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Nanolipidic particles improve the bioavailability and α-secretase inducing ability of epigallocatechin-3-gallate (EGCG) for the treatment of Alzheimer's disease

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ABSTRACT

Prevention of amyloidogenic processing of amyloid precursor protein with the use of natural phytochemicals capable of enhancing α-secretase activity may be a therapeutic approach for treatment of neurodegenerative diseases including Alzheimer's disease (AD) and HIV-associated dementia (HAD). We have recently shown promising preclinical results with the use of green tea polyphenol, (−)-epigallocatechin-3-gallate (EGCG) in mouse models of both diseases, however the translation into clinical use has been problematic primarily as a result of poor bioavailability and inefficient delivery to the central nervous system (CNS). While the antioxidant properties of EGCG are well known, we have shown that it is able to promote non-amyloidogenic processing of amyloid precursor protein (APP) by upregulating α-secretase, thus preventing brain beta amyloid plaque formation, a hallmark of AD pathology and common finding in HIV infection. In this preliminary study, we investigated the ability of one preformulation method to improve the oral bioavailability of EGCG. We found that forming nanolipidic EGCG particles improves the neuronal (SweAPP N2a cells) α-secretase enhancing ability *in vitro* by up to 91% ($P < 001$) and it's oral bioavailability *in vivo* by more than two-fold over free EGCG.

1. Introduction

The deterioration, malfunction, or death of neurons is a common etiological factor in several diseases including Alzheimer's disease (AD) and HIV-associated dementia (HAD) (Alisky, 2007; Wojtera et al., 2005). As the number of elderly individuals continues to rapidly increase, neurodegenerative disease, marked by progressive loss of mnemonic and higher cortical functions, has led to a massive socioeconomic burden which is projected to worsen (Tarkowski et al., 2003). Specifically, some 15% of the population greater than 65 years of age suffers from dementia (Meeuwsen et al., 2009). Its presentation is heterogeneous as it is caused by multiple disorders. Alzheimer's disease (AD) and vascular dementia (VaD) are the two main causes of dementia affecting between 25–45% and 15–35%, respectively, of all patients suffering from dementia (Burns and Iliffe, 2009). Among dementias where brain infectious viruses are etiologic, human immunodeficiency virus type 1 (HIV-1) associated dementia (HAD) is the most common cause of dementia (Ghafouri et al., 2006). We have previously shown that modulation of apoptosis cascades (Giunta et al., 2006, 2008), and APP (amyloid precursor protein) processing (Obregon et al., 2006; Rezai-Zadeh et al., 2005, 2009) with the green tea polyphenol, (−)-epigallocatechin-3-gallate (EGCG) is a plausible therapy in mouse models of AD and HAD. In spite of these preclinical works, translating them to a human clinical trial has presented problems, primarily as a result of inefficient systemic delivery and bioavailability issues. To achieve maximum response of a neuroprotective agent, novel strategies are required to enhance the oral bioavailability of potentially useful agents. Self-assembled polymer

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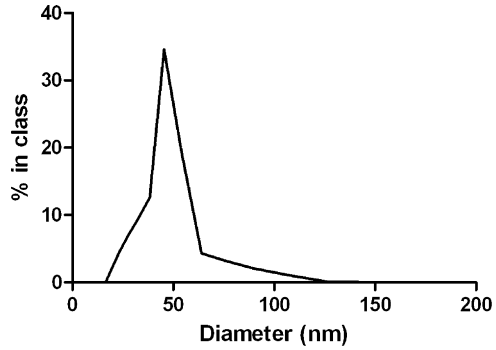

Fig. 1. Dynamic light scattering data. A Wyatt DynaPro Multiwell Reader was used to characterize the diameter of the NanoEGCG particles. The data indicates a narrow size distribution, with a cumulants mean of 49.5 nm and polydispersity of 0.052.

micelles based on amphiphilic block copolymers have attracted substantial interest as delivery vehicles particularly for anti-cancer drugs (Bontha et al., 2006; Kim et al., 2009; Liu et al., 2006; Tian et al., 2007; Yokoyama et al., 1998; Huynh et al., 2009; Siddiqui et al., 2009). Additionally, lipid carriers with incorporated drugs have been demonstrated to increase the absorption and circulation time in the body versus stand-alone compounds secondary to minimized renal clearance (Huynh et al., 2009; Maeda, 2001). This study investigated the ability of nanolipidic particle complexes for increasing the oral bioavailability of EGCG. These nanoparticles (NanoEGCG) differ from traditional liposomes because they do not require micelle formation. Rather, they are drug:lipid complexes. This enables the formation of smaller diameter particles that we hypothesized would be useful for increasing the oral bioavailability of EGCG.

2. Materials and methods

2.1. Reagents and materials

Green tea-derived EGCG (>95% purity by HPLC) was purchased from www.herbs-tech.com. The BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Anti-human amyloid-β antibodies 4G8 and 6E10 were obtained from Signet Laboratories (Dedham, MA, USA) and Biosource International (Camarillo, CA, USA), respectively.

2.2. Preparation of nanolipidic EGCG particles (NanoEGCG)

Nanolipidic particles (NanoEGCG) were prepared using a proprietary (US Provisional Patent Application #61/238,381) co-solubilization methodology involving use of monophasic liquid preparations developed by Nature's Defense Systems, Tampa, FL. These particles have a defined size range from 30 to 80 nm. Six nanoparticle formulations were prepared for the study with various ratios of lipid carrier to EGCG. Formulations prepared for this study were 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 (Nanocarrier material to EGCG on a mg/mg basis). To form NanoEGCG co-solubilization methodology involving use of monophasic liquid preparations were employed with proprietary starting materials. These materials are first solubilized into a water-in-ethanol solution (step 1). Anhydrous EGCG was added to the materials in step 1 and co-solubilized by mixing at room temperature (step 2). NanoEGCG particles were formed by the addition of distilled water while mixing materials (step 3). The final preparation of NanoEGCG particles was stirred for an additional 10 min prior to subjecting the preparation to sizing analysis with a Wyatt DynaPro Multiwell Reader (Wyatt Technology Corporation, Santa Barbara, CA). Fig. 1 shows a representative analysis of the size distribution of the 1:8 NanoEGCG formulation. The stock formulations were stored at 20 °C and protected from light until needed.

2.3. Neuronal sAPP-α ELISA

Murine neuroblastoma cells that were stably transfected with the human APP gene (APP; SweAPP N2a cells) were cultured in 24-well tissue-culture plates at 1×10^5 cells/well ($n = 2$ for each condition) with 0.5 mL of complete medium (MEM medium with 10% fetal calf serum). Prior to treatment, the MEM was aspirated and replaced with 0.5 mL of neurobasal media and differentiated with cAMP for 4 h. Following differentiation, the cells were treated with various nanoparticle formulations and controls (25–3 μM) for 18 h. Controls included two formulations of EGCG without a lipid carrier: one dissolved in water and another in ethanol and water at the same ratio that the nanoparticles were formed (described above). The conditioned media was collected and sAPP-α levels were quantified using a sAPP-α sandwich ELISA protocol as previously described (Bailey et al., 2008). High binding 96-well plates (Nunc, Denmark) were coated with monoclonal antibody 22C11 diluted in 100 μL (1 μg/mL) of carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plate was washed five times with PBS-Tween buffer (0.05% Tween 20) and blocked with 300 μL of blocking buffer (1% BSA and 5% Horse Serum in PBS) for 2 h at 37 °C. Synthetic sAPP-α protein (Abgent, San Diego, CA) was used as the positive control for this ELISA. All samples were analyzed in duplicate. 100 μL samples of conditioned media were added to each well of the plate. The plate was incubated for 2 h at 37 °C. After washing five times, 100 μL of goat anti-human antibody 6E10 (Biosource; diluted 1:3000 in reagent diluent) was added to each well of the plate. Following 2 h-incubation at 37 °C and five times washing, 100 μL of anti-goat IgG conjugated with HRP (1:1500) was added to each well of the plate. The plate was incubated for 1 h at 37 °C. Following five times washing, 100 μL of substrate solution (TMB) was added to each well and plate was incubated at room temperature. Twenty minutes later, 50 μL of stop solution (2N H_2SO_4) was added to each well of the plate. The optical density was determined using a microplate reader at 450 nm. Data were reported as ng of sAPP-α/mg of total intracellular protein produced per well. Total intracellular protein was quantified using a BCA kit (Pierce Biotechnology, Rockford, IL) in accordance with the manufacturer's instructions.

2.4. Pharmacokinetic screening of EGCG formulations in rats

Male Sprague Dawley rats weighing 200–250 g were purchased from Harlan Laboratories (Indianapolis, IN). The rats were pre-cannulated by Harlan. The rounded tip catheters were surgically implanted into the jugular vein of the rats making multiple, precise blood draws painless to the animal. The rats were food (not water) deprived for 18 h prior to the start of the experiment. The EGCG formulations were delivered *via* oral gavage at a dosage of 100 mg EGCG/kg body weight. Blood was collected at the following time points: 0, 5, 10, 30, 60, 120, 240, and 480 min. Because heparin was kept in the catheter lines to prevent clotting, a small amount of blood was drawn and discarded before collecting each sample. Approximately 300 μL of blood was collected in EDTA tubes for each time point. The samples were kept on ice to preserve their integrity, then centrifuged at 4000 rpm for 10 min, after which the plasma was transferred to sterile centrifuge tubes. A preservative solution was added to each plasma sample at 10% (v/v) concentration to ensure the integrity of the EGCG during storage (Lambert et al., 2006a). This preservative was comprised of 20% ascorbic acid (to prevent oxidation of EGCG) and 0.1% EDTA (to scavenge any metal contaminants). The samples were stored at −80 °C until they were analyzed for EGCG content.

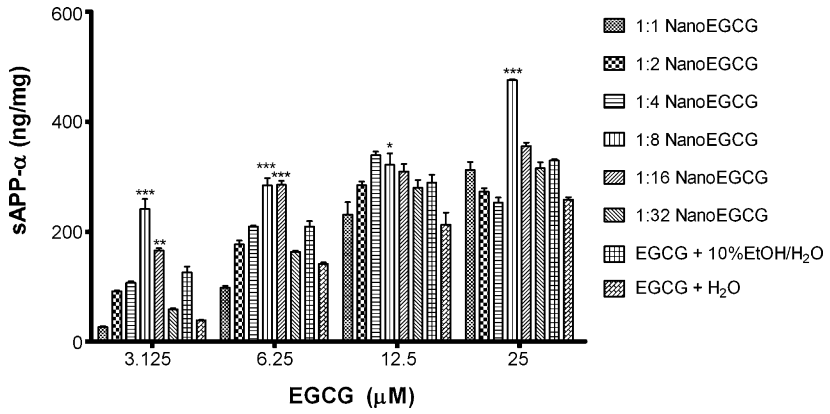

Fig. 2. Estimated sAPP-α generation for each treatment group. The sAPP-α concentration (ng/ml) was normalized to the total protein content (mg/ml). Data are presented as mean ng of sAPP-α per mg of total protein produced ± standard deviation. The 1:8 and 1:16 formulations were superior to the other formulations, with the 1:8 showing 92% improvement in α-secretase activity over the EtOH control at the 3 μM concentration. The 1:8 NanoEGCG formulation was statistically higher than the control at all concentrations tested. The 1:16 NanoEGCG formulation was statistically higher at the lower two concentrations (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

2.5. Quantification of EGCG in rat plasma

The plasma samples were blinded and sent to be analyzed for EGCG content by the Burnham Institute for Medical Research Pharmacology Core (Orlando, FL). To accurately quantify the concentration of EGCG in the plasma, a previously described method was employed using liquid chromatography with tandem mass spectrometry (Sparidans et al., 2007; Wang and Miksa, 2007; Wang et al., 2004, 2000).

2.5.1. Stock preparation

Accurately prepared a 2.00 mg/mL stock solution in DMSO of EGCG. The standard spiking solutions were prepared by diluting the stock solution to 1000 and 100 μg/mL using acetonitrile:water (1:1, v:v). Both solutions were protected from light using amber vials and all solutions were stored at −20 °C.

2.5.2. Standard curve preparation

For this analysis two standard curves were prepared one with a higher (10–0.100 μg/ml) dynamic range the other a lower (1000–10 ng/ml). Both standard curves were prepared using the appropriate blank rat plasma containing the preservative. The results indicated that the standard curve performance was within acceptable range for bioanalytical method acceptance ($R^2 > 0.99$) (Sparidans et al., 2007; Wang and Miksa, 2007; Wang et al., 2000).

2.6. Pharmacokinetic calculations

Mean plasma EGCG concentrations ± the standard error in the mean (SEM) were calculated using GraphPad PRISM software (GraphPad Software, Inc.). Pharmacokinetic graphs and parameters were determined using GraphPad PRISM. Pharmacokinetic parameters included C_{max}, T_{max}, area under curve (AUC), and relative bioavailiblity. Relative bioavailibility was determined by dividing the AUC of each NanoEGCG formulation by the AUC of the control.

2.7. Statistical analysis

2.7.1. sAPP-α ELISA

A two-way ANOVA was performed using GraphPad PRISM software (GraphPad Software, Inc.). This was followed by Bonferonni post-tests to assess the significance of each NanoEGCG formulation versus the EGCG/10%EtOH/H2O control at each concentration.

2.7.2. Pharmacokinetics

A two-way ANOVA was performed using GraphPad PRISM software (GraphPad Software, Inc.). This was followed by Bonferonni post-tests to assess the significance of the 1:16 NanoEGCG formulation versus the EGCG/10%EtOH/H2O control at each time point.

3. Results

3.1. Encapsulating EGCG increases sAPP-α generation in cultured SweAPP N2a cells

We utilized an *in vitro* model for Alzheimer's disease to test the hypothesis that formation of nanoparticle complexes would increase the bioactivity of EGCG by promoting α-secretase activity in cultured SweAPP N2a cells. These cells overproduce human APP, making them ideal for screening compounds that modulate APP processing (Obregon et al., 2006). Additionally, we used this assay as a criterion to select the most effective nanoparticle formulations to carry through to the pharmacokinetic pilot study. Fig. 2 shows the mean ng of sAPP-α per mg of total protein produced ± standard deviation for all EGCG formulations. Because ethanol was used to solubilize the lipid carrier and EGCG during the NanoEGCG productions process, it was appropriate to include a similarly formulated EGCG solution (10% EtOH solution v/v) to rule out any potential gains in α-secretase activity being due to the alcohol content of the nanoparticle formulations.

From Fig. 2, not all NanoEGCG formulations were effective. In fact, the 1:1 and 1:2 formulations were outperformed by the EGCG and 10% EtOH/H2O control at all concentrations tested. The 1:8 and 1:16 NanoEGCG formulations were selected to be advanced to the pharmacokinetics phase of the study because they outperformed the control at all concentrations tested. The 1:8 formulation was statistically significant at all concentrations, whereas the 1:16 was only statistically significant at the lower two concentrations. Not only did these formulations show marked increases in sAPP-α generation but, perhaps more importantly, they continued to promote enhanced levels of α-secretase activity even at the lowest EGCG concentration tested.

3.2. Encapsulation improves the bioavailability of EGCG in rats

There have been numerous groups to report the poor oral bioavailability of EGCG (Feng, 2006; Cai et al., 2002; Chan et al., 2007; Henning et al., 2008; Lambert et al., 2004, 2006b; Lin et al., 2007; Zhang et al., 2004). Recent reports suggest that this poor oral bioavailability is mostly due to factors such as poor absorp-

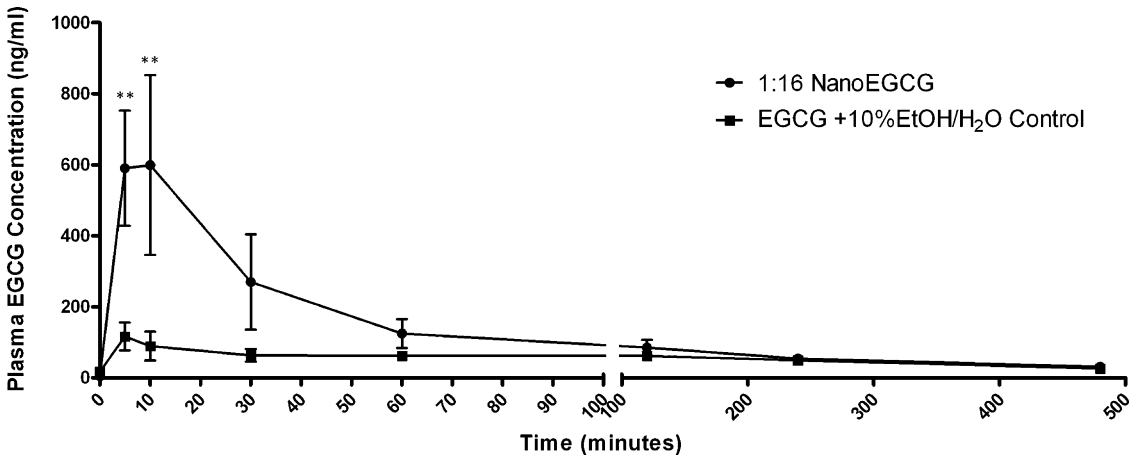

Fig. 3. EGCG pharmacokinetic curve (mean plasma concentration ± SEM versus time) for the 1:16 NanoEGCG formulation ($n = 3$) and free EGCG in 10% EtOH solution ($n = 3$). The nanoparticle formulation resulted in substantial increase in systemic EGCG absorption. Statistical significance ($^{**}P < 0.01$) was observed at the 5 and 10 min time points. The 10% EtOH control had very poor absorption, with plasma concentration peaking at 116.57 ng/ml. In comparison, the 1:16 NanoEGCG reached a maximum plasma concentration of 599.33 ng/ml.

tion and intestinal metabolism, rather than elimination via first pass metabolism (Cai et al., 2002). Larger lipid-based bilayer delivery systems have been shown to increase the absorption of poorly permeable compounds (Allen, 1998). This preliminary study evaluated the ability of proprietary lipid nanoparticle complexes to increase the oral bioavailability of EGCG in rats. Our results indicate that nanoparticles are highly effective at increasing the absorption of EGCG into systemic circulation. Fig. 3 shows a compilation of the mean pharmacokinetic curves for the nanoparticle formulation tested and the control. Because EGCG is poorly water soluble, 10% EtOH was added to fully solubilize the EGCG at a concentration equivalent to the NanoEGCG stock (50 mg/ml) and ensure accurate dosing in the control. Our data suggests that the nanoparticle formulations result in substantial increases in the absorption of EGCG. Although Fig. 3 indicates only one NanoEGCG curve, both 1:8 and 1:16 formulations were tested. However, both nanoparticle formulations were similarly absorbed and not statistically different, so the 1:16 preperation was selected to represent the NanoEGCG pharmacokinetic curve. The control was very poorly absorbed in comparison to the NanoEGCG. Statistical significance ($^{**}P < 0.01$) was observed at the 5 and 10 min time points. Table 1 shows some important pharmacokinetic parameters: C_{max}, T_{max}, AUC, and relative bioavailiblity. The relative bioavailability (defined by the AUC) of the NanoEGCG was 2.31 and 2.50 for the 1:16 and 1:8 formulations, respectively, in comparison to the free EGCG in 10% EtOH solution (10% EtOH control).

4. Discussion

Nanoparticles and larger liposomes have been investigated extensively for increasing the oral bioavailability of poorly absorbed compounds (Frezard et al., 2008; He et al., 2007; Kumar et al., 2007; Pandey et al., 2005; Rao et al., 2008). It has been recently reported that encapsulating EGCG into liposomes can improve its anti-cancer efficacy (Siddiqui et al., 2009) and antioxidant capacity (Italia et al., 2008), probably by increasing its bioavailability. However, these studies utilized larger diameter particles (>100 nm) and focused primarily on improved efficacy of EGCG for specific disease modifying parameters. Here, we have tested the ability of small diameter nanolipidic particle formation as a method for increasing not only the α-secretase inducing ability of EGCG, but also its oral bioavailability.

It has been shown that an oral dose of 800 mg/70 kg/day provides approximately 400 ng/ml EGCG in human plasma (Chow et al., 2001). Given that we have recently shown that 1000–2000ng/mL of free EGCG is necessary for promoting APP α-secretase cleavage in SweAPP N2a cells (Rezai-Zadeh et al., 2005), using linear approximation, an oral dose of EGCG of 1800 mg/70 kg/day would be required to reach therapeutically effective plasma concentrations of EGCG. From a safety and practicality point of view, this dose might be unacceptable for clinical trials (Ullmann et al., 2004, 2003). Since the oral EGCG dosage in most clinical trials for cancer therapy is typically not more than 800 mg/day (Chow et al., 2001) regimens which enhance EGCG bioavailability, effecting reductions in neuropathology and cognitive decline at minimum doses, are very desirable. Thus, the bioavailability of EGCG is an important issue for oral administration of EGCG to clinical trials.

It has been previously reported that decreased bioavailability of EGCG is greatly associated with the glucuronidated form, which is largely present in the plasma of treated mice (Lambert et al., 2003). Additionally, it has been shown that piperine, an alkaloid derived from black pepper, enhances the bioavailability of EGCG by inhibiting glucuronidation (Lambert et al., 2004). Unfortunately the consumption of piperine also influences the metabolism of all other ingested food and drugs (Khajuria et al., 2002). For example it increases the plasma concentration of phenytoin (Pattanaik et al., 2006), propanalol, and theophylline in healthy volunteers (Bano et al., 1991) and plasma concentrations of rifamipicin (rifampin^{TM}) in patients with pulmonary tuberculosis (Zutshi et al., 1985). By forming EGCG nanolipidic complexes as we have, it is possible to increase the oral bioavailability of EGCG as well as its AD and HAD preventative and therapeutic actions, without affecting the absorption of other ingested compounds. This may be an important factor

Table 1
Pharmacokinetic parameters.

Treatment	C_{max}	T_{max}	AUC (0–240 min)	Relative bioavailability
EGCG + 10% EtOH (control)	116.57	5	14621	1
NanoEGCG (1:16)	599.33	10	33722	2.31
NanoEGCG (1:8)	704.67	5	36524	2.50

to consider when bringing an EGCG therapeutic into the clinical setting.

In this study, we have modified EGCG such that it requires no co-administration of other drugs. Rather, it is co-solubilized with a lipid carrier using proprietary methodology to form 30–80 nm diameter nanoparticle complexes. The importance of particle diameter for drug delivery is particularly important for delivery of drugs to the brain (Wissing et al., 2004). Previously, even smaller diameter liposomes (100 nm) have had trouble penetrating the tight junctions between the endothelial cells of the blood brain barrier without osmotic disruption (Sakamoto and Ido, 1993). This highlights an important distinction between this nanoparticle technology and previous liposomal technologies, which require micelle formation. NanoEGCG does not involve encapsulating the EGCG into a micelle. Instead, lipid:EGCG complexes are formed. Because the EGCG is not fully encased in a micelle structure, it is possible to achieve smaller diameter particles without compromising the stability of the carrier. Although this preliminary study has demonstrated the ability of nanoparticles to increase the systemic absorption of EGCG taken orally, it is likely that the small diameter of these particles will also lead to improved blood brain barrier penetration. Further studies will be performed to investigate the possibility that nanolipidic particles can be used to enhance the delivery of poorly absorbed drugs to the brain.

This study provides important preliminary evidence that nanolipidic particles might be useful for safely translating EGCG into human clinical trials. Not only did NanoEGCG more than double the oral bioavailability of EGCG in rats (Fig. 3) but also was more effective at promoting α-secretase activity *in vitro*, even at reduced concentrations (Fig. 2). Taken together, it is possible that NanoEGCG will be therapeutically effective at doses that would be considered acceptable in the clinical setting.

Conflict of interest

The investigators have filed a patent application for this technology.

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