

Alzheimer's disease, the nematode *Caenorhabditis elegans*, and ginkgo biloba leaf extract

Yuan Luo*

Department of Biological Sciences, The University of Southern Mississippi, 118 College drive, Hattiesburg, MS 39406. USA

Received 21 March 2005; accepted 7 December 2005

Abstract

Alzheimer's disease (AD) is affecting larger and larger proportions of our population as lifespan increases. Thus, the means to prevent or reduce the rate of this disorder is a high priority for medical research. A standardized extract of *Ginkgo biloba* leaves EGb 761 is a popular dietary supplement taken by the general public to enhance mental focus and by the elderly to delay onset of age-related loss of cognitive function. EGb 761 has been used for treatment of certain cerebral dysfunctions and dementias associated with aging and AD. Substantial evidence indicates that EGb 761 has neuroprotective effects. But, mechanisms of action of the components of the extract are, unfortunately, poorly understood. Research in my laboratory focuses on understanding mechanisms of action of the components of the herbal extract EGb 761 in protection against Alzheimer's disease. We have demonstrated that EGb 761 inhibited amyloid beta aggregation in vitro and attenuates reactive oxidative species (ROS) in a model organism — the round worm *Caenorhabditis elegans*. Furthermore, EGb 761 eased its toxicity in the transgenic *C. elegans*. We also found that only a certain size of the amyloid beta aggregates is toxic to the worms. These findings suggest that EGb 761 has a clear therapeutic potential for prevention and/or treatment of AD. A better understanding of the mechanisms of neuroprotection by EGb 761 will be important for designing therapeutic strategies, for basic understanding of the underlying neurodegenerative processes, and for a better understanding of the effectiveness and complexity of this herbal medicine.

© 2006 Published by Elsevier Inc.

Keywords: Herbal medicine; Neuroprotective effects; *C. elegans* model of Alzheimer's disease

Introduction

About 50% of all adult Americans have used herbal medicine for their well-being and for prevention or treatment of chronic diseases (Blumenthal, 2000; Eisenberg et al., 1998). *Ginkgo* leaf extracts (such as EGb 761) is on the top of the best-selling herbal medicine product in the United States (Ernst, 2002). It has been used for primary neurodegenerative dementias associated with aging, Alzheimer's disease (AD), peripheral vascular diseases, and neurosensory problems (e.g., tinnitus) (DeFeudis, 1998). The disease prevention theory associated with herbal medicine has the potential to both increase quality of life and reduce health care costs in our society. Substantial experimental evidence supports neuroprotective properties of EGb 761, but the actual mechanisms

remain unknown (Fig. 1). Application of contemporary neuroscience theories and methodologies may provide a better understanding of the efficacy of the herbal extract, which may, in turn, facilitate an explanation of the mechanism by which the disease progresses.

Alzheimer's disease

Alzheimer's disease, an age-related brain dysfunction, is widely recognized as a serious public health problem (Brookmeyer et al., 1998). Currently more than 5 million Americans are affected. For population over 80 years old, half of them are suffering from this disorder. Memory impairment progressing to dementia is the main clinical symptom of AD, which is thought to be the consequence of the selective degeneration of nerve cells in the brain regions critical for memory, cognitive performance and personality (Price et al., 1998). A common feature of AD shared with other

* Tel.: +1 601 266 5417; fax: +1 601 266 5797.

E-mail address: yuan.luo@usm.edu.

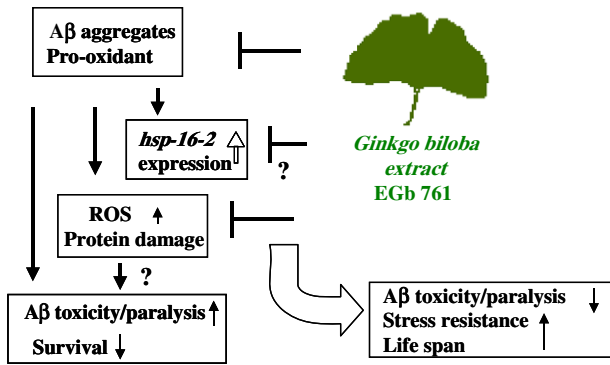


Fig. 1. Summary of neuroprotective mechanisms of EGB 761. The arrows in the box indicate the activation or inhibition effects we have observed. The question marks indicate issues remain to be addressed.

neurodegenerative diseases is the characteristic senile plaques observed in the brain tissues of the cortex, hippocampus, and amygdale. The primary constituents of the plaques are aggregates of amyloid β -peptide ($A\beta$, a 4 kD peptide cleaved by β and γ secretases from the amyloid precursor protein (APP) (Selkoe, 1997). The $A\beta$ monomers form oligomers and polymers, which assemble into protofilaments and then fibrils (Lansbury, 1999).

An “amyloid cascade” hypothesis states that accumulation of $A\beta$ deposition initiates a series of downstream neurotoxic events, which result in neuronal dysfunction and death (Hardy and Selkoe, 2002). The strongest evidence supporting this hypothesis comes from molecular genetic studies. Patients with Down’s syndrome, with extra copy of chromosome 21 containing the APP gene, always develop AD and the formation of $A\beta$ deposits is their early sign of brain lesion (Mann, 1989). All familiar forms of AD (FAD)-linked mutations, in the APP gene or two presenilin genes (PS1 and PS2), result in increased production of $A\beta_{42}$ which is the more amyloidogenic form (Sherrington et al., 1995). Furthermore, transgenic mice over-expressing the mutant APP develop $A\beta$ -containing amyloid plaques similar to those found in AD. In addition, other structure lesions, including neurofibrillary tangles and apoE might contribute to an imbalance between $A\beta$ production and clearance (Hardy and Selkoe, 2002). Therefore, modulation of $A\beta$ production and clearance in the brain is becoming one of the rationale approach for treatment of AD.

Despite large body of experimental evidence supporting the hypothesis that $A\beta$ deposition is critical in the pathogenesis of AD, the theory remains controversial. An intriguing question is whether the $A\beta$ fibrils or the oligomers are the cause of neuronal death in AD, which is critical to determining the mechanism of $A\beta$ toxicity and the specific therapeutic strategies. The controversial theories are: (1) neurotoxicity of $A\beta$ is directly linked to its state of aggregation in that only fibrillar $A\beta$ is toxic (Blanchard et al., 2004; Lorenzo and Yankner, 1994; Pike et al., 1995), (2) fibrils are not necessary for neurotoxicity, rather the intracellular (Oddo et al., 2003; Skovronsky et al., 1998; Takahashi et al., 2002), small aggregates or oligomers of the soluble $A\beta$ are the neurotoxic species (Hsia et al., 1999; Koistinaho et al., 2001; Lambert et

al., 1998; Walsh and Selkoe, 2004), and (3) generation of oxidative stress by $A\beta$ is a possible cause for neurodegenerative diseases in AD (Butterfield, 2003; Goto, 2003; McLellan et al., 2003). The evidence for or against these hypotheses is critical for determining the mechanism of $A\beta$ toxicity and the specific therapeutic strategies.

Many attempts have been made to generate transgenic mouse models of AD for a mechanistic approach to $A\beta$ toxicity in vivo (Higgins and Jacobsen, 2003; Price et al., 1998). The double transgenic mice co-expressing the Swedish mutation APP^{swe} and PS1 (Borchelt et al., 1997) exhibit enhanced production of $A\beta$ than the transgenic mice of single APP mutation (Citron et al., 1992). The APP^{swe}/PS1 mice have accelerated earlier deposition of $A\beta$ in hippocampus and cortex regions (Borchelt et al., 1996, 1997), compared with those found in the single mutant APP, and also exhibit a sex-specific learning deficiency (at 18 months) in the Morris Water maze test. Although these mice do not model the full phenotype of AD (Sommer et al., 2000), in conjunction with a recently developed triple transgenic mice model of AD (Oddo et al., 2003), they represent useful tools for the investigation of $A\beta$ toxicity and of cognitive impairment (van Leuven, 2000).

The nematode *C. elegans*

Use of the round worm *C. elegans*, a simple microscopic organism, to model AD is relatively new (Link et al., 2001). The worms were genetically engineered to carry the human gene for $A\beta_{42}$. The “sick” worms show amyloid aggregates of different sizes, just as seen in Alzheimer’s brain. Instead of cognitive impairment observed in the transgenic mice, the worms become paralyzed. In addition, the worms only live for 20 days, allowing us to evaluate the time sequence of events in these worms during their entire life. Thus, the transgenic *C. elegans* expressing $A\beta_{42}$ has been used extensively for a mechanistic study of $A\beta$ toxicity because of its ability to express muscle-specific human $A\beta$ peptide which forms intracellular $A\beta$ deposits (Link, 1995; Link et al., 2001) and exhibits increased protein carbonyl levels, a biomarker for protein oxidation (Yatin et al., 1999), similar to those observed in AD brain (Hensley et al., 1995). The transgenic strain also develops concomitant progressive paralysis phenotype (CL4176) (Drake et al., 2003). DNA microarray assay of the transgenic strain indicates that several stress-related genes were up-regulated, particularly two genes homologous to human α B-crystalline and tumor-necrosis-factor-induced protein, which were also found up-regulated in the postmortem AD brain (Link et al., 2003). Even though this is an invertebrate system and thus may not have relevance to AD pathology, it is a well-suited model for correlating $A\beta$ expression and toxicity in an in vivo model organism.

To understand the early events in development of AD and to develop therapeutic strategies, it is fundamentally important to determine the temporal sequence of events leading to neurodegeneration. *C. elegans* is a suitable tool for mechanistic examination of the transgene products as well as for pharmacological analysis of time course and kinetics of drug

effect (Driscoll and Gerstbrein, 2003). For example, a relationship between A β amino-acid sequence, amyloid formation and oxidative damage was established using this model. Yatin et al. (1999) showed both in vitro and in the *C. elegans* model that methionine (Met³⁵) is critical for free radical production by A β _{1–42}, and it is also critical for β -sheet formation in the transgenic *C. elegans* lines (Fay et al., 1998). A correlation between a progressed paralysis phenotype with an increased levels of protein carbonyls in CL4176 (Drake et al., 2003) supports the advanced “amyloid hypothesis,” which states that A β -induced oxidative stress leads to neuronal cell death seen in AD (Butterfield, 1997). It is likely that the temporal sequence of events manifested in the transgenic worms is the same as the one demonstrated in a *Drosophila* model of AD (Iijima et al., 2004) that accumulation of A β ₄₂ in the brain is sufficient to cause cognitive impairment and neurodegeneration. A challenging subject for the future studies is to determine whether this sequence of events occurs in human.

A Ginkgo biloba leaf extract

The *Ginkgo biloba* tree has a life span of more than 4000 years due to its leaves which are resistant to infection and diseases (DeFeudis, 1998). The leaves have been recorded in ancient and modern Chinese herbal pharmacopoeia as treatment for dysfunctions of heart and lung and as a promoter of longevity (DeFeudis and Drieu, 2000). The standardized *Ginkgo biloba* leaf extract (EGb 761) was developed and put on market in the early 1970s by IPSEN in France and Dr. Willmar Schwabe Pharmaceuticals in Germany. EGb 761 contains 24% flavonol glycosides (the flavonoid fraction) and 6% terpene lactones (terpenoid fraction). The flavonoid fraction is primarily composed of quercetin, kaempferol and isorhamnetin. The terpenoid fraction primarily contains ginkgolides A, B, C, J and M, as well as bilobalide. The chemical structure of flavonoids preferentially reacts with hydroxyl radicals (Zimmermann et al., 2002) and chelate pro-oxidant transition heavy metal ions (Gohil and Packer, 2002), which consequently inhibits the formation of new hydroxyl radicals. The ginkgolides are known to be platelet activating factor (PAF) antagonists, able to improve blood circulation (DeFeudis, 2002).

During the past decade, in vivo and in vitro experiments in mammalian systems and clinical studies in humans demonstrated that EGb 761 exhibits a range of biochemical and pharmacological effects (DeFeudis, 1998). Major biochemical and pharmacological activities of EGb 761 include: free-radical scavenger activities (Lien et al., 1999), inhibition of membrane lipid peroxidation (DeFeudis and Drieu, 2000), cognition enhancement particularly in aging rats and alleviating stress in the experimental animals (Blavet, 1992; Winter, 1991, 1998), anti-PAF activity contributing to improvements in cerebral insufficiency (Smith et al., 1996), enhancing neuronal plasticity (Gohil and Packer, 2002), anti-inflammatory effects (Oberpichler et al., 1990), and anti-apoptotic activities in neuronal cells (Bastianetto et al., 2000; Luo et al., 2002; Smith et al., 2002). As summarized by Christen at a recent conference (“*Ginkgo biloba*

Extract: From Traditional Medicine To A Medicine Of The Future,” Berlin, 2002), EGb 761 seems to act at all levels of life: from molecules, cells, tissues, to the entire organism (Christen and Maixent, 2002).

In human studies, more than a dozen of clinic trial have supported the clinical efficacy of EGb 761 in primary degenerative dementia of Alzheimer’s type (Le Bars et al., 1997, 2000; Oken et al., 1998). The evidence supporting EGb 761 enhancement of learning in healthy humans is inconclusive (Curtis-Prior et al., 1999; Mix and David Crews, 2002; Solomon et al., 2002). Currently the NIH-supported Ginkgo Evaluation of Memory (GEM) study in the United States is underway to test the efficacy of EGb 761 as a potential preventive treatment for dementia in the normally aging population and in Alzheimer’s disease (Christen et al., 2002). Other clinical effects of EGb 761 include improvements in peripheral arterial insufficiency, in cerebral disorders including cognitive decline, short-term memory, tinnitus, acute cochlear deafness, disturbance in equilibrium (Meyer, 1986), and cognitive deficits that follow stress or traumatic brain injury (DeFeudis and Drieu, 2000). Upon considering all studies conducted to date, it appears that EGb 761 has a beneficial effect on brain functions (DeFeudis, 2002).

Accumulating evidence suggests that many of the actions of EGb 761 are so-called “polyvalent” actions, i.e., the therapeutic activity of EGb 761 is the net effect of interactions between various biological activities of the individual substances in the extract. Presumably, this is one of the advantages of using chemicals obtained from natural products for the prevention and treatment of infirmity, as well as the maintenance of health (NIH, 2002; Normile, 2003). As opposed to pharmacologically manufactured or synthetic drugs, which provide a single target for a single receptor as its mechanism of action, EGb 761 is able to up- or down-regulate signaling pathways, gene transcription, and cellular metabolism, i.e., the general physiological states of the cell and organism in response to both normal and stressed conditions (Luo, in press). At the same time, it appears that it is the multiplicity of effects by EGb 761, or the “polyvalent” action, that complicates the mechanistic studies. Twenty-first century molecular medicine will likely be based on decoding complexity. Genomic and proteomic microarray methods provide researchers with the tools to decode the diverse effects of complex natural substances on biological systems. Gene microarray assays yielded molecular evidence for the neuro-modulatory action of EGb 761 in separate brain regions of mice fed with EGb 761 for 4 weeks (Watanabe et al., 2001). In these mice, transcription of transthyretin and several other molecules with neuroprotective roles were all significantly up regulated. Thus, the therapeutic effects of EGb 761 on cognitive impairment (dementia) probably involve modification of the expression of many genes by actions involving several of its active constituents (DeFeudis, 2002). Combining functional genomic and behavioral analysis to yield an objective assessment of the in vivo effects of EGb 761 is certainly meritorious and is worthy to be considered for future studies (Gohil and Packer, 2002) and for therapeutic development (Smith and Luo, 2004).

Neuroprotective mechanisms of EGb 761

To determine the “polyvalent” activities of EGb 761 reflected in global gene expression changes, we first compared the transcriptional profiles in a neuronal cell line (PC12) treated with or without EGb 761 using the DNA microarray technique. We discovered that multiple gene transcripts (more than 70 out of 816 aging-related genes) are altered more than 2-fold in EGb 761-treated PC12 cells (Table 1). The transcript level for an anti-apoptotic Bcl-2-like protein was elevated, whereas the transcript level for pro-apoptotic caspase 12 was decreased in EGb 761-treated cells. We confirmed it by the biochemical assays (Luo et al., 2002; Smith et al., 2002) and indicated that the protective action of EGb 761 may be carried out, at least in part, by modulating cellular apoptotic machinery. Similarly, expression of genes encoding transcriptional factors, antioxidant defenses and stress response is strongly modified by EGb 761 treatment of human hNT neurons (Soulie et al., 2002). In mice fed with EGb 761, the hippocampus and cerebral cortex regions displayed up-regulation of more than

10 neuromodulatory genes, especially that for transthyretin which could be involved in neuroprotection (Watanabe et al., 2001). However, interpretation of microarray results from post-mortem AD brain tissue (Ginsberg and Che, 2002; Pasinetti, 2001) is often complicated by the genetic and environmental heterogeneity of the sample, time lag between the onset of pathology and tissue recovered, and the DNA chips selected for hybridization.

For these reasons, Link et al. (2003) used DNA microarray analysis to look at changes in gene expression resulting from the induction of human A β expression in a transgenic strain CL4176 and identified 67 candidate up-regulated and 240 down-regulated genes, of which 40% of these genes have recognizable human homology. Among them, the small heat-shock protein gene *hsp-16*, which is closely co-localized with intracellular A β (Fonte et al., 2002) is particularly interesting. *Hsp-16* was reported to be up-regulated in A β -expressing *C. elegans* (Link et al., 2003). Using the GFP-reporter transgenic *C. elegans* (*hsp-16/GFP*) to visualize the expression of *hsp-16* in vivo, we found that in these worms fed with EGb 761 the expression of the *hsp:GFP* gene in response to oxidative stresses was significantly suppressed (Strayer et al., 2003). These data support the action of EGb 761 on heat shock protein, which further support the microarray data that EGb 761 regulates transcription of multiple antioxidant and stress-response genes (Soulie et al., 2002). Our finding that EGb 761 significantly attenuated the *hsp-16* gene expression in the *C. elegans* was unexpected (Strayer et al., 2003). Whether *hsp-16* expression is protective (Kudva et al., 1997), or toxic (Stegé et al., 1999) is a question that remains to be addressed. We speculate that the presence of EGb 761 reduces the cellular flux of free radicals, leading to a concomitant decrease in damaged proteins, and a reduced requirement for the stress-response gene produce, such as *hsp-16*. Consistent with this notion, we demonstrated that treatment of the nematodes with EGb 761 increased their resistance to an acute oxidative stress by 33%, and their thermo-tolerance by 25% (Wu et al., 2002) suggesting that EGb 761 can successfully counteract oxidative and thermal stress. As a consequence, the wild type *C. elegans* fed with EGb 761 lived longer than their untreated controls (Wu et al., 2002).

In order to demonstrate a possible link between oxidative stress and A β -expression we established an assay for the measurement of intracellular levels of H₂O₂-related reactive oxygen species (ROS), using 2',7'-dichlorofluorescein (DCF) methods. Our data indicates that EGb 761 significantly attenuates both A β and Juglone-induced ROS production in the transgenic A β -expressing neuroblastoma cells (Smith and Luo, 2003). We then modified the assay to be used in the *C. elegans* and showed that the intracellular level of ROS is significantly higher in the mutant *C. elegans* in an AD-associated strain CL2006 than in the wild-type (Smith and Luo, 2003) supporting the free-radical hypothesis of A β toxicity (Butterfield, 1997). We further demonstrated that EGb 761 attenuates elevated levels of ROS in the transgenic *C. elegans*. Flavonoid components, kaempferol and quercetin provided most significant effect. As a comparison, the known antioxidant ascorbic acid (vitamin C) also attenuated elevated ROS in *C.*

Table 1
Representative transcriptional effects of EGb 761 on NGF differentiated PC12 cells

Gene/function	Gene ID	Clone description	Fold change
<i>Apoptosis</i>			
Bcl-2 interacting protein	H3103B07	<i>Mus musculus</i> Bcl2/adenovirus E1B 19 kDa-interacting protein 3-like	1.95
Tumor necrosis factor	H3091D11	<i>Mus musculus</i> tumor necrosis factor superfamily member 19	1.86
Caspase 12	H3131G02	<i>Mus musculus</i> caspase 12	-1.74
Apoptosis regulator	H3038E03	PRKC, apoptosis, WT1, regulator	-1.84
<i>Other Mitochip clones</i>			
ATPase like proton channel	H3027A10	<i>Mus musculus</i> ATPase-like vacuolar proton channel (Atp1)	1.89
Choline transporter	H3102C06	Choline transporter (CHOT1)	1.76
Brain cDNA clone MNCb-0663	H3122H03	<i>Mus musculus</i> brain cDNA, clone MNCb-0663, liver regeneration-like	1.71
Voltage-gated-sodium channel	H3149E11	<i>Homo sapiens</i> sodium channel, voltage-gated type II	-1.71
Glutathione-S-transferase	H3111F09	Rat Y-b3 glutathione-S-transferase mRNA	-1.81
Glycogen phosphorylase	H3117G06	Rat glycogen phosphorylase brain isozyme mRNA	-1.91
Serine protease	H3045E05	<i>Mus musculus</i> serine protease OMI	-2.02
Glycerol-3-phosphate dehydrogenase	H3005G01	Mouse mRNA for glycerol-3-phosphate dehydrogenase	-2.09
Mitotic arrest deficient	H3124D11	<i>Mus musculus</i> mitotic arrest deficient 1-like	-3.16

Total mRNA was extracted from the PC12 cells treated with or without EGb 761 (100 μ g/ml for 48 h). cDNAs for array printing were amplified by PCR. Mouse Mitochip array consists of 816 cDNA clones originated from the NIA 15k mouse cDNA library. Positive numbers and negative numbers indicate up-regulation or down-regulation of the transcription by EGb 761 treatment (Smith et al., 2002).

elegans, but to a lesser extent than the flavonoids (Smith and Luo, 2003). This assay further allowed us to monitor the anti-oxidative effect of EGb 761 kinetically using the temperature inducible CL4176 (unpublished results).

The amyloid hypothesis remains controversial partly because the specific neurotoxic species of A β and the nature of its effects in vivo have not been defined. In an A β -expressing neuronal cell line, we demonstrated that EGb 761 appears to inhibit formation of an extracellular, SDS-stable A β species of molecular weight around 7–10 kD, which corresponds to an A β dimer (Luo et al., 2002). The SDS-stable A β oligomers (Mr ~8–12 kD) have been detected by Western blotting in the soluble fraction of Alzheimer's diseased cortex (McLean et al., 1999), in certain cultured cells (Morishima-Kawashima and Ihara, 1998; Walsh et al., 2000), and have been shown to inhibit hippocampal long-term potentiation (Walsh et al., 2002).

To verify this inhibitory effect of EGb 761 in vivo, we have analyzed tissue samples from an intracellular A β -expressing *C. elegans* strain CL2006 (Lim et al., 2001) fed with or without EGb 761 by Western blotting with an anti-A β antibody for A β species. Our data show that multiple A β immunoreactive bands were detected in the A β -expressing *C. elegans*, and an A β species with molecular weight at around 14 kD (oligomers) were decreased in EGb 761-fed *C. elegans* (unpublished). Interestingly, Congo red also decreased this A β species. The 14 kD species inhibited by EGb 761 is very similar to, in terms of its size, the neurotoxic small diffusible A β oligomers referred to as ADDLs, for A β -derived diffusible ligands, which were found to kill mature neurons in cultured hippocampal slices at nanomolar concentrations (Lambert et al., 1998) and can be inhibited in vitro by EGb 761 in a dose-dependent manner (Yao et al., 2001).

To determine whether EGb 761-induced inhibition of A β oligomerization in the *C. elegans* is associated with cellular function, we further examined the motility of transgenic CL2006 strain across their life span. Our results show that EGb 761 delays a muscle-specific motility decline in transgenic CL2006 and this effect is specific against A β -toxicity. To establish a temporal relationship between onset of A β oligomerization and A β -induced toxicity, we measured the temperature-inducible paralysis in CL4176 strain. We observed a convincing delay of paralysis in the worms fed with EGb 761. Congo red which was shown to inhibit the A β oligomers in CL2006, did not generate significant decrease in A β -induced paralysis in CL 4176 (unpublished). We reasoned that EGb 761 offers more protective activities than anti-A β aggregation alone.

Using the “wonder” worms, we correlated A β aggregates with their toxicity. Identifying the toxic form of A β in an organism is novel. Our findings strongly support the current theory that the small amyloid beta aggregates are more risky than the larger aggregates, which were originally thought to be the main cause of Alzheimer's disease. Interestingly, a known antioxidant vitamin C alone was not sufficient to ease the toxicity in the paralyzed worms. We assume that it is the combined properties (anti-oxidative and anti-amyloidogenic) of the *Ginkgo biloba* extract that protect the brain against cognitive

dysfunction. In further research, we hope to differentiate effects of individual components of the *Ginkgo biloba* extract and to determine the optimal concentration of the extract in the organism.

Summary

We demonstrated that EGb 761 exerts an anti-A β aggregation effect in a neuroblastoma cell line expressing A β (Luo et al., 2002). The anti-apoptotic properties of EGb 761 are supported by DNA microarray method in PC 12 cells (Table 1 and Smith et al., 2002). Furthermore, EGb 761 exhibits anti-stress effects in wild type *C. elegans* (Wu et al., 2002), reduces intracellular free-radical production in the transgenic *C. elegans* (Smith and Luo, 2003) and significantly attenuates expression of stress-response protein hsp16-2 in *C. elegans* (Strayer et al., 2003). These observations suggest a functional linkage between anti-oxidative and stress-response pathways in EGb 761 neuroprotection. We have recently observed that EGb 761 decreases A β oligomerization and A β induced paralysis in transgenic *C. elegans* (unpublished result). These findings suggest that A β oligomers and A β -induced oxidative stress are crucial for A β toxicity, and EGb 761 has a clear therapeutic potential for prevention and treatment of AD.

Acknowledgment

Special thanks to my collaborators Dr. Chris Link and Dr. Ikhtas Khan, and members in my lab who contributed to this study: Marishka Brown, Adam Burdick, Astrid Gutierrez-Zepeda, Julie Smith, Amy Strayer, Yanjue Wu, Zhixin Wu and Yanan Xu. This study is supported by NIH grant R01AT001928-01A1 from the National Center for Complementary and Alternative Medicine (NCCAM), and by the IPSEN, Paris, France.

References

- Bastianetto, S., Ramassamy, C., Dore, S., Christen, Y., Poirier, J., Quirion, R., 2000. The Ginkgo biloba extract (EGb 761) protects hippocampal neurons against cell death induced by beta-amyloid. *European Journal of Neuroscience* 12 (6), 1882–1890.
- Blanchard, B.J., Chen, A., Rozeboom, L.M., Stafford, K.A., Weigle, P., Ingram, V.M., 2004. Efficient reversal of Alzheimer's disease fibril formation and elimination of neurotoxicity by a small molecule. *Proceedings of the National Academy of Sciences of the United States of America* 101 (40), 14326–14332.
- Blavet, N., 1992. Effects of Ginkgo Biloba Extract (EGb 761) on the Central Nervous System. Elsevier, Paris.
- Blumenthal, M., 2000. Preface. In: Weiss, R.F., Fintemann, V. (Eds.), *Herbal Medicine*, 2nd Ed. Thieme Stuttgart, New York.
- Borchelt, D.R., Ratovitski, T., van Lare, J., Lee, M.K., Gonzales, V., Jenkins, N.A., Copeland, N.G., Price, D.L., Sisodia, S.S., 1997. Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* 19 (4), 939–945.
- Brookmeyer, R., Gray, S., Kawas, C., 1998. Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. *American Journal of Public Health* 88 (9), 1337–1342.
- Butterfield, D.A., 1997. beta-Amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease. *Chemical Research in Toxicology* 10 (5), 495–506.

- Butterfield, D.A., 2003. Amyloid beta-peptide [1–42]-associated free radical-induced oxidative stress and neurodegeneration in Alzheimer's disease brain: mechanisms and consequences. *Current Medicinal Chemistry* 10 (24), 2651–2659.
- Christen, Y., Maixent, J.M., 2002. What is Ginkgo biloba extract EGb 761? An overview—from molecular biology to clinical medicine. *Cellular and Molecular Biology* 48 (6), 601–611.
- Christen, Y., Olano-Martin, E., Packer, L., 2002. EGb 761 in the postgenomic era: new tools from molecular biology for the study of complex products such as Ginkgo biloba extract. *Cellular and Molecular Biology* 48 (6), 593–599.
- Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., Selkoe, D.J., 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* 360 (6405), 672–674.
- Curtis-Prior, P., Vere, D., Fray, P., 1999. Therapeutic value of Ginkgo biloba in reducing symptoms of decline in mental function. *Journal of Pharmacy and Pharmacology* 51, 535–541.
- DeFeudis, F.V., 1998. Ginkgo Biloba Extract (EGb 761): from Chemistry to Clinic. *Publi Ullstein Med.* Weisbaden, Germany.
- DeFeudis, F.V., 2002. Effects of Ginkgo biloba extract (EGb 761) on gene expression: possible relevance to neurological disorders and age-associated cognitive impairment. *Drug Development Research* 57, 214–235.
- DeFeudis, F.V., Drieu, K., 2000. Ginkgo biloba extract (EGb 761) and CNS functions: basic studies and clinical applications. *Current Drug Targets* 1 (1), 25–58.
- Drake, J., Link, C.D., Butterfield, D.A., 2003. Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1–42) in a transgenic *Caenorhabditis elegans* model. *Neurobiology of Aging* 24 (3), 415–420.
- Driscoll, M., Gerstbrein, B., 2003. Dying for a cause: invertebrate genetics takes on human neurodegeneration. *Nature Reviews. Genetics* 4 (3), 181–194.
- Eisenberg, D.M., Davis, R.B., Ettner, S.L., Appel, S., Wilkey, S., Van Rompay, M., Kessler, R.C., 1998. Trends in alternative medicine use in the United States, 1990–1997: results of a follow-up national survey. *JAMA* 280 (18), 1569–1575.
- Ernst, E., 2002. The risk–benefit profile of commonly used herbal therapies: Ginkgo, St. John's Wort, Ginseng, Echinacea, Saw Palmetto, and Kava. *Annals of Internal Medicine* 136 (1), 42–53.
- Fay, D.S., Fluet, A., Johnson, C.J., Link, C.D., 1998. In vivo aggregation of beta-amyloid peptide variants. *Journal of Neurochemistry* 71 (4), 1616–1625.
- Fonte, V., Kapulkin, V., Taft, A., Fluet, A., Friedman, D., Link, C.D., 2002. Interaction of intracellular beta amyloid peptide with chaperone proteins. *Proceedings of the National Academy of Sciences of the United States of America* 99 (14), 9439–9444.
- Ginsberg, S.D., Che, S., 2002. RNA amplification in brain tissues. *Neurochemical Research* 27 (10), 981–992.
- Gohil, K., Packer, L., 2002. Global gene expression analysis identifies cell and tissue specific actions of Ginkgo biloba extract, EGb 761. *Cellular and Molecular Biology (Noisy-le-grand)* 48 (6), 625–631.
- Goto, S., 2003. *Biological Implications of Protein Oxidation*. World Scientific Publishing, New Jersey.
- Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297 (5580), 353–356.
- Hensley, K., Hall, N., Subramaniam, R., Cole, P., Harris, M., Aksenov, M., Aksenova, M., Gabbita, S.P., Wu, J.F., Carney, J.M., et al., 1995. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. *Journal of Neurochemistry* 65 (5), 2146–2156.
- Higgins, G.A., Jacobsen, H., 2003. Transgenic mouse models of Alzheimer's disease: phenotype and application. *Behavioural Pharmacology* 14 (5–6), 419–438.
- Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., Mucke, L., 1999. Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proceedings of the National Academy of Sciences of the United States of America* 96 (6), 3228–3233.
- Iijima, K., Liu, H.P., Chiang, A.S., Hearn, S.A., Konsolaki, M., Zhong, Y., 2004. Dissecting the pathological effects of human A β 40 and A β 42 in *Drosophila*: a potential model for Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America* 101 (17), 6623–6628.
- Koistinaho, M., Ort, M., Cimadevilla, J.M., Vondrou, R., Cordell, B., Koistinaho, J., Bures, J., Higgins, L.S., 2001. Specific spatial learning deficits become severe with age in beta-amyloid precursor protein transgenic mice that harbor diffuse beta-amyloid deposits but do not form plaques. *Proceedings of the National Academy of Sciences of the United States of America* 98 (25), 14675–14680.
- Kudva, Y.C., Hiddinga, H.J., Butler, P.C., Mueske, C.S., Eberhardt, N.L., 1997. Small heat shock proteins inhibit in vitro A beta(1–42) amyloidogenesis. *FEBS Letters* 416 (1), 117–121.
- Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., Klein, W.L., 1998. Diffusible, nonfibrillar ligands derived from A β 1–42 are potent central nervous system neurotoxins. *Proceedings of the National Academy of Sciences of the United States of America* 95 (11), 6448–6453.
- Lansbury Jr., P.T., 1999. Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. *Proceedings of the National Academy of Sciences of the United States of America* 96 (7), 3342–3344.
- Le Bars, P.L., Katz, M.M., Berman, N., Itil, T.M., Freedman, A.M., Schatzberg, A.F., 1997. A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo biloba for dementia. North American EGb study group. *JAMA* 278 (16), 1327–1332.
- Le Bars, P.L., Kieser, M., Itil, K.Z., 2000. A 26-week analysis of a double-blind, placebo-controlled trial of the ginkgo biloba extract EGb 761 in dementia. *Dementia and Geriatric Cognitive Disorders* 11 (4), 230–237.
- Lien, E.J., Ren, S., Bui, H.H., Wang, R., 1999. Quantitative structure–activity relationship analysis of phenolic antioxidants. *Free Radical Biology & Medicine* 26 (3–4), 285–294.
- Lim, G.P., Yang, F., Chu, T., Gahtan, E., Ubeda, O., Beech, W., Overmier, J.B., Hsiao-Ashec, K., Frautschy, S.A., Cole, G.M., 2001. Ibuprofen effects on Alzheimer pathology and open field activity in APPsw transgenic mice. *Neurobiology of Aging* 22 (6), 983–991.
- Link, C.D., 1995. Expression of human beta-amyloid peptide in transgenic *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America* 92 (20), 9368–9372.
- Link, C.D., Johnson, C.J., Fonte, V., Paupard, M., Hall, D.H., Styren, S., Mathis, C.A., Klunk, W.E., 2001. Visualization of fibrillar amyloid deposits in living, transgenic *Caenorhabditis elegans* animals using the sensitive amyloid dye, X-34. *Neurobiology of Aging* 22 (2), 217–226.
- Link, C.D., Taft, A., Kapulkin, V., Duke, K., Kim, S., Fei, Q., Wood, D.E., Sahagan, B.G., 2003. Gene expression analysis in a transgenic *Caenorhabditis elegans* Alzheimer's disease model. *Neurobiology of Aging* 24 (3), 397–413.
- Lorenzo, A., Yankner, B.A., 1994. Beta-amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. *Proceedings of the National Academy of Sciences of the United States of America* 91 (25), 12243–12247.
- Luo, Y. *Contemporary Neuroscience Meets Traditional Medicine—Towards Understanding Ginkgo Biloba Neuroprotection*. *Current Topics in Nutra-ceutical Research* (in press).
- Luo, Y., Smith, J.V., Paramasivam, V., Burdick, A., Curry, K.J., Buford, J.P., Khan, I., Netzer, W.J., Xu, H., Butko, P., 2002. Inhibition of amyloid-beta aggregation and caspase-3 activation by the Ginkgo biloba extract EGb761. *Proceedings of the National Academy of Sciences of the United States of America* 99 (19), 12197–12202.
- Mann, D.M., 1989. Cerebral amyloidosis, ageing and Alzheimer's disease; a contribution from studies on Down's syndrome. *Neurobiology of Aging* 10 (5), 397–399 (discussion 412–4).
- McLean, C.A., Cherny, R.A., Fraser, F.W., Fuller, S.J., Smith, M.J., Beyreuther, K., Bush, A.I., Masters, C.L., 1999. Soluble pool of A β amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Annals of Neurology* 46 (6), 860–866.

- McLellan, M.E., Kajdasz, S.T., Hyman, B.T., Bacskai, B.J., 2003. In vivo imaging of reactive oxygen species specifically associated with thioflavine S-positive amyloid plaques by multiphoton microscopy. *Journal of Neuroscience* 23 (6), 2212–2217.
- Meyer, B., 1986. A Multicenter Study of Tinnitus Epidemiology and Therapy. *Ann. Otolaryngol. Chir. Cervicofac.*, vol. 103(3), pp. 185–188.
- Mix, J.A., David Crews Jr., W., 2002. A double-blind, placebo-controlled, randomized trial of Ginkgo biloba extract EGb 761(R) in a sample of cognitively intact older adults: neuropsychological findings. *Human Psychopharmacology* 17 (6), 267–277.
- Morishima-Kawashima, M., Ihara, Y., 1998. The presence of amyloid beta-protein in the detergent-insoluble membrane compartment of human neuroblastoma cells. *Biochemistry* 37 (44), 15247–15253.
- NIH, 2002. Basic and Preclinical Research on Complementary and Alternative Medicine PA-02-124.
- Normile, D., 2003. Asian medicine. The New Face of Traditional Chinese Medicine Science, vol. 299(5604), pp. 188–190.
- Oberpichler, H., Sauer, D., Rossberg, C., Mennel, H.D., Krieglstein, J., 1990. PAF antagonist ginkgolide B reduces postischemic neuronal damage in rat brain hippocampus. *Journal of Cerebral Blood Flow and Metabolism* 10 (1), 133–135.
- Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kaye, R., Metherate, R., Mattson, M.P., Akbari, Y., LaFerla, F.M., 2003. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular A β and synaptic dysfunction. *Neuron* 39 (3), 409–421.
- Oken, B.S., Storzbach, D.M., Kaye, J.A., 1998. The efficacy of Ginkgo biloba on cognitive function in Alzheimer disease. *Archives of Neurology* 55, 1409–1415.
- Pasinetti, G.M., 2001. Use of cDNA microarray in the search for molecular markers involved in the onset of Alzheimer's disease dementia. *Journal of Neuroscience Research* 65 (6), 471–476.
- Pike, C.J., Walencewicz-Wasserman, A.J., Kosmoski, J., Cribbs, D.H., Glabe, C.G., Cotman, C.W., 1995. Structure–activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity. *Journal of Neurochemistry* 64 (1), 253–265.
- Price, D.L., Sisodia, S.S., Borchelt, D.R., 1998. Alzheimer disease—when and why? *Nature Genetics* 19 (4), 314–316.
- Selkoe, D.J., 1997. Alzheimer's disease: genotypes, phenotypes, and treatments. *Science* 275 (5300), 630–631.
- Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., et al., 1995. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375 (6534), 754–760.
- Skovronsky, D.M., Doms, R.W., Lee, V.M., 1998. Detection of a novel intraneuronal pool of insoluble amyloid beta protein that accumulates with time in culture. *Journal of Cell Biology* 141 (4), 1031–1039.
- Smith, J.V., Luo, Y., 2003. Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by Ginkgo biloba Extract EGb 761. *Journal of Alzheimer's Disease* 5, 287–300.
- Smith, J.V., Luo, Y., 2004. Studies on molecular mechanisms of Ginkgo biloba extract. *Applied Microbiology and Biotechnology* 64 (4), 465–472.
- Smith, P.F., Maclennan, K., Darlington, C.L., 1996. The neuroprotective properties of the Ginkgo biloba leaf: a review of the possible relationship to platelet-activating factor (PAF). *Journal of Ethnopharmacology* 50 (3), 131–139.
- Smith, J.V., Burdick, A.J., Golik, P., Khan, I., Wallace, D., Luo, Y., 2002. Anti-apoptotic properties of Ginkgo biloba extract EGb 761 in differentiated PC12 cells. *Cellular and Molecular Biology* 48 (6), 699–707.
- Solomon, P.R., Adams, F., Silver, A., Zimmer, J., DeVeaux, R., 2002. Ginkgo for memory enhancement: a randomized controlled trial. *JAMA* 288 (7), 835–840.
- Sommer, B., Sturchler-Pierrat, C., Abramowski, D., Wiederhold, K.H., Calhoun, M., Jucker, M., Kelly, P., Staufenbiel, M., 2000. Transgenic approaches to model Alzheimer's disease. *Reviews in the Neurosciences* 11 (1), 47–51.
- Soulie, C., Nicole, A., Christen, Y., Ceballos-Picot, I., 2002. The Ginkgo biloba extract EGb 761 increases viability of hnt human neurons in culture and affects the expression of genes implicated in the stress response. *Cellular and Molecular Biology (Noisy-le-grand)* 48 (6), 641–646.
- Stege, G.J., Renkawek, K., Overkamp, P.S., Verschuure, P., van Rijk, A.F., Reijnen-Aalbers, A., Boelens, W.C., Bosman, G.J., de Jong, W.W., 1999. The molecular chaperone α B-crystallin enhances amyloid beta neurotoxicity. *Biochemical and Biophysical Research Communications* 262 (1), 152–156.
- Strayer, A., Wu, Z.-X., Christen, Y., Link, C.D., Luo, Y., 2003. Expression of small heat-shock protein Hsp16-2 in *Caenorhabditis elegans* is suppressed by Ginkgo Biloba Extract EGb 761. *FASEB Journal* (Oct 2).
- Takahashi, R.H., Milner, T.A., Li, F., Nam, E.E., Edgar, M.A., Yamaguchi, H., Beal, M.F., Xu, H., Greengard, P., Gouras, G.K., 2002. Intraneuronal Alzheimer a β 42 accumulates in multivesicular bodies and is associated with synaptic pathology. *American Journal of Pathology* 161 (5), 1869–1879.
- van Leuven, F., 2000. Single and multiple transgenic mice as models for Alzheimer's disease. *Progress in Neurobiology* 61 (3), 305–312.
- Walsh, D.M., Selkoe, D.J., 2004. Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. *Protein Peptide Letters* 11 (3), 213–228.
- Walsh, D.M., Tseng, B.P., Rydel, R.E., Podlisny, M.B., Selkoe, D.J., 2000. The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. *Biochemistry* 39 (35), 10831–10839.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., Selkoe, D.J., 2002. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416 (6880), 535–539.
- Watanabe, C.M., Wolfram, S., Ader, P., Rimbach, G., Packer, L., Maguire, J.J., Schultz, P.G., Gohil, K., 2001. The in vivo neuromodulatory effects of the herbal medicine ginkgo biloba. *Proceedings of the National Academy of Sciences of the United States of America* 98 (12), 6577–6580.
- Winter, E., 1991. Effects of an extract of Ginkgo biloba on learning and memory in mice. *Pharmacology, Biochemistry and Behavior* 38 (1), 109–114.
- Winter, J.C., 1998. The effects of an extract of Ginkgo biloba, EGb 761, on cognitive behavior and longevity in the rat. *Physiology & Behavior* 63 (3), 425–433.
- Wu, Z., Smith, J.V., Paramasivam, V., Butko, P., Khan, I., Cypser, J.R., Luo, Y., 2002. Ginkgo biloba extract EGb 761 increases stress resistance and extends life span of *Caenorhabditis elegans*. *Cellular and Molecular Biology* 48 (6), 725–731.
- Yao, Z., Driue, K., Papadopoulos, V., 2001. 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 Research* 889 (1–2), 181–190.
- Yatin, S.M., Varadarajan, S., Link, C.D., Butterfield, D.A., 1999. In vitro and in vivo oxidative stress associated with Alzheimer's amyloid beta-peptide (1–42). *Neurobiology of Aging* 20 (3), 325–330 (discussion 339–42).
- Zimmermann, M., Colciaghi, F., Cattabeni, F., Di Luca, M., 2002. Ginkgo biloba extract: from molecular mechanisms to the treatment of Alzheimer's disease. *Cellular and Molecular Biology (Noisy-le-grand)* 48 (6), 613–623.