hop extracts might prevent emotional disturbances in AD as well.

Finally, we examined whether Hop extracts were indeed able to reduce A $\beta$  depositions in the brain of our AD model mice. We sacrificed 16-month old mice and examined their brains by immunohistochemical analyses (**Fig. 5**). On first inspection, we had the impression that the brains of water-drinking AD mice were smaller than those of the others, but the differences were not significant. In the cerebral cortex of the frontal lobe (areas indicated by black boxes in **Fig. 5A**), we could not observe any clear difference of A $\beta$  deposition among water-drinking AD mice, Hop extract-drinking AD mice, and age-matched wild-type mice. In the cerebral cortex of the parietal lobe (areas indicated by red boxes in **Fig. 5A**), very large amounts of A $\beta$  deposition were observed in water-drinking AD mice. However, the A $\beta$  deposition was greatly reduced in

age-matched Hop extract-drinking AD mice. Slight A $\beta$  deposition was observed in the cerebral cortex of the parietal lobe in the age-matched wild-type mice (**Fig. 5B and C**). Similar results were observed by FSB staining (**Fig. 5C and Fig. S4**). Furthermore, in the hippocampus (areas indicated by blue boxes in **Fig. 5A**), A $\beta$  deposition was much reduced in Hop extract-drinking AD mice compared with water-drinking AD mice. It is notable that in water-drinking AD mice, A $\beta$  deposition was clearly observed around certain artery walls, albeit infrequently, which is termed “amyloid angiopathy” [34], but such “amyloid angiopathy” was barely observed in age-matched Hop extract-drinking AD mice or wild-type mice (**Fig. 5D**). In Hop extract-drinking AD mice as well as the other two groups of mice, we did not observe any obvious skin problems, e.g. acne inversa [35,36], skin cancers [37,38], etc. up to ages of 18 months (see Discussion).

## Discussion

The “amyloid hypothesis” predicts that reduction of A $\beta$  production or accumulation would be an effective strategy for the treatment or prevention of AD. Accordingly, one approach has focused on the development of inhibitors of  $\gamma$ -secretase, whose activity is crucial to overall A $\beta$  production as well as the A $\beta$ 40/A $\beta$ 42 ratio. Indeed, Semagacestat®, an investigational compound, was developed and was tested in clinical trials on AD patients, but the trials did not show any significant slowing of AD phenotypes, or even worsened the symptoms in a phase III clinical study [38]. In addition, an increased risk of skin cancers has been reported [38], which might be due to inhibition of Notch signaling pathways by the  $\gamma$ -secretase inhibitor [37]. Recently, it has been proposed that enhanced activation of  $\gamma$ -secretase may promote the degradation of A $\beta$  and thus lead to reduced A $\beta$  accumulation [39]. An alternative approach was to neutralize A $\beta$  by A $\beta$  immunization or with A $\beta$ -specific antibodies. Such biological drugs, e.g. Bapineuzumab®, were developed, and their efficacies were tested in large clinical trials, but no benefit was observed [40,41]. In addition, during anti-A $\beta$  immunotherapies, several patients incurred intracranial hemorrhaging or meningoencephalitis as a complication, which prompted the discontinuation of the clinical trials [42]. The hemorrhaging was assumed to be caused by the A $\beta$  antibody reacting with amyloid angiopathy. The failures of these trials for AD treatment notwithstanding, a recent cohort study in Iceland brought new strong evidence supporting the idea that reducing A $\beta$  indeed reduces AD risk. The study identified an

AD-resistant mutation (A673T) in APP near the  $\beta$  cleavage site [19]. In cell culture experiments, the mutated APP produced approximately half the amount of  $A\beta$  than non-mutated APP. Thus, individuals possessing one allele of the mutated APP gene should have a 25% reduction of  $A\beta$  production [19]. Given that such individuals showed very low risk of AD, mild inhibition of  $A\beta$  production might be optimal for avoiding AD. As shown in this study, Hop-extracts contain mild activities that partially inhibit  $A\beta$  production in cultured cells, and indeed they proved to be markedly effective in preventing not only learning and memory impairment but also  $A\beta$  depositions in AD model mice. These results support the “amyloid hypothesis” and are consistent with the idea that mild inhibition of  $A\beta$  production is a plausible strategy to reduce AD risk. Moreover, we did not observe any skin problems throughout the administration of Hop extracts up to 18 months, indicating that mild inhibition of  $\gamma$ -secretase is not deleterious.

In humans, it is generally believed that memory disturbance in AD patients occurs long after the completion of  $A\beta$  deposition. By contrast, in our AD mice, neurological phenotypes apparently precede the completion of  $A\beta$  deposition. In 16-month old AD mice, administration of Hop extracts was still visibly apparent as reduced  $A\beta$  deposition, but at that age, the Hop extract-drinking mice had already lost memory ability to levels similar to those of water-drinking AD mice. In the cell culture experiments in neuronal cells,  $A\beta$  oligomers showed much stronger toxicity than  $A\beta$  aggregates, and thus  $A\beta$  oligomers have been proposed to be the causative agent for neuronal dysfunction or cell death [43]. Even if this might be the case in humans as well, partial inhibition of  $A\beta$  production appears to be a good strategy to reduce AD risk.

In this study, we also succeeded in purifying a major active component in Hop extracts that inhibits  $\gamma$ -secretase. As often observed in the purification procedures of plant extracts, specific activities were not substantially increased during the purification. This result raises the possibility that the inhibitory activity of the purified compound might be enhanced synergistically with other components in the hop extracts. As is often observed in Chinese medicine, the single usage of the purified compound by itself might not be effective for AD. By mass analysis, its molecular weight was determined to be 416, and its chemical structure was solved by NMR analysis and was found to be Garcinielliptone HC [33]. This compound was originally isolated from *Garcinia*, suggesting that *Garcinia* extracts might also have the potential to reduce  $A\beta$

production or accumulation. This possibility remains to be tested. Future studies are necessary to reveal the mechanism of inhibition.

In addition to the observed A $\beta$ -reducing activity, Hop-extracts have been reported to contain other interesting properties, including anti-inflammatory activities [44], estrogen-like activities [45,46], anti-atherosclerotic activities [47], etc. It has long been argued that chronic inflammation may be a mechanism underlying AD [48,49], and recent clinical studies indicate that estrogen therapy reduced the risk of AD, especially for young postmenopausal women [50,51]. These additional Hop activities might also add some benefits to the prevention or retardation of AD progression. Hop extracts have long been used in Chinese medicine for sedation, calming gastric and intestinal disorders, diuretics, etc. In Europe, Hop flower has been used as an herb for the treatment of insomnia, neuralgia, and menopausal disorders [52,53]. Furthermore, from their estrogen-like activities, Hop extracts have been tested in several clinical trials for reducing post-menopausal complications, at a maximal dose of 300 mg/day, and no obvious side effects were reported [54]. Recently,  $\gamma$ -secretase mutations have been reported to cause acne inversa [35,36]. In our mouse experiments, more than one and a half years of daily administration of Hop extracts did not manifest acne inversa nor elicit any overt deleterious effects. These lines of evidence encourage us to further investigate Hop extracts as safe and promising anti-AD substances. Finally, it should be mentioned that Hop sub-species contain different levels of  $\gamma$ -secretase inhibitory activities, and some have no measurable activity at all. Growing locales and conditions might also affect the activities. Thus, quality control of the extracts will be critical for maximizing the prophylactic effects on AD.

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## References

1. Wang XP, Ding HL (2008) Alzheimer's disease: epidemiology, genetics, and beyond. *Neurosci Bull* 24: 105-109.
2. Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, et al. (2005) Global prevalence of dementia: a Delphi consensus study. *Lancet* 366: 2112-2117.
3. Nelson PT, Head E, Schmitt FA, Davis PR, Neltner JH, et al. (2011) Alzheimer's disease is not "brain aging": neuropathological, genetic, and epidemiological human studies. *Acta Neuropathol* 121: 571-587.
4. Howard R, McShane R, Lindesay J, Ritchie C, Baldwin A, et al. (2012) Donepezil and memantine for moderate-to-severe Alzheimer's disease. *N Engl J Med* 366: 893-903.
5. Raschetti R, Maggini M, Sorrentino GC, Martini N, Caffari B, et al. (2005) A cohort study of effectiveness of acetylcholinesterase inhibitors in Alzheimer's disease. *Eur J Clin Pharmacol* 61: 361-368.
6. Selkoe DJ (1996) Amyloid  $\beta$ -protein and the genetics of Alzheimer's disease. *J Biol Chem* 271: 18295-18298.
7. O'Brien RJ, Wong PC (2011) Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci* 34: 185-204.
8. Sambamurti K, Greig NH, Utsuki T, Barnwell EL, Sharma E, et al. (2011) Targets for AD treatment: conflicting messages from  $\gamma$ -secretase inhibitors. *J Neurochem* 117: 359-374.
9. Iqbal K, Grundke-Iqbal I (2008) Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention. *J Cell Mol Med* 12: 38-55.
10. Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297: 353-356.
11. Morelli L, Perry G, Tagliavini F (2012) The contribution of the amyloid hypothesis to the understanding of Alzheimer's disease: a critical overview. *Int J Alzheimers Dis* 2012: 709613.
12. Neugroschl J, Sano M (2010) Current treatment and recent clinical research in Alzheimer's disease. *Mt Sinai J Med* 77: 3-16.
13. Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, et al. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface

- receptor. *Nature* 325: 733-736.
14. Murrell J, Farlow M, Ghetti B, Benson MD (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 254: 97-99.
  15. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349: 704-706.
  16. Selkoe DJ (2012) Preventing Alzheimer's disease. *Science* 337: 1488-1492.
  17. Thomas P, Fenech M (2007) A review of genome mutation and Alzheimer's disease. *Mutagenesis* 22: 15-33.
  18. Annaert W, Cupers P, Saftig P, De Strooper B (2000) Presenilin function in APP processing. *Ann N Y Acad Sci* 920: 158-164.
  19. Jonsson T, Atwal JK, Steinberg S, Snaedal J, Jonsson PV, et al. (2012) A mutation in *APP* protects against Alzheimer's disease and age-related cognitive decline. *Nature* 488: 96-99.
  20. Braak H, Braak E (1997) Frequency of stages of Alzheimer-related lesions in different age categories. *Neurobiol Aging* 18: 351-357.
  21. Funato H, Yoshimura M, Kusui K, Tamaoka A, Ishikawa K, et al. (1998) Quantitation of amyloid  $\beta$ -protein ( $A\beta$ ) in the cortex during aging and in Alzheimer's disease. *Am J Pathol* 152: 1633-1640.
  22. Godbolt AK, Cipolotti L, Watt H, Fox NC, Janssen JC, et al. (2004) The natural history of Alzheimer disease: a longitudinal presymptomatic and symptomatic study of a familial cohort. *Arch Neurol* 61: 1743-1748.
  23. Yamamoto Y, Hasegawa H, Tanaka K, Kakizuka A (2001) Isolation of neuronal cells with high processing activity for the Machado-Joseph disease protein. *Cell Death Differ* 8: 871-873.
  24. Saitou M, Narumiya S, Kakizuka A (1994) Alteration of a single amino acid residue in retinoic acid receptor causes dominant-negative phenotype. *J Biol Chem* 269: 19101-19107.
  25. Martínez-Aguilar JF, Peña-Álvarez A (2009) Characterization of five typical agave plants used to produce mezcal through their simple lipid composition analysis by gas chromatography. *J Agric Food Chem* 57: 1933-1939.
  26. Kumari P, Reddy CRK, Jha B (2011) Comparative evaluation and selection of a



- method for lipid and fatty acid extraction from macroalgae. *Anal Biochem* 415: 134-144.
27. Koike M, Fukushi J, Ichinohe Y, Higashimae N, Fujishiro M, et al. (2010) Valosin-containing protein (VCP) in novel feedback machinery between abnormal protein accumulation and transcriptional suppression. *J Biol Chem* 285: 21736-21749.
  28. Mori-Konya C, Kato N, Maeda R, Yasuda K, Higashimae N, et al. (2009) p97/valosin-containing protein (VCP) is highly modulated by phosphorylation and acetylation. *Genes Cells* 14: 483-497.
  29. Saitou M, Sugai S, Tanaka T, Shimouchi K, Fuchs E, et al. (1995) Inhibition of skin development by targeted expression of a dominant-negative retinoic acid receptor. *Nature* 374: 159-162.
  30. Janus C (2004) Search strategies used by *APP* transgenic mice during navigation in the Morris water maze. *Learn Mem* 11: 337-346.
  31. Crusio WE (2001) Genetic dissection of mouse exploratory behaviour. *Behav Brain Res* 125: 127-132.
  32. Vom Berg J, Prokop S, Miller KR, Obst J, Kälin RE, et al. (2012) Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. *Nat Med* 18: 1812-1819.
  33. Lu YH, Wei BL, Ko HH, Lin CN (2008) DNA strand-scission by phloroglucinols and lignans from heartwood of *Garcinia subelliptica* Merr. and *Justicia* plants. *Phytochemistry* 69: 225-233.
  34. Viswanathan A, Greenberg SM (2011) Cerebral amyloid angiopathy in the elderly. *Ann Neurol* 70: 871-880.
  35. Wang B, Yang W, Wen W, Sun J, Su B, et al. (2010)  $\gamma$ -secretase gene mutations in familial acne inversa. *Science* 330: 1065.
  36. Kelleher RJ 3rd, Shen J (2010)  $\gamma$ -secretase and human disease. *Science* 330: 1055-1056.
  37. Xia X, Qian S, Soriano S, Wu Y, Fletcher AM, et al. (2001) Loss of presenilin 1 is associated with enhanced  $\beta$ -catenin signaling and skin tumorigenesis. *Proc Natl Acad Sci U S A* 98:10863-10868.
  38. Extnance A (2010) Alzheimer's failure raises questions about disease-modifying strategies. *Nat Rev Drug Discov* 9: 749-751.

39. Okochi M, Tagami S, Yanagida K, Takami M, Kodama TS, et al. (2013)  $\gamma$ -secretase modulators and presenilin 1 mutants act differently on presenilin/ $\gamma$ -secretase function to cleave A $\beta$ 42 and A $\beta$ 43. *Cell Rep* 3: 42-51.
40. Blennow K, Zetterberg H, Rinne JO, Salloway S, Wei J, et al. (2012) Effect of immunotherapy with bapineuzumab on cerebrospinal fluid biomarker levels in patients with mild to moderate Alzheimer disease. *Arch Neurol* 69: 1002-1010.
41. Delrieu J, Ousset PJ, Caillaud C, Vellas B (2012) 'Clinical trials in Alzheimer's disease': immunotherapy approaches. *J Neurochem* 120: 186-193.
42. von Bernhardi R (2010) Immunotherapy in Alzheimer's disease: where do we stand? Where should we go? *J Alzheimers Dis* 19: 405-421.
43. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, et al. (2002) Naturally secreted oligomers of amyloid  $\beta$  protein potently inhibit hippocampal long-term potentiation *in vivo*. *Nature* 416: 535-539.
44. Akazawa H, Kohno H, Tokuda H, Suzuki N, Yasukawa K, et al. (2012) Anti-inflammatory and anti-tumor-promoting effects of 5-deprenyllupulonol C and other compounds from Hop (*Humulus lupulus* L.). *Chem Biodivers* 9: 1045-1054.
45. Choi Y, van Breemen RB (2008) Development of a screening assay for ligands to the estrogen receptor based on magnetic microparticles and LC-MS. *Comb Chem High Throughput Screen* 11: 1-6.
46. Chadwick LR, Pauli GF, Farnsworth NR (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. *Phytomedicine* 13: 119-131.
47. Desai A, Darland G, Bland JS, Tripp ML, Konda VR (2012) META060 attenuates TNF- $\alpha$ -activated inflammation, endothelial-monocyte interactions, and matrix metalloproteinase-9 expression, and inhibits NF- $\kappa$ B and AP-1 in THP-1 monocytes. *Atherosclerosis* 223: 130-136.
48. Prasad S, Sung B, Aggarwal BB (2012) Age-associated chronic diseases require age-old medicine: role of chronic inflammation. *Prev Med* 54: S29-37.
49. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, et al. (2000) Inflammation and Alzheimer's disease. *Neurobiol Aging* 21: 383-421.
50. Levine AJ, Battista M (2004) Estrogen replacement therapy: effects on the cognitive functioning and clinical course of women with Alzheimer's disease. *Arch Clin Neuropsychol* 19: 769-778.

51. Wharton W, Baker LD, Gleason CE, Dowling M, Barnet JH, et al. (2011) Short-term hormone therapy with transdermal estradiol improves cognition for postmenopausal women with Alzheimer's disease: results of a randomized controlled trial. *J Alzheimers Dis* 26: 495-505.
52. Erkkola R, Vervarcke S, Vansteelandt S, Rompotti P, De Keukeleire D, et al. (2010) A randomized, double-blind, placebo-controlled, cross-over pilot study on the use of a standardized hop extract to alleviate menopausal discomforts. *Phytomedicine* 17: 389-396.
53. Van Cleemput M, Cattoor K, De Bosscher K, Haegeman G, De Keukeleire D, et al. (2009) Hop (*Humulus lupulus*)-derived bitter acids as multipotent bioactive compounds. *J Nat Prod* 72: 1220-1230.
54. Heyerick A, Vervarcke S, Depypere H, Bracke M, De Keukeleire D (2006) A first prospective, randomized, double-blind, placebo-controlled study on the use of a standardized hop extract to alleviate menopausal discomforts. *Maturitas* 54: 164-175.

## Figure legends

### Fig. 1. Hop extracts inhibit A $\beta$ production.

(A) Schematic drawing of the luciferase-based  $\gamma$ -secretase assay. The  $\beta$ -C-terminal fragment of APP ( $\beta$ CTF) was expressed in HEK293A cells as a fusion protein with Gal4VP16, a chimeric transcriptional factor, together with a luciferase-expressing reporter plasmid containing the thymidine kinase (TK) promoter with 4 copies of a Gal4-responsive element (GalRE) [23]. As an internal control of transfection, a  $\beta$ -galactosidase-expressing plasmid was also co-transfected. When  $\beta$ CTF-Gal4VP16 was cleaved by  $\gamma$ -secretase, the APP intracellular domain (AICD)-Gal4VP16 fusion was released and translocated into the nucleus, where it activated transcription of the luciferase reporter gene. Luciferase and  $\beta$ -galactosidase activities in whole cell extracts were measured.

(B) Quantification of relative luciferase activities. Twenty-four hours after transfection with the plasmids described in A, HEK293A cells were treated with or without different amounts of DAPT, a commercially available  $\gamma$ -secretase inhibitor in DMSO (D), or HOP flower extracts (HFE) in methanol (M). Twenty-four hours after the treatments, whole cell extracts were prepared and the luciferase and  $\beta$ -galactosidase activities were measured. Mean values of relative luciferase activities, after normalization with  $\beta$ -galactosidase activities, are shown, with values for DMSO alone (D) set at 1.0. Error bars indicate standard deviations. \*  $p < 0.05$ , \*\* $p < 0.01$

(C) Quantification of relative A $\beta$ 40 and A $\beta$ 42 amounts by ELISA. The amounts of A $\beta$ 40 and A $\beta$ 42 released into the culture medium in B were measured by ELISA. Mean values of relative ELISA values, after normalization with  $\beta$ -galactosidase activities, are shown, with values for DMSO alone (D) set at 1.0. Error bars indicate standard deviations. \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$

### Fig. 2 Purification of a major component in Hop extract that inhibits A $\beta$ production.

(A) Results of the luciferase assay after solid phase extraction. Three hundreds and thirty mg of the lipid-soluble fraction (Fraction 2) from the Bligh-Dyer method was applied to a Sep-Pak Vac Silica Cartridge, and was eluted with 100% chloroform (fraction 2-1), followed by 100% methanol (fraction 2-2). Each fraction was dried and dissolved in methanol (M), then assayed for inhibition of A $\beta$  production using the

luciferase assay, as described in Fig. 1B. Mean values of relative luciferase activities, after normalization with  $\beta$ -galactosidase activities, are shown, with values for DMSO alone (D) set at 1.0. Bars denote standard deviations. \*  $p < 0.05$ , \*\* $p < 0.01$

(B) A representative chromatogram from the first reverse-phase HPLC is shown (upper panel). One hundred and eighty mg of Fractions 4-6 from the normal-phase HPLC (Fig. S1 and S3) were applied to a COSMOSIL 5C18-AR-II column, and were eluted with a linear gradient of acetonitrile, starting from 80% to 100% for 20 min, followed by continuous flow with 100% acetonitrile. Effluent fractions were collected every minute. The results of the luciferase assay for each fraction are shown (lower panel). Fractions 9-12 were pooled and used for further purification.

(C) A representative chromatogram from the second reverse-phase HPLC is shown (upper panel). Twenty-six mg of pooled fractions 9-12 in (B) was applied to a symmetry shield C18 column, and was eluted following the same procedures as in (B). Effluent fractions were collected at 1-minute intervals. The results of the luciferase assay for each fraction are shown (lower panel). Fractions 15-16 were pooled and used for further purification.

### **Fig. 3 Characterizations of the purified compound**

(A) A representative chromatogram from the third reverse-phase HPLC is shown (upper panel). Seven point three mg of pooled fractions 15-16 in Fig. 2C were applied to a COSMOSIL  $\pi$ -NAP column, and were eluted by continuous flow with 65% acetonitrile. The compound was eluted at 15 min as a single peak.

(B) Comparison of DAPT and the purified compound by the luciferase assay.

(C) Optical absorbance profile of the purified compound.

(D) LC/MS profile of the purified compound. The molecular mass of the purified compound was determined as 416 g/mol; obtained value of M+H was 417.2632. The molecular architecture of the purified compound, determined by NMR, is also shown. Note that the chemical formula of the compound was  $C_{25}H_{36}O_5$ .

### **Fig. 4 Mitigation of memory impairment in AD mice by oral administration of Hop extracts**

(A) Average daily water consumption per mouse, per experimental group. Decrease of water volume in each water bottle was measured every 7 days. No significant difference

was observed in the amount of water consumed, comparing the three groups.

(B) Body weights of wild-type mice, water-drinking AD mice, and Hop extract-drinking AD mice, from age 3 months to 18 months. Bars represent standard deviations. No significant difference was observed among the groups of mice.

(C) Quantitative RT-PCR analysis of transgene mRNA in the brains of water-drinking AD mice and Hop extract-drinking AD mice. Brain mRNAs from 9-month old water-drinking AD mice and Hop extract-drinking AD mice were analyzed by quantitative RT-PCR to determine the expression levels of the transgene mRNA. No significant difference was observed among the groups.

(D) Morris water-maze test. The test measured the time required for mice to locate a hidden platform. At ages of 2 months, no significant difference was observed among the groups of mice. At ages of 9 and 12 months, Hop extract-drinking AD mice (n=7), required significantly shorter times to reach the hidden platform at days 3, 4, 5 compared with water-drinking AD mice (n=7). Bars indicate standard deviation. \* p<0.05, \*\*p<0.01

(E) Prolonged stay in the quadrant, where the hidden platform had been placed in the Morris water-maze test by oral administration of Hop extracts. At ages of 2 months, there was no significant difference in time spent in the quadrant where the hidden platform had been placed, comparing water-drinking and Hop extract-drinking mice, At ages of 9 and 12 months, Hop extract-drinking AD mice (n=7) showed a significantly prolonged stay in the quadrant where the hidden platform had been placed, compared to water-drinking AD mice (n=7). Bars indicate standard deviation. \* p<0.05, \*\*p<0.01

(F) Open field test for basal activity and anxiety. At ages of 6, 12, 15, and 18 months, no significant differences in basal activity were observed among the three groups. However, at 18 months, water-drinking AD mice (n=7) stayed a significantly longer period in zone 2 or zone 3, as compared with age-matched wild-type (n=8) or Hop extract-drinking AD mice (n=7), indicating that 18-month old water-drinking AD mice experienced abnormally reduced anxiety, and that Hop extracts prevented the decrease.

### **Fig. 5 Mitigation of A $\beta$ depositions in AD mice by oral administration of Hop extracts**

(A) Histochemical analyses of mouse brains stained by an anti-A $\beta$  antibody. Sagittal sections of brains of 16-month old mice (n=3 in each) were stained with an anti-A $\beta$

antibody. A $\beta$  depositions were visualized (brown color) by the ABC method. Scale bars, 200  $\mu$ m.

(B) Quantification of A $\beta$  depositions in sections of parietal lobes (areas indicated by red boxes in A) with a BZ-9000 Generation II microscope (KEYENCE) and image analysis application programs. Bars indicate standard deviation. \*\* $p < 0.01$  (n=15 for each, see details in **Materials and methods**)

(C) Histochemical analysis of sections in the cerebral cortex of the parietal lobe of mouse brains stained by FSB, an amyloid sensitive fluorescent dye. Scale bars, 200  $\mu$ m.

(D) A representative photo of amyloid angiopathy. At 16 months, the A $\beta$  deposition in the artery wall was specifically observed in the brains of water-drinking AD mice, but not in those of age-matched wild-type and Hop extract-drinking AD mice. Scale bars, 50  $\mu$ m.

## Supporting Information Figure Legends

**Fig. S1. A schematic flow diagram of the purification procedures.**

**Fig. S2. Quantification of inhibition of A $\beta$  production by fractions from the Bligh-Dyer method.**

Mean values of relative luciferase activities from cells treated with DAPT and Fractions 1 and 2 of Bligh-Dyer method, after normalization with  $\beta$ -galactosidase activities, are shown. Values in the absence of test compounds (0) are set at 1.0. Error bars indicate standard deviations. \*  $p < 0.05$ , \*\*  $p < 0.01$

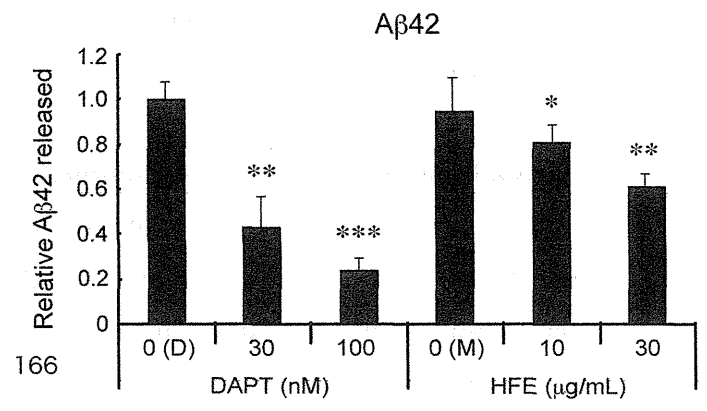
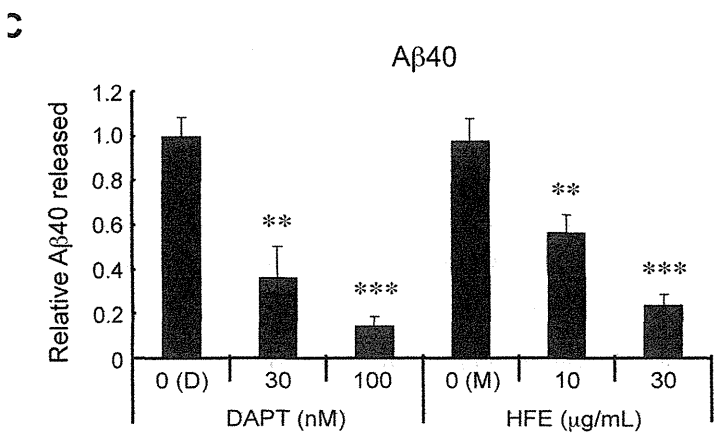
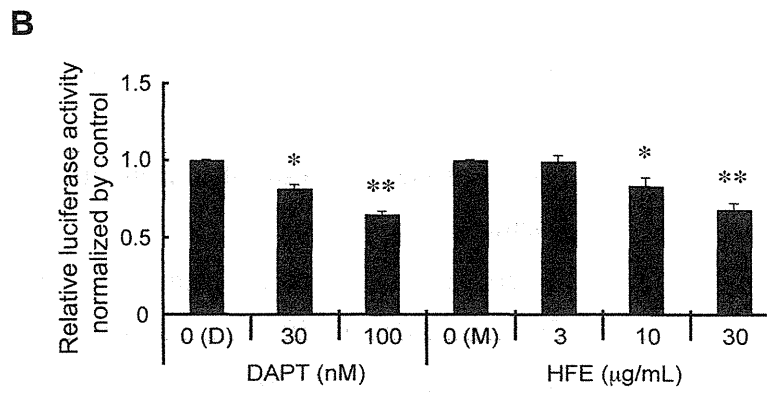
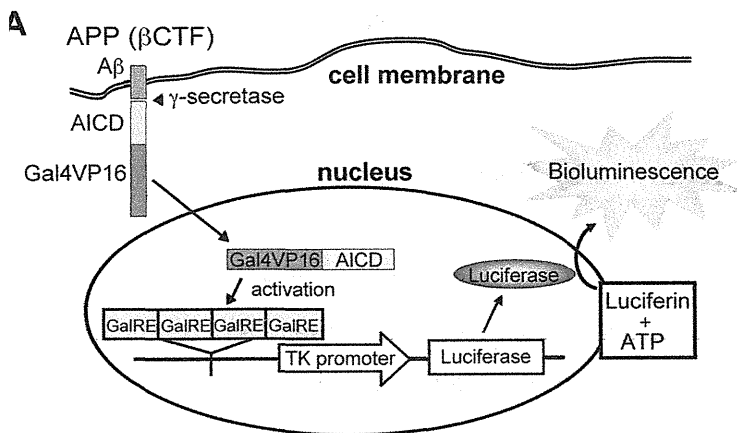
**Fig. S3. Quantification of inhibition of A $\beta$  production by fractions from the first normal-phase HPLC.**

A representative chromatogram of the normal-phase HPLC is shown (upper panel). Two hundreds and fifty mg of Fraction 2-2 from the solid phase extraction (**Fig. 2A**) were applied to a COSMOSIL 5CN-MS column, and were eluted by a linear gradient of methanol, from 0% to 15% over 15 min, in hexane : chloroform (1:1), followed by continuous flow of 15% methanol in Hexane : chloroform (1:1). Effluent fractions were collected every minute. The results of the luciferase assay on each fraction are shown (lower panel).

**Fig. S4. Enlarged images of FSB staining.**

Sections of the cerebral cortex of the parietal lobe (CTX) and the hippocampus (HC), from 16-month old mice, were stained by FSB. Scale bars, 20  $\mu$ m. Stronger FSB signals were observed in the sections from water-drinking AD mice (TG) than those from age-matched wild-type mice (WT).





166

