Published in final edited form as:

Chem Biol Drug Des. 2017 April ; 89(4): 475–481. doi:10.1111/cbdd.12883.

Identification of ginkgolide targets in brain by photoaffinity-labeling

Akira Kawamura, Ph.D.^{1,2,*}, Ilyas Washington³, Doina M. Mihai³, Francesca Bartolini⁴, Gregg G. Gundersen⁴, Milica Tesic Mark⁵, and Koji Nakanishi⁶

¹Department of Chemistry, Hunter College, 695 Park Avenue, New York, NY 10065, USA.

²Program in Chemistry, The Graduate Center of the City University of New York, 365 Fifth Avenue, New York, New York 10016, USA.

³Department of Ophthalmology, Columbia University Medical Center, 160 Fort Washington Ave, New York, NY, 10032, USA.

⁴Department of Pathology and Cell Biology, Columbia University, 630 West 168th Street, New York, NY, 10032, USA.

⁵Proteomics Resource Center, The Rockefeller University, 1230 York Avenue, New York, NY, 10065, USA.

⁶Department of Chemistry, Columbia University, 3000 Broadway, New York, NY, 10027, USA.

Abstract

Ginkgolides are terpene trilactones in *Ginkgo biloba*, a popular medicinal herb for memory disorders. Although ginkgolides are known for various neurobiological effects, their macromolecular target in brain is unknown. In this work, we employed benzophenone derivatives of ginkgolides to identify their binding-target in brain. Photolabeling of bovine hippocampus homogenates identified a series of α -tubulin isoforms. Selective photolabeling of α -tubulin over β -tubulin, which is equally abundant in brain, suggested that ginkgolides might modulate microtubule (MT) biology differently than typical MT-binding agents, such as taxol. In fact, ginkgolide A did not affect MT polymerization or cell proliferation; instead, it inhibited detyrosination of α -tubulin and reorientation of microtubule-organizing centers (MTOCs). Taken together, the current findings indicate that ginkgolides constitute a new class of MT-binding agents with distinct effects on α -tubulin biology.

Graphical Abstract

*Corresponding author: Akira Kawamura, +1-212-650-3095 (tel), +1-212-772-5332 (fax), akawamura@hunter.cuny.edu.

Conflict of Interest: The authors declare no conflict of interest.

Supporting Information: Additional Supporting Information may be found online in the supporting information tab for this article.

Photoactive analogues of ginkgolide selectively photolabeled α -tubulin in bovine hippocampus homogenate. Furthermore, ginkgolides exhibited unique effects on tubulin biology, including inhibition of microtubule detyrosination. The finding heralds a new chapter of the research on ginkgolides whose biological potential is yet to be discovered.

Keywords

Ginkgo biloba; benzophenone; mass spectrometry; α -tubulin; detyrosination

Introduction

Ginkgo biloba has been used for more than 3,500 years to improve cognitive functions (1, 2). Although its short-term clinical benefit remains controversial (3–9), a recent long-term population-based study indicated that *G. biloba* helped non-demented people maintain cognitive functions (10). The clinical benefit of *G. biloba* is in agreement with its pharmacological effects. *G. biloba* protects neurons from free radical damages (11) and A β -mediated neurotoxicity (12–14); it also promotes neurogenesis (15) and improves neurotransmission (16–18). These pharmacological effects are attributed to two major groups of chemical constituents, namely, flavonoids and terpene trilactones. Flavonoids in *G. biloba*, such as quercetin, are potent antioxidants (19), which may contribute to the neuroprotective effect. However, flavonoids are not unique to *G. biloba*; they exist in many other plants. The chemical constituents unique to *G. biloba* are terpene trilactones, such as ginkgolides. Ginkgolides are known as antagonists of the platelet-activating factor (PAF) receptor (2). In addition, they exhibit various pharmacological activities, including neuroprotective and anxiolytic effects (20–24).

Although ginkgolides exhibit diverse effects on neurons, their macromolecular target in brain is unknown. This is an important problem, because, without the knowledge of binding target, it would not be possible to understand the mechanism by which ginkgolides modulate neuronal and cognitive functions. Here, we report that benzophenone-derivatives of ginkgolides selectively photocrosslink α -tubulin in brain. In addition, ginkgolides modulate MT biology in a manner that is distinctly different from taxol, which is a prototypical MT-binding agent targeting β -tubulin. These findings provide new clues to understand the neurobiological effects of ginkgolides and *G. biloba*.

Methods and Materials

Photoaffinity-labeling of hippocampus tissues

Ginkgolide photoprobes **1–3** were synthesized previously (25). Hippocampus tissues were obtained from mouse and bovine cadavers. Tissues were homogenized in M-PER Mammalian Protein Extraction Reagent (Pierce) (tissue:buffer=1:20) using a dounce tissue homogenizer on ice. Homogenates were centrifuged at 10,000×g for 5 min. The supernatant was transferred to a new tube, and the concentration of total protein was adjusted to 4 µg/µL. Photolabeling was carried out using a reported protocol (26) with a minor modification. Briefly, 2 mM of ginkgolide-photoprobe in DMSO was added to the homogenate (photoprobe solution:homogenate=1:50). The mixture was incubated on ice for 30 min and then subjected to UV irradiation under six Sylvania 350 Blacklight lamps (15 W, λ_{max} 350 nm), in which samples were kept on ice and placed approximately 5 cm below the lamps.

Western blot of photolabeled hippocampus homogenate

The photolabeled mouse hippocampus homogenates were mixed with Laemmli Sample Buffer (BioRad) 5%(v/v) containing 2-mercaptoethanol, denatured at 80 °C for 5 min, and separated on SDS-PAGE (10% Tris-HCl gel, 200 V, 1 h) in 1×Tris-Glycine-SDS buffer (BioRad). Gel was blotted onto PVDF membrane (200 mA, 2h) in a cold transfer buffer (20% methanol in 1×Tris-Glycine buffer). Blotted membrane was blocked with 5% non-fat milk in 50 mL TBS-T for 1 h. Blocked membrane was rinsed with TBS-T (5 min×3), treated with avidin-horseradish peroxidase (HRP) (BioRad, 1:3000 dilution in 5% non-fat milk in TBS-T, 50 ml for 30 minutes), and washed with TBS-T (20 min×4). The washed membrane was treated with the ImmobilonTM Western-chemiluminescent HRP substrate for 5 min. Bands were observed with the BioRad ChemiDoc gel documentation system.

Affinity-purification of photolabeled proteins and SDS PAGE

Affinity purification of photochemically biotinylated proteins was carried out with ImmunoPure immobilized tetrameric avidin resin (Pierce). The photolabeled bovine hippocampus homogenates were mixed with the resin and incubated overnight at 4 °C. The supernatant was removed and the gel was washed with 1×TBS-T (30 min × 5). The washed resin was mixed with Laemmli Sample Buffer (BioRad) containing 5%(v/v) 2-mercaptoethanol, denatured at 80 °C for 5 min, and subjected to SDS-PAGE (10% Tris-HCl gel, 200 V, 1 h) in 1×Tris-Glycine-SDS buffer (BioRad). Coomassie blue stain was used to visualize the 50 kDa band, which was excised and subjected to the MS analyses.

Mass spectrometry

Proteins in the gel slice were reduced, alkylated and subjected to in-gel proteolytic digestion with trypsin. Resulting peptides were extracted two times with 40% acetonitrile and 2% formic acid mixture, and dried down. Peptides were re-suspended in sample loading buffer that contained 5% acetonitrile and 2% formic acid. Peptides were separated and analyzed using Ultimate 3000 nano-HPLC system coupled to the LTQ-Orbitrap XL mass spectrometer from Thermo Scientific. Following the loading on the C₁₈ trap column (5 µm ID beads, Thermo Scientific) at the flow rate of 3 µL/min, peptide separation was achieved

during a 50 min gradient (buffer A was 0.1% formic acid in water, buffer B was 0.1% formic acid in acetonitrile) using a C₁₈ analytical column (3 µm ID beads, Nikkyo Technologies) at the flow rate of 0.3 µL/min. Mass spectra were recorded in a 300–1800 m/z mass range, with 60,000 resolution for precursor scan and 30,000 resolution for fragment ions. Data were recorded in profile mode. Ten precursors from each scan were selected for fragmentation. Dynamic exclusion was used to resolve the less intense components of the sample with the following parameters: exclusion list size 500, duration, 60 seconds, exclusion by mass with both high and low exclusion mass widths of 5 ppm. The normalized collision energy in the ion trap was 35. Data were extracted and searched against Bovine Uniprot database using Proteome Discoverer 1.4 (Thermo Scientific) and Mascot 2.4 (Matrix Science). Potential common fetal bovine serum and human contaminants were included in the search (see Supplementary table) and labeled CON in the accession number. Identified peptides were filtered using 1% false discovery rate on Percolator (27). In the table, proteins were sorted according to their relative abundance, which is represented by the peak area. The protein area was calculated as an average of the areas of the three most abundant peptides for that protein.

Tubulin-polymerization assay

The effect of ginkgolide A on tubulin polymerization was examined with a commercial tubulin-polymerization assay kit (BK006P) from Cytoskeleton (Denver, CO). This assay is based on the fact that light scattering by MTs is proportional to the concentration of MTs. The assay followed the protocol provided by the manufacturer. Duplicate experiments were carried out, and representative results are shown in Figure 2a; the two sets of experiments were nearly identical.

Cell proliferation assay

Human ARPE-19 cells (a human retinal pigment epithelial cell line) (ATCC) were grown in Dulbecco's Modified Eagle medium/nutrient mixture F12 without glucose (DMEM/F12, US Biological, MA, USA) with 10% fetal bovine serum (Fetal Bovine Serum, Standard Quality, PAA Laboratories Inc, Etobicoke, Ontario, Canada), 5.5 mM glucose, 5 mM HEPES, 1 mM sodium pyruvate and 1% L-glutamine-penicillin-streptomycin (Sigma, St. Louis, MO, USA) at 37 °C and 5% CO₂. Cells were plated into 96-well plates at ca. 50 % confluency. Cells were treated with DMSO (vehicle control) and samples (5 nM, 100 nM and 500 nM taxol and 5 nM, 25 nM, 100 nM, 500 nM, 1 µM, 10 µM, 25 µM and 50 µM of ginkgolide A). Three and five days after treatment, resazurin reduction rates were measured. Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) was prepared as a stock solution of 75 µg/mL in calcium- and magnesium-free PBS and added to wells to give a final concentration of 10 µg/mL. Resorufin fluorescence was measured over two hours at ten minute intervals, at 37°C using SpectraMax Paradigm Multi-Mode Microplate Detection Platform (Molecular Devices LLC, Sunnyvale, CA.) by exciting at 530 nm and recording emission at 590 nm. The rate of resazurin reduced was calculated by fitting the rate of resorufin appearance to a line, the slopes of which were defined as resazurin reduction rates. For determining P values, we compared the resazurin reduction rates for control with treated groups using 2-tailed, unpaired t-tests: * p<0.05.

Effects of ginkgolide A on LPA-induced MT detyrosination and MTOC reorientation in motile fibroblasts

Serum-starved NIH3T3 fibroblasts were treated with 10 μ M LPA and/or ginkgolide A (20 or 50 μ M) for 2 hr and then fixed in methanol at -20°C . Fixed cells were immunofluorescently stained for detyrosinated (Glu) α -tubulin, tyrosinated (Tyr) α -tubulin (a marker of dynamic MTs), pericentrin (a marker of the centrosome/MTOC), and DAPI to stain nuclei. Alternatively, treated cells were fixed in 4% paraformaldehyde and stained with fluorescently labeled phalloidin to visualize actin filaments. The extent of detyrosinated MT formation was scored by determining the percentage of cells with detyrosinated MTs (Y-MTs): cells were scored positive if >10 MTs were brightly stained by the Glu antibody. In the case of MTOC reorientation, cells were scored positive if the centrosome was in the pie-shaped sector facing the leading edge as previously described (28). Scoring was performed blinded, and results were quantified and expressed as the means $\pm$ standard deviation from three independent experiments ($n>100$ per experiment). P values were derived from unpaired, two-tailed student t-test: n.s. $p>0.05$; *** $p<0.0005$; ** $p<0.005$.

Results and Discussion

In order to identify the target of ginkgolides in brain, we employed their derivatives with benzophenone and biotin that can photochemically biotinylate the binding target (Figure 1A) (25). Homogenates of mouse hippocampus were photolabeled with two photoactive analogs of ginkgolide A, **1** and **2**. Biotinylated proteins were visualized by Western blot, which revealed a distinct band near 50 kDa (Figure 1B, lanes 3 and 5). The fact that both **1** and **2** gave the same 50-kDa band, which was not observed in the background (lane 2), suggested that this 50-kDa band was the binding-target of ginkgolide A. The intensity of this band diminished in the presence of free ginkgolide A ($10\times$ higher concentration than **2**) (29) (lane 6), indicating that the 50-kDa protein(s) recognized the ginkgolide framework.

To identify the protein(s) in the 50-kDa band, photolabeling was repeated in a large scale using bovine hippocampus homogenates (Supplementary Scheme S1). The homogenate was photolabeled by **2**. Photochemically biotinylated proteins were purified by streptavidin-affinity resin. Affinity-purified sample was separated by SDS PAGE and visualized by Coomassie blue stain. The 50-kDa band was excised and subjected to mass spectrometry (MS) for protein identification. Table 1 summarizes the most abundant proteins identified in this study (see Supplementary Table for the complete list). It turned out that the top proteins were all α -tubulin isotypes: the “uncharacterized” proteins on the table were all classified as α -tubulin proteins (by sequence). This is not nonspecific photolabeling of abundant proteins in brain because the photolabeling occurred selectively with α -tubulin over β -tubulin, which is equally abundant in brain homogenate.

To verify the finding, photolabeling was repeated with a purified bovine MT protein (purity $>99\%$), in which the polymerized form of MT was stabilized by taxol. The MT protein was subjected to photolabeling with **2** and **3**, which were photoprobes of ginkgolides A and B, respectively (Figure 1A). Both **2** and **3** photolabeled MT efficiently as demonstrated by dot blot analyses (Figure 1C,D). Collectively, our photoaffinity-labeling studies indicated that ginkgolides selectively target α -tubulin in MTs.

The identification of α -tubulin raised a possibility that ginkgolides might modulate MT-biology differently than typical MT-binding agents targeting β -tubulin (30), such as vinblastine and taxol. As such, we conducted preliminary biochemical and biological studies on ginkgolide A. To examine the effect of ginkgolides on MT-assembly, ginkgolide A was subjected to a tubulin-polymerization assay (31), in which the polymerization was monitored by the increase in optical density at 340 nm. While 10 μ M taxol (“Taxol10” in Figure 2A; the positive control) rapidly polymerized the $\alpha\beta$ -tubulin dimer into MTs, ginkgolide A at 10 and 100 μ M (“GA10” and “GA100”) gave the results that were identical to the profile of DMSO vehicle control (Figure 2A). The result indicated that ginkgolide A had no effect on the assembly of tubulin. Furthermore, ginkgolide A at 50 μ M did not affect the proliferation of ARPE-19 cells (a human retinal pigment epithelial cell line), whereas 5 nM taxol significantly inhibited the growth of the same cell line (Figure 2B). Taken together, these results suggested that, although ginkgolide A targets α -tubulin, it has no effect on the cytoskeletal functions of MT.

Although ginkgolide A did not affect cytoskeletal functions of MT, there remained a possibility that it could still modulate the biological processes involving α -tubulin. α -tubulin has recently emerged as hotspots for post-translational modifications (PTMs), such as, the deetyrosination-tyrosination cycle (Figure 3A) (32). The PTMs of α -tubulin are pronounced in polarized cells, including neurons, and believed to control cell polarity and polarized cargo transport (33–37). As such, we wondered if ginkgolides might modulate α -tubulin PTMs.

To examine this possibility, we turned to a well-established model of α -tubulin deetyrosination in motile NIH3T3 fibroblasts (38). In this model, a monolayer of serum-starved fibroblasts is wounded, which is followed by wound-closure by migrating fibroblasts in the presence of lysophosphatidic acid (LPA), a serum mitogen (Supplementary Figure S1). Wounding in the presence of LPA induced deetyrosinated MTs (Y-MTs) in the fibroblasts along the wound (the wound is the dark area in the image) (Figure 3B, left panel; Y-MTs are highlighted by white triangles). When the cells were treated with both LPA and ginkgolide A (50 μ M), however, MT-deetyrosination was visibly attenuated (Figure 3B, right panel; the wound is on the right edge of this image). Quantitation of Y-MTs revealed significant inhibition ($p<0.005$) of the LPA-induced MT-deetyrosination by ginkgolide A (Supplementary Figure S2).

The fibroblast-wounding assay also revealed another unique effect of ginkgolide A. Ginkgolide A inhibited the reorientation of microtubule-organizing centers (MTOCs) in polarizing cells. Cells treated with LPA and/or ginkgolide were immunofluorescently stained for pericentrin, a marker of MTOC (centrosome) (Figure 3C). LPA induced clustering (reorientation) of MTOCs in the cells along the wound (the left panel; clustered MTOCs are highlighted by white arrows). On the other hand, the reorientation was visibly attenuated in the cells treated with both LPA and 50 μ M ginkgolide A (the right panel). Again, the quantitation of the cells with oriented MTOCs confirmed the significant inhibition ($p<0.05$) of MTOC reorientation by ginkgolide A (Supplementary Figure S3).

It is noted that the observed effect of ginkgolide A is not the consequence of non-specific disruption of cytoskeletal structures. When tyrosinated MT (Tyr) and filamentous actin were visualized in fibroblasts, ginkgolide A had no effect on the distribution of MTs or actin (Supplementary Figures S3 and S4). Taken together, ginkgolide A selectively inhibited MT detyrosination and MTOC reorientation without affecting the overall cytoskeletal structures.

Although further studies are needed to clearly define the effects of ginkgolides on α -tubulin biology, the current study suggests that ginkgolides constitute a new class of MT-modulating agents. The photoaffinity-labeling study suggests that ginkgolides selectively bind to α -tubulin in bovine hippocampus homogenates. Although photoaffinity-labeling has its own limitations such as off-target interactions, the current finding of α -tubulin does not appear to be non-specific photocrosslinking of abundant proteins for the following two reasons: (i) the excess amount of free ginkgolide A attenuated the photolabeling of α -tubulin (10 $\times$ higher concentration than **2**) and (ii) photolabeling selectively occurred on α -tubulin over equally abundant β -tubulin. Unlike typical MT-binding agents targeting β -tubulin (30, 39), ginkgolide A does not affect MT-polymerization or cell proliferation. Instead, it inhibits α -tubulin detyrosination and reorientation of MTOCs. As noted earlier, PTMs of α -tubulin play important roles in cell polarization and polarized cargo transport (33–37). Reorientation of MTOCs is the hallmark of various biological processes, including cell division, cell polarization, axonal specification in neurons, and interaction between T cells and antigen-presenting cells (40–43). As such, the current findings warrant further studies to define the scope of this newly discovered biological effects of ginkgolides.

The current study also raises several new questions regarding ginkgolides' mechanism of action *in vivo*. The first question is whether the brain is the only organ that mediates their beneficial effects. The expression of α -tubulin is not limited to the central nervous system (CNS), although it is abundant in neuronal cells. In addition, a previous biodistribution study of ^{18}F -labeled ginkgolide B (GB) indicated that GB, when injected in rats, rapidly accumulated in the liver and the intestine (44); on the other hand, GB exhibited low blood-brain barrier permeability. The accumulation of ginkgolides in the intestine might be relevant to their biological effects because recent studies revealed the emerging roles of enteric nervous system (ENS) in neurological disorders including Alzheimer's disease (45). The second question is whether α -tubulin is really the only major target of ginkgolides *in vivo*. This question stems from the observation that ginkgolides exists in two forms *in vivo*: namely, (i) the original form with its lactone rings closed and (ii) a second form with one of the lactone rings open ("the open form") (44). Further studies are needed to determine whether the open form also binds to α -tubulin or it has some other target.

Conclusion

In conclusion, the current work suggests that ginkgolides are a new class of MT-modulating agents with distinct effects on α -tubulin biology. The finding heralds a new chapter of the research on ginkgolides whose biological potential is yet to be discovered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Eisuke Kato, Rachel Howitt, and Sergei V. Dzyuba for for the synthesis of photoprobes 1–3. This study was supported in part by NIMHD/NIH G12 MD007599-27. NIH/NIGMS R01GM062939 and NIH/NIGMS R01GM099481 to GGG and PSC CUNY grant to AK are also acknowledged.

References

1. Brondino N, De Silvestri A, Re S, Lanati N, Thiemann P, Verna A, Emanuele E, Politi P. A Systematic Review and Meta-Analysis of Ginkgo biloba in Neuropsychiatric Disorders: From Ancient Tradition to Modern-Day Medicine. *Evid Based Complement Alternat Med*. 2013; 2013:915691. [PubMed: 23781271]
2. Nakanishi K. Terpene trilactones from Ginkgo biloba: from ancient times to the 21st century. *Bioorg Med Chem*. 2005; 13:4987–5000. [PubMed: 15990319]
3. Andrieu S, Gillette S, Amouyal K, Nourhashemi F, Reynish E, Ousset PJ, Albarede JL, Vellas B, Grandjean H, study E. Association of Alzheimer's disease onset with ginkgo biloba and other symptomatic cognitive treatments in a population of women aged 75 years and older from the EPIDOS study. *J Gerontol A Biol Sci Med Sci*. 2003; 58:372–377. [PubMed: 12663701]
4. Herrschaft H, Nacu A, Likhachev S, Sholomov I, Hoerr R, Schlaefke S. Ginkgo biloba extract EGb 761® in dementia with neuropsychiatric features: a randomised, placebo-controlled trial to confirm the efficacy and safety of a daily dose of 240 mg. *J Psychiatr Res*. 2012; 46:716–723. [PubMed: 22459264]
5. Ihl R, Tribanek M, Bachinskaya N. Baseline neuropsychiatric symptoms are effect modifiers in Ginkgo biloba extract (EGb 761®) treatment of dementia with neuropsychiatric features. Retrospective data analyses of a randomized controlled trial. *J Neurol Sci*. 2010; 299:184–187. [PubMed: 20837354]
6. Schneider LS, DeKosky ST, Farlow MR, Tariot PN, Hoerr R, Kieser M. A randomized, double-blind, placebo-controlled trial of two doses of Ginkgo biloba extract in dementia of the Alzheimer's type. *Curr Alzheimer Res*. 2005; 2:541–551. [PubMed: 16375657]
7. Weinmann S, Roll S, Schwarzbach C, Vauth C, Willich SN. Effects of Ginkgo biloba in dementia: systematic review and meta-analysis. *BMC Geriatr*. 2010; 10:14. [PubMed: 20236541]
8. DeKosky ST, Williamson JD, Fitzpatrick AL, Kronmal RA, Ives DG, Saxton JA, Lopez OL, Burke G, Carlson MC, Fried LP, Kuller LH, Robbins JA, Tracy RP, Woolard NF, Dunn L, Snitz BE, Nahin RL, Furberg CD. Investigators G.E.O.M.G.S. Ginkgo biloba for prevention of dementia: a randomized controlled trial. *JAMA*. 2008; 300:2253–2262. [PubMed: 19017911]
9. Vellas B, Coley N, Ousset PJ, Berrut G, Dartigues JF, Dubois B, Grandjean H, Pasquier F, Piette F, Robert P, Touchon J, Garnier P, Mathiex-Fortunet H, Andrieu S, Group GS. Long-term use of standardised Ginkgo biloba extract for the prevention of Alzheimer's disease (GuidAge): a randomised placebo-controlled trial. *Lancet Neurol*. 2012; 11:851–859. [PubMed: 22959217]
10. Amieva H, Meillon C, Helmer C, Barberger-Gateau P, Dartigues JF. Ginkgo biloba extract and long-term cognitive decline: a 20-year follow-up population-based study. *PLoS One*. 2013; 8:e52755. [PubMed: 23326356]
11. Rösler M, Retz W, Thome J, Riederer P. Free radicals in Alzheimer's dementia: currently available therapeutic strategies. *J Neural Transm Suppl*. 1998; 54:211–219. [PubMed: 9850930]
12. Wu Y, Wu Z, Butko P, Christen Y, Lambert MP, Klein WL, Link CD, Luo Y. Amyloid-beta-induced pathological behaviors are suppressed by Ginkgo biloba extract EGb 761 and ginkgolides in transgenic *Caenorhabditis elegans*. *J Neurosci*. 2006; 26:13102–13113. [PubMed: 17167099]
13. Bastianetto S, Ramassamy C, Dore S, Christen Y, Poirier J, Quirion R. The Ginkgo biloba extract (EGb 761) protects hippocampal neurons against cell death induced by beta-amyloid. *Eur J Neurosci*. 2000; 12:1882–1890. [PubMed: 10886329]

14. Yao Z, Drieu K, Papadopoulos V. The Ginkgo biloba extract EGb 761 rescues the PC12 neuronal cells from beta-amyloid-induced cell death by inhibiting the formation of beta-amyloid-derived diffusible neurotoxic ligands. *Brain Res.* 2001; 889:181–190. [PubMed: 11166702]
15. Tchanchou F, Xu Y, Wu Y, Christen Y, Luo Y. EGb 761 enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer's disease. *FASEB J.* 2007; 21:2400–2408. [PubMed: 17356006]
16. Kehr J, Yoshitake S, Ijiri S, Koch E, Nöldner M, Yoshitake T. Ginkgo biloba leaf extract (EGb 761®) and its specific acylated flavonol constituents increase dopamine and acetylcholine levels in the rat medial prefrontal cortex: possible implications for the cognitive enhancing properties of EGb 761®. *Int Psychogeriatr.* 2012; 24(Suppl 1):S25–S34. [PubMed: 22784425]
17. Williams B, Watanabe CM, Schultz PG, Rimbach G, Krucker T. Age-related effects of Ginkgo biloba extract on synaptic plasticity and excitability. *Neurobiol Aging.* 2004; 25:955–962. [PubMed: 15212849]
18. Yoshitake T, Yoshitake S, Kehr J. The Ginkgo biloba extract EGb 761(R) and its main constituent flavonoids and ginkgolides increase extracellular dopamine levels in the rat prefrontal cortex. *Br J Pharmacol.* 2010; 159:659–668. [PubMed: 20105177]
19. Rendeiro C, Guerreiro JD, Williams CM, Spencer JP. Flavonoids as modulators of memory and learning: molecular interactions resulting in behavioural effects. *Proc Nutr Soc.* 2012; 71:246–262. [PubMed: 22414320]
20. Wu X, Su J, Chen L, Ma B, Gu X, Zhu L. Ginkgolide B Protects Neurons from Ischemic Injury by Inhibiting the Expression of RTP801. *Cell Mol Neurobiol.* 2015; 35:943–952. [PubMed: 25869596]
21. Kaur N, Dhiman M, Perez-Polo JR, Mantha AK. Ginkgolide B revamps neuroprotective role of apurinic/apyrimidinic endonuclease 1 and mitochondrial oxidative phosphorylation against Abeta25-35 -induced neurotoxicity in human neuroblastoma cells. *J Neurosci Res.* 2015; 93:938–947. [PubMed: 25677400]
22. Wang X, Jiang CM, Wan HY, Wu JL, Quan WQ, Wu KY, Li D. Neuroprotection against permanent focal cerebral ischemia by ginkgolides A and B is associated with obstruction of the mitochondrial apoptotic pathway via inhibition of c-Jun N-terminal kinase in rats. *J Neurosci Res.* 2014; 92:232–242. [PubMed: 24327346]
23. Wu X, Zhou C, Du F, Lu Y, Peng B, Chen L, Zhu L. Ginkgolide B preconditioning on astrocytes promotes neuronal survival in ischemic injury via up-regulating erythropoietin secretion. *Neurochem Int.* 2013; 62:157–164. [PubMed: 23201340]
24. Kuribara H, Weintraub ST, Yoshihama T, Maruyama Y. An anxiolytic-like effect of Ginkgo biloba extract and its constituent, ginkgolide-A, in mice. *J Nat Prod.* 2003; 66:1333–1337. [PubMed: 14575433]
25. Kato E, Howitt R, Dzyuba SV, Nakanishi K. Synthesis of novel ginkgolide photoaffinity--biotin probes. *Org Biomol Chem.* 2007; 5:3758–3761. [PubMed: 18004454]
26. Kawamura A, Mihai DM. Probing proteomes with benzophenone photoprobes. *Methods Mol Biol.* 2012; 803:65–75. [PubMed: 22065219]
27. Kall L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat Methods.* 2007; 4:923–925. [PubMed: 17952086]
28. Palazzo AF, Joseph HL, Chen YJ, Dujardin DL, Alberts AS, Pfister KK, Vallee RB, Gundersen GG. Cdc42, dynein, and dynactin regulate MTOC reorientation independent of Rho-regulated microtubule stabilization. *Curr Biol.* 2001; 11:1536–1541. [PubMed: 11591323]
29. Higher concentrations of ginkgolide A, which would be needed to completely block photolabeling, could not be tested due to its poor solubility in aqueous media.
30. Dumontet C, Jordan MA. Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nat Rev Drug Discov.* 2010; 9:790–803. [PubMed: 20885410]
31. Shelanski ML, Gaskin F, Cantor CR. Microtubule assembly in the absence of added nucleotides. *Proc Natl Acad Sci U S A.* 1973; 70:765–768. [PubMed: 4514990]

32. Gundersen GG, Khawaja S, Bulinski JC. Postpolymerization detyrosination of alpha-tubulin: a mechanism for subcellular differentiation of microtubules. *J Cell Biol.* 1987; 105:251–264. [PubMed: 2886509]
33. Janke C, Kneussel M. Tubulin post-translational modifications: encoding functions on the neuronal microtubule cytoskeleton. *Trends Neurosci.* 2010; 33:362–372. [PubMed: 20541813]
34. Konishi Y, Setou M. Tubulin tyrosination navigates the kinesin-1 motor domain to axons. *Nat Neurosci.* 2009; 12:559–567. [PubMed: 19377471]
35. Li R, Gundersen GG. Beyond polymer polarity: how the cytoskeleton builds a polarized cell. *Nat Rev Mol Cell Biol.* 2008; 9:860–873. [PubMed: 18946475]
36. Sirajuddin M, Rice LM, Vale RD. Regulation of microtubule motors by tubulin isotypes and post-translational modifications. *Nat Cell Biol.* 2014; 16:335–344. [PubMed: 24633327]
37. Sugioka K, Sawa H. Formation and functions of asymmetric microtubule organization in polarized cells. *Curr Opin Cell Biol.* 2012; 24:517–525. [PubMed: 22704717]
38. Nagasaki T, Gundersen GG. Depletion of lysophosphatidic acid triggers a loss of oriented detyrosinated microtubules in motile fibroblasts. *J Cell Sci.* 1996; 109(Pt 10):2461–2469. [PubMed: 8923207]
39. Rohena CC, Mooberry SL. Recent progress with microtubule stabilizers: new compounds, binding modes and cellular activities. *Nat Prod Rep.* 2014; 31:335–355. [PubMed: 24481420]
40. Segal M, Bloom K. Control of spindle polarity and orientation in *Saccharomyces cerevisiae*. *Trends Cell Biol.* 2001; 11:160–166. [PubMed: 11306295]
41. Sancho D, Vicente-Manzanares M, Mittelbrunn M, Montoya MC, Gordon-Alonso M, Serrador JM, Sanchez-Madrid F. Regulation of microtubule-organizing center orientation and actomyosin cytoskeleton rearrangement during immune interactions. *Immunol Rev.* 2002; 189:84–97. [PubMed: 12445267]
42. Cowan CR, Hyman AA. Asymmetric cell division in *C. elegans*: cortical polarity and spindle positioning. *Annu Rev Cell Dev Biol.* 2004; 20:427–453. [PubMed: 15473847]
43. Gundersen GG, Gomes ER, Wen Y. Cortical control of microtubule stability and polarization. *Curr Opin Cell Biol.* 2004; 16:106–112. [PubMed: 15037313]
44. Suehiro M, Simpson NR, Underwood MD, Castrillon J, Nakanishi K, van Heertum R. In vivo biodistribution of ginkgolide B, a constituent of *Ginkgo biloba*, visualized by MicroPET. *Planta Med.* 2005; 71:622–627. [PubMed: 16041647]
45. Rao M, Gershon MD. The bowel and beyond: the enteric nervous system in neurological disorders. *Nat Rev Gastroenterol Hepatol.* 2016; 13:517–528. [PubMed: 27435372]

Figure 1. Photoaffinity-labeling of ginkgolide target. (A) Three photoprobes used in this study: **1** and **2** were derivative of ginkgolide A; **3** was derived from ginkgolide B. (B) Photolabeling of mouse hippocampus homogenate. Lane 1: ladders (L); lane 2: background (B) (The hippocampus homogenate); lanes 3 and 4: the homogenate photolabeled with **1** in the absence (-) or presence (+) of ginkgolide A as the blocker, respectively; lanes 5 and 6: the homogenate photolabeled with **2** in the absence (-) or presence (+) of ginkgolide A, respectively. The black arrow indicates the 50-kDa band photolabeled by **1** and **2**. (C) and

(D) Photolabeling of purified MTs with **2** and **3**, respectively. Purified bovine MT sample (>99%) was mixed with photoprobes and incubated with (left) or without (right) UV irradiation. Samples were analyzed by dot blot.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2.

Ginkgolide A does not affect tubulin-polymerization or cell proliferation. (A) The extent of tubulin-polymerization was monitored by the increase in optical density at 340 nm.

Duplicate experiments were carried out for each condition; the two sets of experiments were nearly identical. DMSO (vehicle control), Taxol10 (10 μM taxol: positive control), GA10 (10 μM ginkgolide A), and GA100 (100 μM ginkgolide A). (B) Cell proliferation after 3 and 5 days of incubation with ginkgolide A (GA) and taxol as measured by resazurin reduction rates. Mean resazurin reduction rates with 95% confidence intervals are shown. P values were derived from unpaired, two-tailed student t-test: * p<0.05.

Figure 3.

Ginkgolide A inhibits lysophosphatidic acid (LPA) induced formation of detyrosinated MTs (Δ Y-MTs) and reorientation of the centrosome. (A) The detyrosination-tyrosination cycle of α -tubulin. (B) Δ Y-MTs in serum-starved fibroblasts treated with LPA (10 μ M) and LPA +GA50 (10 μ M LPA and 50 μ M ginkgolide A). Δ Y-MTs were visualized by immunofluorescence using an antibody against detyrosinated (Glu) α -tubulin. The dark area in each image is the wounded space. Δ Y-MTs can be seen in the polarizing fibroblasts near the wound in the presence of LPA (marked by white triangles). Bar, 20 μ m. (C) Pericentrin

in serum-starved fibroblasts treated with with LPA (10 μ M) and LPA+GA50 (10 μ M LPA and 50 μ M ginkgolide A). MTOCs were visualized by the immunofluorescence of pericentrin. Reorientation of MTOCs can be seen in the polarizing fibroblasts along the wound in the presence of LPA (marked by white arrows). Bar, 20 μ m.

Table 1

Bovine hippocampus proteins identified by photoprobe 2.

Accession ^a	Protein name	Area ^b	Score	kDa
P81947	Tubulin alpha-1B	1.131E9	5970	50.1
F2Z4C1	Uncharacterized protein ^c	1.131E9	6332	50.1
Q3ZCJ7	Tubulin alpha-1C	1.131E9	5078	49.8
Q2HJ86	Tubulin alpha-1D	1.131E9	5870	50.3
F1MNF8	Uncharacterized protein ^c	1.131E9	5132	49.9
F2Z4KO	Uncharacterized protein ^c	9.806E8	4468	49.9
Q32KN8	Tubulin alpha-3	9.806E8	4461	49.9
F6RP72	Uncharacterized protein ^c	9.806E8	4468	49.8
P81948	Tubulin alpha-4A	8.981E8	4031	49.9
Q2HJB8	Tubulin alpha-8	7.857E8	3057	50.0

^aUniprot accession.^bArea (abundance) was calculated as an average of the areas of the three most abundant peptides for that protein.^cAll uncharacterized proteins are classified as α -tubulin proteins (by sequence).