Transgenic mice. The AD mice were established following a standard procedure using the C57BL/6 strain [27,29]. The mice expressed a FLAG-tagged C-terminal portion of human APP (βCTF) with the Indiana mutation (V717F) in neuronal cells, driven by a neuron-specific enolase (NSE) promoter. Homozygous transgenic mice were maintained and bred to achieve numbers sufficient for the experiments. All animal studies were approved by the Review Board of Kyoto University.

Real-time RT-PCR. Total mouse brain RNA was purified from 9-month old mice (n=3 for each genotype), using TRIsure (BIOLINE), following the manufacturer's protocol. Three hundreds ng of the purified RNA was used for cDNA synthesis with random primers and M-MLV reverse transcriptase (Promega). Real-time quantitative PCR was performed on a LightCycler System (Roche Diagnostics) using DNA Master SYBR Green 1 (TOYOBO). The PCR primers used were the following:
mouse GAPDH/F: 5'-CCTGCACCACCAACTGCTTA-3'
mouse GAPDH/R: 5'-TGAGCCCTTCCACAATGCCAAA-3'
human APP/F: 5'-GAAGAAGAAACAGTACACATCCAT-3'
human APP/R: 5'-CCGTTCTGCTGCATCTTGGA-3'

Mouse behavioral studies. Morris water maze behavioral analysis was performed as described previously [30]. An escape platform (6 x 6 cm) was submerged 0.5 cm under the surface of the 21-22 °C water in a circular tank (0.7 m in diameter and 0.2 m high). A DCR-TRV20 (SONY) camera was placed above the tank, and recorded the movements of the mice. The first 2 days of testing consisted of non-spatial training and acclimating the mice to the water and the platform. The next 5 days consisted of memory training with 4 trials each day, with the platform placed in the same quadrant. The latencies were determined by measuring the time to reach the platform. Twenty-four hours after the final trial, the platform was removed and the mice were given probe trials to test their memory of the hidden platform. The lengths of time that mice stayed in each quadrant were calculated over the 5-minute duration of the test. The open field test was performed as described previously [31]. Briefly, each mouse was placed in the center of a circular box (75 cm in diameter) and was allowed to freely explore for 5 min, under a standard fluorescent light. Three zones were set in the box (zone 3, center of the circle, with a 15 cm diameter; zone 1, the outer-most annulus,

with a width of 15 cm; zone 2, the annulus between zone 1 and zone 3, with a width of 15 cm. See **Fig. 4F**). A DCR-TRV20 (SONY) camera was placed above the field, and recorded the movements of the mice. During the test, the time spent in zones 2 and 3 was calculated using SMART software (version 2.0, Panlab).

Histochemical analyses. Mouse brains were fixed with 4% paraformaldehyde overnight and then embedded in paraffin, and sagittal sections were prepared with a thickness of 3 μm. The sections were treated with a monoclonal anti-Aβ antibody (1-16) diluted 1:200, (6E10, Covance). Immune signals were detected with a Vectostain elite ABC standard kit (Vector laboratories), following the manufacturer's protocol. The stereomorphological analyses were performed on 5 randomly selected regions (40,000 μm^2 for each) of the each section of the parietal lobe from the mouse brain (n=3 for each genotypes) by BZ-9000 Generation II microscopy (KEYENCE) with its analysis application programs [32]. FSB staining was performed following the manufacturer's protocol. Briefly, the sagittal sections of brains were deparaffinized and immersed in 0.01% FSB in 50% ethanol for 30 min. Then, the sections were differentiated in saturated Li_2CO_3 and rinsed in 50% ethanol. The fluorescence images were obtained with AxioVision (Zeiss).

Statistical analysis. The statistical significance was analyzed using Student's *t* test.

Results

Screening of plant extracts that inhibit Aβ production

We previously developed an assay in which proteolytic activities directed at membrane proteins can be converted to a transcriptional activity readout [23]. We constructed a similar system to monitor the cleavage of APP by γ-secretase (**Fig. 1A**). In this system, Gal4VP16, a chimeric transcription factor, was fused to the FLAG-tagged C-terminal end of the β-C-terminal fragment of APP (βCTF), and the resulting FLAG-βCTF-Gal4VP16 fusion protein was expressed in HEK293A cells, together with a luciferase reporter plasmid, which contains 4 copies of GalRE [23], a Gal4-responsive element in the promoter region. We also co-transfected a plasmid expressing β-galactosidase for the normalization of transfection efficiencies.

Twenty-four hours after the transfection, measurable levels of both luciferase and β-galactosidase were observed. In the presence of DAPT, a commercially available γ-secretase inhibitor, luciferase but not β-galactosidase activity was decreased, indicating that this system can monitor the inhibition of γ-secretase (**Fig. 1B**). By ELISA, we confirmed the decrease of Aβ 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 Aβ40 and Aβ42. These results indicated that ethanol-extracts of Hop flowers (referred to as "Hop extracts" hereafter) contain molecule(s) that inhibit γ-secretase.

Isolation and identification of a major γ-secretase-inhibitory component from Hop extracts

In order to isolate major components of Hop extracts that inhibit γ-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 π-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 Aβ 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 α, β- and α, β, γ, δ-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 $C_{25}H_{36}O_5$ (**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 β-C-terminal fragment of APP (βCTF) 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 Aβ depositions in the brain of our AD model mice. We sacrificed 16-month old mice and examined their brains by immunohisotchemical 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 Aβ 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 Aβ deposition were observed in water-drinking AD mice. However, the Aβ deposition was greatly reduced in

age-matched Hop extract-drinking AD mice. Slight Aβ 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**), Aβ 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, Aβ 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 Aβ 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 γ-secretase, whose activity is crucial to overall Aβ production as well as the Aβ40/Aβ42 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 γ-secretase inhibitor [37]. Recently, it has been proposed that enhanced activation of γ-secretase may promote the degradation of Aβ and thus lead to reduced Aβ accumulation [39]. An alternative approach was to neutralize Aβ by Aβ immunization or with Aβ-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-Aβ 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 Aβ 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 Aβ indeed reduces AD risk. The study identified an

AD-resistant mutation (A673T) in APP near the β cleavage site [19]. In cell culture experiments, the mutated APP produced approximately half the amount of Aβ than non-mutated APP. Thus, individuals possessing one allele of the mutated APP gene should have a 25% reduction of Aβ production [19]. Given that such individuals showed very low risk of AD, mild inhibition of Aβ production might be optimal for avoiding AD. As shown in this study, Hop-extracts contain mild activities that partially inhibit Aβ production in cultured cells, and indeed they proved to be markedly effective in preventing not only learning and memory impairment but also Aβ depositions in AD model mice. These results support the "amyloid hypothesis" and are consistent with the idea that mild inhibition of Aβ 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 γ-secretase is not deleterious.

In humans, it is generally believed that memory disturbance in AD patients occurs long after the completion of Aβ deposition. By contrast, in our AD mice, neurological phenotypes apparently precede the completion of Aβ deposition. In 16-month old AD mice, administration of Hop extracts was still visibly apparent as reduced Aβ 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, Aβ oligomers showed much stronger toxicity than Aβ aggregates, and thus Aβ 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 Aβ 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 γ-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 Aβ

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

In addition to the observed Aβ-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, γ-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 γ-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.

Acknowledgements

We thank Professor James A. Hejna (Kyoto University) for critical reading of the manuscript, and Professor Hitoshi Takahashi (Niigata University) for the gift of the *neuron-specific enolase* promoter.

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 β-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 γ-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 β-protein (Aβ) 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) γ-secretase gene mutations in familial acne inversa. Science 330: 1065.
36. Kelleher RJ 3rd, Shen J (2010) γ-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 β-catenin signaling and skin tumorigenesis. Proc Natl Acad Sci U S A 98:10863-10868.
38. Extance 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) γ-secretase modulators and presenilin 1 mutants act differently on presenilin/γ–secretase function to cleave Aβ42 and Aβ43. 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 β 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-α-activated inflammation, endothelial-monocyte interactions, and matrix metalloproteinase-9 expression, and inhibits NF-κB 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 Aβ production.

(A) Schematic drawing of the luciferase-based γ-secretase assay. The β-C-terminal fragment of APP (βCTF) 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 β-galactosidase-expressing plasmid was also co-transfected. When βCTF-Gal4VP16 was cleaved by γ-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 β-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 γ-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 β-galactosidase activities were measured. Mean values of relative luciferase activities, after normalization with β-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 Aβ40 and Aβ42 amounts by ELISA. The amounts of Aβ40 and Aβ42 released into the culture medium in **B** were measured by ELISA. Mean values of relative ELISA values, after normalization with β-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 Aβ 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 Aβ production using the

luciferase assay, as described in Fig. 1B. Mean values of relative luciferase activities, after normalization with β-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 π-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 Aβ depositions in AD mice by oral administration of Hop extracts

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

antibody. Aβ depositions were visualized (brown color) by the ABC method. Scale bars, 200 μm.

(B) Quantification of Aβ 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 μm.

(D) A representative photo of amyloid angiopathy. At 16 months, the Aβ 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 μm.

Supporting Information Figure Legends

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

Fig. S2. Quantification of inhibition of Aβ production by fractions from the Bligh-Dyer method.

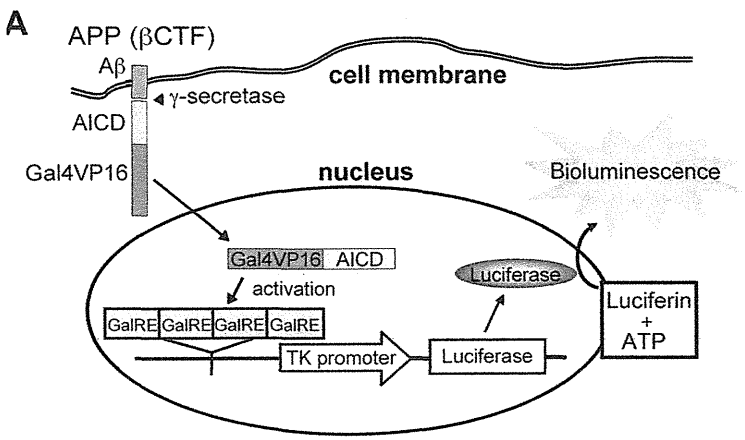
Mean values of relative luciferase activities from cells treated with DAPT and Fractions 1 and 2 of Bligh-Dyer method, after normalization with β-galactosidase activities, are shown. Values in the absence of test compounds (0) are set at 1.0. Error bars indicate standard deviations. * $p<0.05$, ** $p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

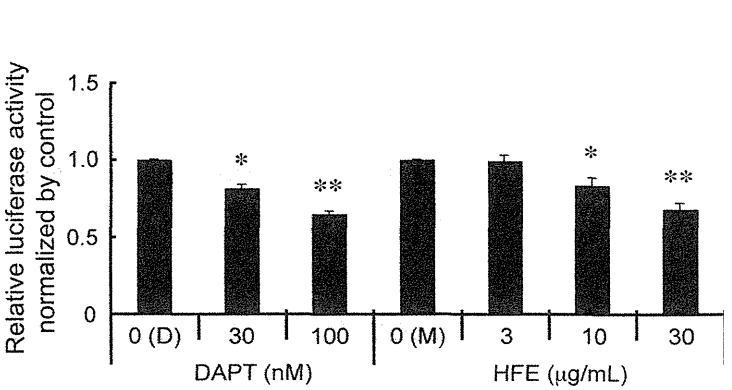
Fig. S3. Quantification of inhibition of Aβ production by fractions from the first normal-phase HPLC.

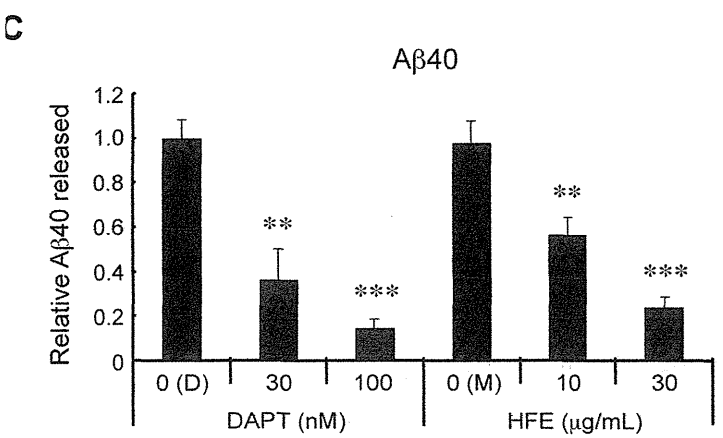
A representative chromatogram of the normal-phase HPLC is shown (upper panel). Two hundreds and fifty mg of Fraction 2-2 from the solid phase extraction (**Fig. 2A**) were applied to a COSMOSIL 5CN-MS column, and were eluted by a linear gradient of methanol, from 0% to 15% over 15 min, in hexane : chloroform (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).

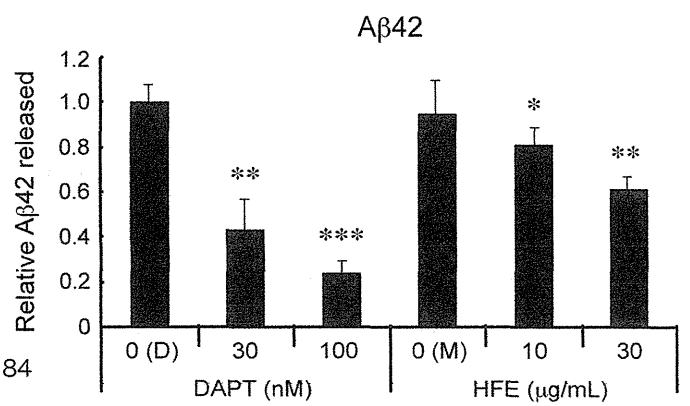
Fig. S4. Enlarged images of FSB staining.

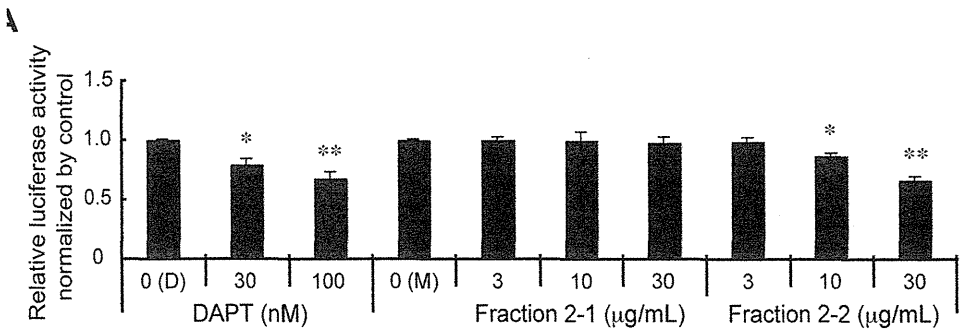
Sections of the cerebral cortex of the parietal lobe (CTX) and the hippocampus (HC), from 16-month old mice, were stained by FSB. Scale bars, 20 μm. Stronger FSB signals were observed in the sections from water-drinking AD mice (TG) than those from age-matched wild-type mice (WT).

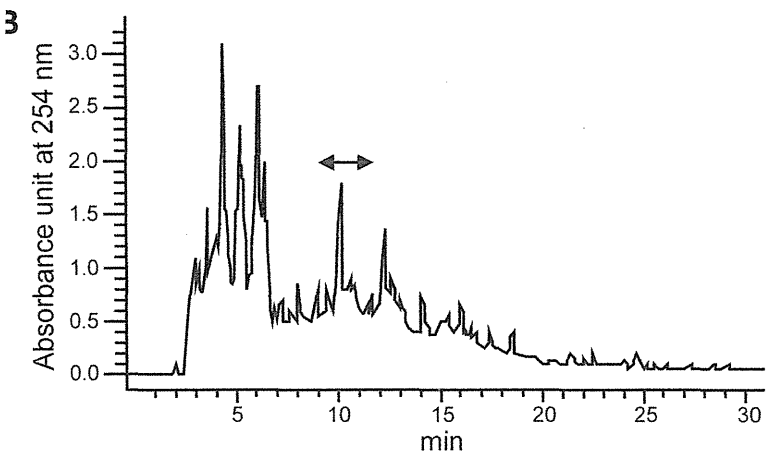

A
APP (βCTF)
Aβ
γ-secretase
cell membrane
AICD
Gal4VP16
nucleus
Bioluminescence
Gal4VP16 AICD
activation
GalRE GalRE GalRE GalRE
TK promoter
Luciferase
Luciferase
Luciferin
+
ATP

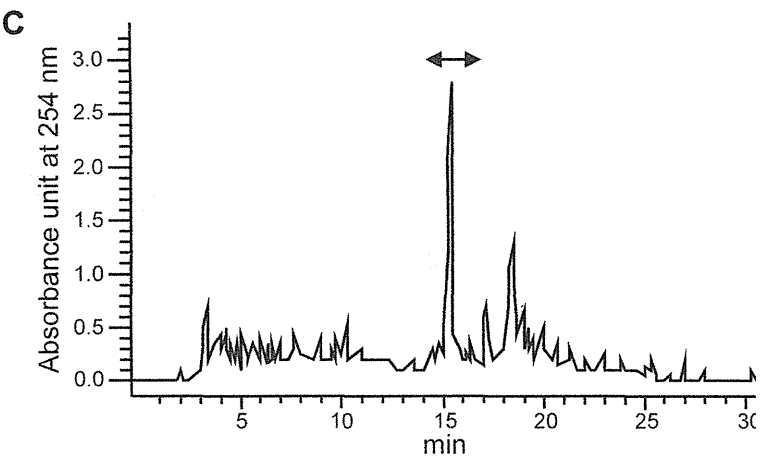

B
Relative luciferase activity normalized by control
1.5
1.0
0.5
0
*
**
*
**
0 (D)
30
100
0 (M)
3
10
30
DAPT (nM)
HFE (μg/mL)

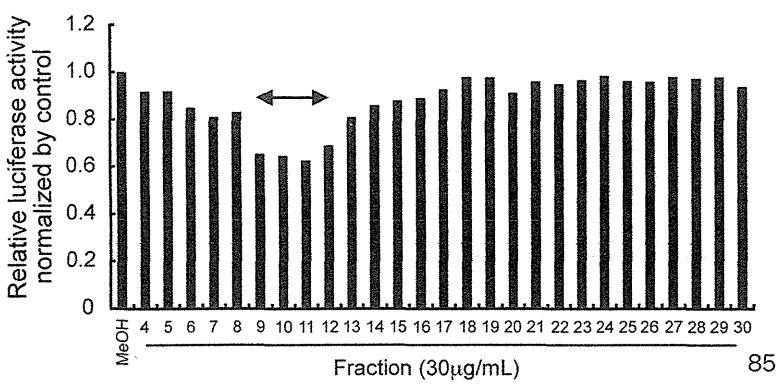

C
Aβ40
Relative Aβ40 released
1.2
1.0
0.8
0.6
0.4
0.2
0
**
***
**
***
0 (D)
30
100
0 (M)
10
30
DAPT (nM)
HFE (μg/mL)

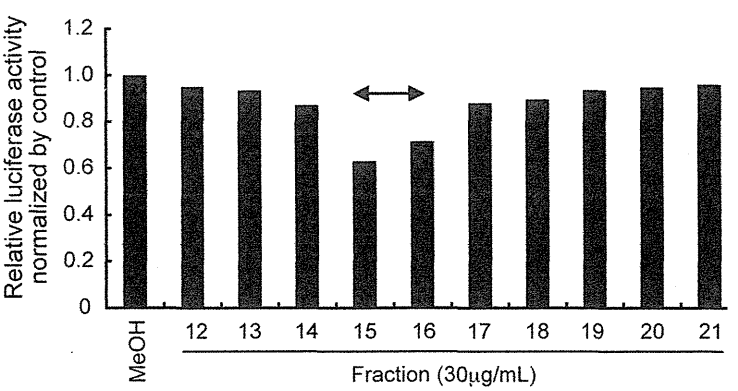

Aβ42
Relative Aβ42 released
1.2
1.0
0.8
0.6
0.4
0.2
0
**
***
*
**
0 (D)
30
100
0 (M)
10
30
DAPT (nM)
HFE (μg/mL)


A
Relative luciferase activity normalized by control
1.5
1.0
0.5
0
*
**
*
**
0 (D)
30
100
0 (M)
3
10
30
3
10
30
DAPT (nM)
Fraction 2-1 (μg/mL)
Fraction 2-2 (μg/mL)


B
Absorbance unit at 254 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
30
min


C
Absorbance unit at 254 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
30
min


Relative luciferase activity normalized by control
1.2
1.0
0.8
0.6
0.4
0.2
0
MeOH
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Fraction (30μg/mL)


Relative luciferase activity normalized by control
1.2
1.0
0.8
0.6
0.4
0.2
0
MeOH
12 13 14 15 16 17 18 19 20 21
Fraction (30μg/mL)