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Diosmin reduces cerebral A β levels, tau hyperphosphorylation, neuroinflammation, and cognitive impairment in the 3xTg-AD Mice

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Abstract

Naturally-occurring bioactive flavonoids such as diosmin significantly reduces amyloid beta (A β) associated pathology in Alzheimer's disease (AD) mouse models. In the present study, oral administration of diosmin reduced cerebral A β oligomer levels, tau-hyperphosphorylation and cognitive impairment in the 3xTg-AD mouse model through glycogen synthase kinase-3 (GSK-3) and transient receptor potential canonical 6-related mechanisms. Diosmetin, one major bioactive metabolite of diosmin, increased inhibitory GSK-3 β phosphorylation, while selectively reducing γ -secretase activity, A β generation, tau hyperphosphorylation and pro-inflammatory activation of

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Competing Interest: JT and RDS are co-inventors on a USF owned US Patent 8,802,638, titled "Flavonoid Treatment of Glycogen Synthase Kinase-based Disease," which covers the use of diosmin for the treatment of Alzheimer's disease.

AUTHOR CONTRIBUTIONS

D.S. (Sawmiller) performed the experiments, assisted in the design of the study, analyzed the data and drafted the manuscript. A.H., S.L. D.D., H.H., J. Tian and A.S. performed experiments (including animal works, ELISA, WB, IP, IHC analyses). D.S. (Shytle) and B.G. assisted in the design of the study and analyzed the data. T.M. assisted in the design of the study, analyzed the data, manuscript composition and editing, and supervised IHC technical issues. J. Tan. designed and supervised the study, analyzed the data, assisted in the composition and editing of the manuscript. All authors discussed the results and commented on the final version of the manuscript.

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microglia *in vitro*, without altering Notch processing. Therefore, both diosmin and diosmetin could be considered as potential candidates for novel anti-AD therapy.

Graphical abstract

Keywords

Alzheimer's disease; A β ; tau; neuroinflammation; γ -secretase; GSK-3; diosmin

1. INTRODUCTION

Amyloid- β (A β) peptide generation and aggregation as plaques are key pathological events in the development of Alzheimer's disease (AD) (Funamoto et al., 2004, Golde et al., 2000, Huse and Doms, 2000, Sambamurti et al., 2002, Selkoe et al., 1996), as they are reported as mediators of apoptosis (LaFerla et al., 1995, Loo et al., 1993), inflammation (Bradt et al., 1998, Suo et al., 1998), and oxidative stress (Hensley et al., 1994, Murakami et al., 2005). A β peptides are produced *via* the amyloidogenic pathway of amyloid precursor protein (APP) proteolysis, which involves the concerted efforts of β and γ -secretases (Schenk et al., 1995, Selkoe, Yamazaki, 1996). Initially, β -secretase (BACE1) cleaves APP, creating an A β -containing carboxyl terminal fragment, known as β -C-terminal fragment (β -CTF) or C99 (Sinha and Lieberburg, 1999, Vassar et al., 1999, Yan et al., 1999), and an amino-terminal, soluble APP- β (sAPP- β) fragment, which is released extracellularly. Intracellularly, β -CTF is then cleaved by a multi-protein γ -secretase complex that results in generation of the A β peptide and a smaller γ -CTF, also known as C57 (De Strooper et al., 1998, Steiner et al., 1999). Neurofibrillary tangles (NFTs), consisting of misfolded and hyperphosphorylated tau (a microtubule protein), have also been implicated as a central pathological feature of AD (Braak et al., 1993, Jellinger, 1998). The accumulation of A β can adversely affect distinct molecular pathways, thus facilitating tau phosphorylation, aggregation, and accumulation of NFTs (Lee and Trojanowski, 2006). Indeed, A β and NFTs synergize to accelerate neurodegenerative processes involved in abnormal metabolism, cellular toxicity, mitochondrial dysfunction, and neuritic plaque formation (Lee and Trojanowski, 2006).

The intense search for small-molecular compounds that may modulate AD pathology has advanced the analysis of specific dietary-derived substances from fruits and vegetables, which have been suggested to be beneficial against neurodegeneration and aging (Barberger-

Gateau et al., 2007, Dai et al., 2006). In this regard, a group of polyphenols categorized as flavonoids has been recently found to be potentially anti-amyloidogenic (Marambaud et al., 2005, Rezai-Zadeh et al., 2005, Yang et al., 2005). We previously found that the flavonoid luteolin significantly reduces A β generation in murine N2a cells expressing the “Swedish” mutant form of APP as well as primary neuronal cells derived from “Swedish” mutant APP overexpressing mice (Tg2576 mouse), *via* selective inactivation of glycogen synthase kinase-3 α (GSK-3 α) (Rezai-Zadeh et al., 2009). Consistent with these *in vitro* findings, oral administration of luteolin to Tg2576 mice similarly reduced A β generation as well as GSK-3 activity (Rezai-Zadeh, Douglas, 2009). This inhibition of GSK-3 increases phosphorylation of presenilin 1, which forms the catalytic core of γ -secretase complex, thereby inhibiting PS1-APP association. In addition, since both isoforms of GSK-3 α/β directly phosphorylate tau on residues specific for hyperphosphorylated paired helical filaments (PHFs) (Ishiguro et al., 1993), mediated by a common binding region on PS1 (Takashima et al., 1998), the GSK-3 inhibiting activity of luteolin may endow this compound the potential to reduce tau phosphorylation as well.

However, luteolin has a poor oral bioavailability (< 2%) and a short half-life in plasma (< 4 hours), which limit the clinical utility of this compound (Shimoi et al., 1998, Wittemer et al., 2005). Therefore, we screened other compounds with 5,7-dihydroxyflavone structural backbone to identify more suitable flavonoids for oral administration (Diagram 1) and found that oral administration of one such compound, diosmin, could reduce A β associated pathology in Tg2576 mice (Rezai-Zadeh, Douglas, 2009). However, the underlying molecular mechanisms and its potential impacts on tau pathology have not been determined. In the present study, we further found that oral diosmin administration could reduce cerebral soluble and oligomer A β levels and AD-like tau pathology and ameliorate cognitive impairment in 3xTg-AD mouse models *via* modulation of GSK-3 activity and transient receptor potential canonical 6 (TRPC6)-related mechanisms. Interestingly, we further found that diosmetin, one major metabolite of diosmin and luteolin (Chen et al., 2011, Rezai-Zadeh, Douglas, 2009), significantly reduced A β generation and tau phosphorylation as well as γ -secretase and GSK-3 activities *in vitro*. Taken together, these results strongly indicate that diosmin reduces cerebral A β levels, tau-related pathology and cognitive impairment in AD mouse models, and diosmetin might be the major bioactive metabolite contributing to the anti-AD activities of diosmin. Therefore, both diosmin and diosmetin could be considered as potential candidates for novel anti-AD therapy.

2. METHODS

2.1 Reagents

Diosmin and diosmetin were obtained from LKT laboratories (St Paul, MN). Antibodies against A β _{1–16} (6E10, 82E1) and A β _{17–24} (4G8) were obtained from Covance Research Products (Emeryville, CA). Antibodies against p-tau (Thr231), p-tau (Ser202), and total tau (tau 46) were obtained from Thermo Fisher Scientific (Waltham, MA). PHF1 antibody was kindly provided by Dr. Peter Davies. Antibodies against p-GSK-3 α (Ser21), p-GSK-3 β (Ser9), pGSK-3 β (Thr390), total GSK-3 α , and total GSK-3 β were obtained from Cell Signaling Technology (Danvers, MA). Antibody against TRPC6 was obtained from Sigma-

Aldrich (St. Louis, MO). Antibody against c-Myc (9E10) was obtained from Abcam (Cambridge, MA). Anti-cleaved Notch1 rabbit antibody (Val1744) was purchased from Cell Signaling Technology. The γ -secretase activity kit was obtained from R&D Systems (Minneapolis, MN).

2.2 Ethics Statement

All experiments were performed in accordance with the guidelines of the National Institutes of Health, and all animal studies were approved by the University of South Florida (USF) Institutional Animal Care and Use Committee. Animals were humanely cared for during all experiments, and all efforts were made to minimize animal suffering. Animals were anesthetized with isoflurane (2 to 3% for induction and 1% for maintenance) (Sigma-Aldrich) and euthanized by transcardial perfusion with physiological saline containing heparin (10 U/mL) (Sigma-Aldrich).

2.3 Animal treatment

Transgenic 3xTg-AD mice (N = 36, 19F/17M) and their wild-type (WT) littermates (B6129SF2/J, N = 30, 15F/15M) were purchased from the Jackson Laboratory (Bar Harbor, ME). 3xTg-AD mice, harboring presenilin-1 (PS1/M146V), APP (KM670/671NL), and tau (P301L) transgenes, progressively develop A β and NFT pathology which potentially synergize to accelerate neurodegeneration by 6 months of age (Oddo et al., 2003). Four-month old 3xTg-AD mice in addition to their WT littermates were orally treated with 0.0005% (1 mg/kg/day) or 0.005% diosmin (10 mg/kg/day) supplemented or control diet for 6 months. All mice were maintained on a 12-hour light/12-hour dark cycle at ambient temperature and humidity and housed in the animal facility at the USF, Morsani College of Medicine (Johnnie B. Byrd Sr. Alzheimer's Center and Research Institute, Tampa, FL).

2.4 Behavioral Tests

Memory performance was tested for 3xTg-AD mice and WT controls after 2-, 4-, and 6-month treatment with diosmin-supplemented or control diets using the fear conditioning test (Wang et al., 2014). Briefly, each mouse was placed in the fear conditioning apparatus (Panlab, Barcelona, Spain) for 2 minutes. Then, a 30-second acoustic conditioned stimulus (CS; 80 dB tone) was delivered paired with a 0.5 mA shock unconditioned stimulus (US) applied to the floor grid during the last 2 seconds of the CS. Training consisted of two CS-US pairings, with a 1.5 minute interval between each. The mice were placed in the chamber and monitored for freezing behavior (*i.e.*, motionless position for at least 2 seconds) to the context 24 hours after training (no shocks or conditioned stimulus given, hippocampal dependent). Immediately after the contextual test, mice were placed into a novel context and exposed to the CS for 3 minutes (cued fear conditioning, hippocampal and amygdala dependent). Learning and memory were assessed by measuring freezing behavior.

2.5 Cell Culture and Treatment

CHO cells expressing human APP695 (CHO/APP695), or CHO/APP695 and Notch- E vector (CHO/APP695/Notch- E, kindly provided by Dr. Peter Klein), HeLa cells expressing human stable tau (HeLa/tau; kindly provided by Dr. Chad Dickey), and human

neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1 mM sodium pyruvate, and 100 U/mL of penicillin/streptomycin. Primary neuronal cells were obtained from cerebral cortices of Tg2576 mouse embryos, between 15 and 17 days *in utero*, as described previously (Rezai-Zadeh, Douglas, 2009). In brief, cortices were incubated for 15 minutes at 37°C and then mechanically dissociated in trypsin (0.25%). Cells were centrifuged at 1,200 rpm, suspended in DMEM supplemented with 10% fetal calf serum, 10% horse serum, uridine (33.6 µg/mL; Sigma-Aldrich), and fluorodeoxyuridine (13.6 µg/mL; Sigma-Aldrich) and seeded in 24-well collagen coated culture plates at 2.5×10^5 cells per well. Cells were treated with diosmetin followed by biochemical analysis of cell lysates and cultured media as indicated.

Murine primary culture microglia were isolated from mouse cerebral cortices as described previously (Zhu et al., 2011a). Briefly, cerebral cortices from newborn mice (1–2-day old) were isolated under sterile conditions and mechanically dissociated at 4°C. Cells were grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 0.1 µg/mL streptomycin, and 0.05 µM 2-mercaptoethanol for 14 days, after which only glial cells remained. Astrocytes were separated from microglial cultures using a mild trypsinization protocol as described (Saura et al., 2003). More than 98% of these glial cells stained positive for Mac-1 (Roche Diagnostics) by flow cytometry (Zhu, Hou, 2011a).

To determine the effect of diosmetin on microglial pro-inflammatory activity and Aβ phagocytosis, primary microglial cells were treated with diosmetin in the presence of IFN-γ (100 U/ml) or/and CD40 ligand (CD40L, 1 µg/mL) for 8 hours, and then pro-inflammatory microglial activation was assessed by flow cytometric (FACS) analysis and ELISA (Zhu, Hou, 2011a, Zhu et al., 2011b). In addition, primary microglia were pretreated with diosmetin at 10 µM or vehicle (1% DMSO) for 6 hours and incubated with 1 µM aged FITC-Aβ₄₂ for 1 hour. Cellular supernatants and lysates were analyzed for extracellular and cell-associated FITC-Aβ₄₂ using a fluorometer. Data are represented as the relative fold of mean fluorescence change, calculated as the mean fluorescence for each samples at 37°C divided by mean fluorescence at 4°C.

2.6 Immunohistochemistry

For frozen brain tissues, we sectioned five coronal sections per region with a 100-µm interval and a thickness of 15-µm for cortex and hippocampus, respectively (Li et al., 2015, Rezai-Zadeh, Douglas, 2009). Immunohistochemical (IHC) staining was conducted according to the manufacturer's protocol using a VECTASTAIN ABC *Elite* kit (Vector Laboratories, Burlingame, CA) coupled with the diaminobenzidine reaction. Images were acquired as digitized tagged-image format files (to retain maximum resolution) using a BX60 microscope with an attached CCD camera system (DP-72, Olympus, Tokyo, Japan), and digital images were routed into a Windows PC for quantitative analyses using SimplePCI software (Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan). We captured images of five 15-µm (3xTg-AD mice) sections through each anatomic region of interest based on anatomical criteria defined by Franklin and Paxinos (Franklin and Paxinos, 2001) and obtained a threshold optical density that discriminated staining from background. Each

anatomic region of interest was manually edited to eliminate artifacts. Selection bias was controlled by analyzing each region of interest in its entirety. Brain sections, mainly CA1 and CA3, were stained with anti-phosphorylated tau (Thr231) [p-tau (Thr231)] or anti-phosphorylated tau (Ser202) [p-tau (Ser202)] antibodies and percentages of anti-p-tau (Thr231) or anti-p-tau (Ser202) positive areas per total area were calculated by quantitative image (40X) analysis.

2.7 WB Analysis

Mouse brains were homogenized in ice-cold 1 × lysis buffer for 30 seconds using a Minilys tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) set at high speed and centrifuged at 15,000 rpm for 30 minutes. Cell culture lysates were routinely prepared and WB analysis was performed as previously described (Li, Hou, 2015, Rezai-Zadeh, Douglas, 2009). Briefly, the proteins from cell lysates, and brain homogenates were electrophoretically separated using 10% bicine/tris gel (8 M urea) for proteins less than 5 kDa or 10% tris-SDS gels for larger proteins. Electrophoresed proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA), washed and blocked for 2 hours at room temperature in tris-buffered saline containing 5% (w/v) nonfat dry milk (TBS/NFDM). After blocking, membranes were hybridized for 2 hours with appropriate primary antibodies, washed and incubated for 1 hour with the appropriate HRP-conjugated secondary antibody in TBS/NFDM. Blots were developed using the luminol reagent (Thermo Fisher Scientific, Waltham, MA). Densitometric analysis was performed as described previously (Rezai-Zadeh, Shytle, 2005) using a FluorS Multiimager with Quantity One software (Bio-Rad, Hercules, CA).

2.8 ELISA

Soluble A β _{1-40, 42} species were directly detected in cultured cell media or brain homogenates using the A β _{1-40, 42} ELISA kit obtained from Invitrogen (Carlsbad, CA). A β oligomer levels in brain homogenates were measured using an A β oligomer ELISA kits (TECAN, Morrisville, NC) accordingly to the procedures provided by kit.

2.9 Statistical Analysis

Data are expressed as mean $\pm$ SEM. Comparison between groups was performed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by LSD or Bonferroni *post hoc* test for equal variance or Dunnett's test for unequal variance. *P* < 0.05 was considered statistically significant.

3. RESULTS

3.1 Diosmin reduces cerebral soluble A β and A β oligomer levels, mediated in part by enhanced TRPC6 levels in 3xTg-AD Mice

Given our previous report showing diosmin decreased AD-like pathology in Tg2576 mice, we further determined the effect of diosmin treatment on AD-related A β and tau pathology in another well-accepted AD rodent model, 3xTg-AD mice (Oddo, Caccamo, 2003). 3xTg-AD mice were treated with 0.0005% (DLO) or 0.005% diosmin (DHI) supplemented or control diet (Ctrl) at 4 months of age, followed by analysis of A β and tau pathologies in

brain sections and homogenates. No notable reduction of A β deposits was observed by IHC using anti-A β _{17–26} (4G8) (Fig. 1a). Diosmin markedly decreased cerebral A β levels, as determined by WB using anti-A β _{1–16} antibody (82E1) (Fig. 1b) as well as soluble A β _{1–40, 42} levels by 37% (DLO) and 51% (DHI), as determined by ELISA (Fig. 1c). Most notably, oral diosmin treatment reduced A β oligomer levels more in female than in male 3xTg-AD mice (26% reduction in females versus no significant difference in males) (Fig. 1d). These results are important because they suggest that diosmin may be particularly effective in treating female AD patients by targeting A β oligomers, the most toxic form of A β . There was no noticeable effect of diosmin on body weight or health of the mice.

Recent studies indicate that transient receptor potential canonical 6 (TRPC6) specifically interacts with APP to inhibit its cleavage by γ -secretase and reduce A β production, without altering Notch cleavage (Wang et al., 2015). Therefore, we examined TRPC6 protein levels by WB following chronic diosmin treatment. Diosmin increased TRPC6 levels by 26% (DLO) and 74% (DHI) (Fig. 1e), thus suggesting that diosmin specifically reduces γ -secretase activity through a TRPC6-related mechanism.

3.2 Diosmin reduces GSK-3 α/β and tau pathology in 3xTg-AD mice

In agreement with the data from our previous study using Tg2576 mice, diosmin increased the levels of inhibitory phospho-GSK-3 α (Ser21) and phospho-GSK-3 β (Ser9) as determined by WB (Fig. 2a & b). Densitometry analysis data showed that diosmin significantly increases the band density ratios of pGSK-3 α (Ser21) to tGSK-3 α (58% DLO, 71% DHI) as well as increased the band density ratios of pGSK-3 β (Ser9) to tGSK-3 β (60% DLO, 62% DHI). In addition, quantitative image analysis shows that oral diosmin treatment reduced p-tau (Thr231) positive area/total area (55% DLO, 86% DHI) and p-tau (Ser202) positive area/total area (42% DLO, 75% DHI) in the hippocampus (CA1 and CA3) and cortex regions, as determined by IHC (Fig. 2c & d). All these *in vivo* data further confirmed that diosmin treatment could reduce tau pathology of AD mouse models through inhibition of GSK-3 α/β .

3.3 Diosmin reduces cognitive impairment

In addition to AD pathology, behavioral analyses were performed after 2, 4, and 6 months of diosmin treatment in 3xTg-AD and WT mice to evaluate cognitive performance, using the contextual and cued fear conditioning tests. Diosmin treatment enhanced contextual and cued fear conditioning between 2 and 6 months of treatment in both male and female 3xTg-AD and WT mice (Fig. 3). Diosmin appeared effective in enhancing cognitive performance in a gender independent manner.

3.4 Diosmetin inhibits γ -secretase cleavage and A β production, without altering Notch processing

CHO/APP695 cells and primary neuronal cells cultured from brain tissues of embryonic Tg2576 mice were treated with diosmetin at 0, 2.5, 5, and 10 μ M for 12 hours followed by analysis of A β _{1–40, 42} levels secreted in the cell culture media by A β ELISA (Fig. 4a & b) and WB (Fig. 4c & d, left panels). The ELISA and WB data showed that diosmetin reduced A β _{1–40} and A β _{1–42} production in CHO/APP695 cells and primary neuronal cells in a dose-

dependent fashion. In addition, given the obvious implications on γ -secretase activity, CHO/APPwt and primary neuronal cells were treated with diosmetin at 0, 2.5, 5, and 10 μ M for 90 minutes followed by analysis of γ -secretase activity using a fluorometric assay. In accordance with expectations, diosmetin reduced γ -secretase activity in a dose-dependent fashion in both cell types, starting at 2.5 μ M and producing maximal inhibition of 30–40% at 10 μ M (Fig. 4c & d, right panels).

Since γ -secretase inhibition (GSIs) can potentially alter Notch processing, CHO/APP695/Notch- E cells were treated with diosmetin at 0, 2.5, 5, and 10 μ M or DFK-167 (a known γ -secretase inhibitor) at 0, 10, 20, and 40 μ M for 18 hours followed by WB and ELISA analysis of A β _{1–40, 42} production (Fig. 4e) and WB analysis of cleaved Notch and c-Myc (Fig. 4f). While DFK-167 dose-dependently inhibited Notch cleavage, reflected as a reduction in levels of cleaved Notch (Fig. 4f, right panel), diosmetin did not alter Notch cleavage (Fig. 4f, left panel). Additionally, diosmetin reduced A β production by WB (data not shown), confirming that diosmetin reduces γ -secretase processing of APP. These results further support our previous observation with luteolin (Rezai-Zadeh, Douglas, 2009).

3.5 Diosmetin inhibits GSK-3 β activity and decreases tau phosphorylation

Since *in vivo* data suggested that diosmin potentially inhibited GSK-3 activity and reduced tau pathology in AD mouse models, we further evaluated these bioactivities of diosmetin *in vitro*. Human neuroblastoma SH-SY5Y cells (Fig. 5a) and primary neuronal cells (Fig. 5b) were treated with diosmetin at 0, 2.5, 5, and 10 μ M for 12 hours followed by analysis of inhibitory phospho-GSK-3 β (Ser9) and total GSK-3 β levels by WB. Diosmetin markedly elevated phospho-GSK-3 β (Ser9) and the band density ratios of phospho-GSK-3 β (Ser9) to total GSK-3 β in a dose-dependent fashion in both cell types (below Fig. 5a & b's each blot). Subsequently, HeLa/tau cells were treated with diosmetin at 0, 2.5, 5, and 10 μ M for 12 hours followed by analysis of inhibitory phospho-GSK-3 β (Ser9) and total GSK-3 β levels as well as PHF-1, phospho-tau (Thr231), and total tau levels (Fig. 5 c & d). As expected, diosmetin also enhanced the level of inhibitory phospho-GSK-3 β (Ser9) and reduced tau phosphorylation in HeLa/tau cells, as determined by WB. Densitometry analysis shows a significant increase in the ratio of pGSK-3 β (Ser9) to total GSK-3 β and decrease in phosphorylated tau (p-tau) to total tau for HeLa/tau cells treated with 10 μ M diosmetin compared with control (0 μ M) below each figure panel.

3.6 Diosmetin inhibits microglial pro-inflammatory activation and enhances A β phagocytosis

Since γ -secretase inhibitors and PS1 dysfunction have been shown to alter the microglial cytoskeleton as well as impair microglial phagocytosis and clearance of A β (Behbahani et al., 2006, Farfara et al., 2011, Pigino et al., 2001), we determined the effect of diosmetin on microglial pro-inflammatory activation and A β phagocytosis *in vitro*. Primary microglial cells were treated with diosmetin at 0, 2.5, 5, 10, and 20 μ M in the presence of IFN γ (100 U/mL) and/or CD40 ligand (CD40L, 1 μ g/mL) for 8 hours followed by analysis of pro-inflammatory activation by FACS and ELISA. Diosmetin reduced IFN γ -induced CD40 expression in a dose-dependent fashion (Fig. 6a) and reduced IFN γ /CD40L-induced production of pro-inflammatory cytokines [TNF α and IL-12 (p70)] (Fig. 6b). In additional

studies, primary microglia were pretreated with diosmetin at 10 μ M or vehicle (1% DMSO) for 6 hours and then incubated with aged FITC-A β_{1-42} for 1 hour, followed by analyses of FITC-A β_{1-42} levels in cell supernatants (extracellular) and lysates (cell-associated) using a fluorimeter. Surprisingly, diosmetin increased FITC-A β_{1-42} levels in cell lysates, while reducing FITC-A β_{1-42} levels in cell supernatants (Fig. 6c). Therefore, diosmetin reduces pro-inflammatory microglial activation, while enhancing A β phagocytosis.

4. DISCUSSION

In the present study, we determined the effects of oral diosmin treatment on β -amyloid/A β - and tau pathologies in the 3xTg-AD mice. Oral diosmin reduces cerebral soluble A β and A β oligomer levels and ameliorates abnormal tau pathology in the 3xTg-AD mouse model as determined by IHC, WB and ELISA. In addition, our results show the biologically active form of diosmin, diosmetin, reduces A β generation, tau-hyperphosphorylation, neuroinflammation, as well as γ -secretase and GSK-3 activities *in vitro*, indicating that the biological effects of oral diosmin may be mediated by this metabolite. These results confirm our previous findings indicating that diosmin and the structurally related compound, luteolin, both reduces β -amyloid pathology in Tg2576 mice, thus strongly suggesting that diosmin should be tested as a disease-modifying treatment for AD (Rezai-Zadeh, Douglas, 2009). Most importantly, diosmin reduced brain A β oligomer levels more in female than in male mice. These results are salient because they suggest that diosmin may be particularly effective in treating female AD patients by targeting the most toxic form of A β . AD has been shown to affect female AD mouse models and patients more than males (Lin and Doraiswamy, 2014) and finding a therapy for the treatment of females with AD has been an important challenge. In addition, our results indicate that these prophylactic effects of diosmin are mediated by increased levels of TPRC6, which has been shown to specifically inhibit APP cleavage by γ -secretase and reduce A β production, without affecting Notch cleavage (Wang, Lu, 2015). These effects also involved inhibitory phosphorylation of GSK-3 α and GSK-3 β . Importantly, these beneficial outcomes resulting from diosmin treatment corresponded with reduced cognitive impairment, as determined by fear conditioning tests.

There may be a dual role of γ -secretase in AD. The cleavage of APP by γ -secretase is key in the pathogenesis of AD and β -amyloid accumulation in the brain. As such, γ -secretase has been targeted for years for development of γ -secretase inhibitors (GSIs) for AD therapies. Most recently, it was shown that microglia deficient in presenilin 1 and 2 have altered γ -secretase activity which may impair phagocytosis of A β and promote A β accumulation (Farfara, Trudler, 2011). Because GSIs may impair salutary microglia activity *via* inhibition of the Notch signaling pathway, we sought to develop a new class of flavonoid GSIs that can reduce γ -APP cleavage yet minimize their potential negative effect on microglial phagocytosis activity. Thus, notably we found that diosmin treatment reduced AD-like pathological changes in AD mice *via* specific inhibition of APP γ -secretase cleavage activity without altering Notch processing.

Flavonoids are widely occurring in our diet and they all, to some degree, inhibit p450 (Androutsopoulos et al., 2010, Li et al., 1994). However it is notable that diosmetin has less

of an inhibitory effect on P450 3A activity compared to luteolin at the same dose range (Tsujimoto et al., 2009), due to the replacement of a hydroxyl group at the 4' position with a methoxy group. Since P450 inhibition by flavonoids is unavoidable, as with many drugs and nutraceuticals, upon clinical translation the provider could simply adjust the dose of drugs metabolized through this system. Further in regard to safety, diosmin containing supplements have been in use in Europe for the past three decades with an excellent safety record. An array of reports demonstrates their safety and efficacy in the treatment of vascular disorders (Casley-Smith and Casley-Smith, 1985, Jantet, 2000, 2002, Pecking, 1995, Pecking et al., 1997) and diabetes (Lacombe et al., 1988, Lacombe et al., 1989).

A micronized nutraceutical formulation of diosmin, under the trade name Daflon[®], has been successfully used to treat chronic venous disease, hemorrhoids and diabetes for over a decade in Europe. Recently, improved formulations of diosmin have been marketed in both Europe and the United States for the treatment of varicose and spider veins. At the same time, these clinical studies evaluating diosmin and its various formulations have laid the groundwork safety data for a future AD clinical trial. Based on the body surface area calculation for translation of mouse to human dose, the high dose of diosmin employed in our oral study (10 mg/kg/day) should be equivalent to a ~50 mg diosmin daily intake in humans (Reagan-Shaw et al., 2008). Clinical trials have used doses of 500 mg to 6 grams per day orally for up to 1 year. Throughout these trials, diosmin has demonstrated an excellent safety profile and was well tolerated. Adverse events with such complexes were rare; and when they occurred, they were always mild, and transient. The side effects typically observed were mild cases of digestive intolerance requiring no changes in treatment.

Ultimately, the identification of compounds that target multiple pathologies is essential for the formulation of effective therapeutic interventions. For these reasons, diosmin may prove to be such a compound. Our future ultimate goal is to obtain an investigational new drug approval for diosmin for the treatment or prevention of AD and mild cognitive impairment (MCI).

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HIGHLIGHTS

- 1.** Oral administration of diosmin reduced cerebral A β oligomer levels, tau-hyperphosphorylation and cognitive impairment in the 3xTg-AD mouse model through glycogen synthase kinase-3 (GSK-3) and transient receptor potential canonical 6-related mechanisms.
- 2.** Diosmetin, one major bioactive metabolite of diosmin, increased inhibitory GSK-3 β phosphorylation, while selectively reducing γ -secretase activity, A β generation, tau hyperphosphorylation and pro-inflammatory activation of microglia *in vitro*, without altering Notch processing.
- 3.** Both diosmin and diosmetin could be considered as potential candidates for novel anti-AD therapy.

Figure 1. Diosmin reduces cerebral soluble Aβ and Aβ oligomer levels in 3xTg-AD mice 3xTg-AD mice at 4 months of age were treated with 0.0005% (diosmin low dose: DLO) or 0.005% diosmin (diosmin high dose: DHI) supplemented or control diet (Ctrl) for 6 months. (a) Representative images of 4G8 Aβ₁₇₋₂₄ antibody immunostaining for Ctrl-, DLO-, or DHI-treated coronal brains. (b) WB with 82E1 Aβ₁₋₁₆ antibody for brain homogenates from Ctrl-, DLO-, or DHI-treated 3xTg-AD mice. Anti-β-actin antibody was used as an internal reference control. (c) Total soluble Aβ_{1-40, 42} levels for brain homogenates from Ctrl-, DLO-, or DHI-treated 3xTg-AD mice. The data are presented as pg/mg of total protein. Diosmin treatment reduced soluble Aβ_{1-40, 42} (37% DLO, 51% DHI). (d) Aβ oligomer levels for brain homogenates from Ctrl-, DLO-, or DHI-treated 3xTg-AD mice. The data are presented as pg/mg of total protein. Oral diosmin treatment reduced Aβ oligomer levels more in female (26% DLO, 31% DHI) than in male 3xTg-AD mice (no change DLO, 26% DHI). (e) WB with transient receptor potential canonical 6 (TRPC6) antibody for brain homogenates from Ctrl-, DLO-, or DHI-treated 3xTg-AD mice. Anti-β-actin antibody was used as an internal reference control. Densitometry analysis shows that diosmin increased TRPC6 levels/β-actin ratios (74% DHI, 26% DLO). Our results suggest that diosmin may specifically reduce APP γ-secretase activity and Aβ pathology through enhancement of the TRPC6 protein. All data are presented as mean ± SEM (**P* < 0.05, ***P* < 0.01; *N* = 6 mice per group).

Fig. 2a & b

Fig. 2c & d

Figure 2. Diosmin reduces GSK-3α/β activity and tau pathology in 3xTg AD-mice

(a) Brain homogenates prepared from 3xTg AD-mice treated with 0.0005% (diosmin low dose: DLO) or 0.005% diosmin (diosmin high dose: DHI) supplemented or control diet (Ctrl) were analyzed by WB for determination of inhibitory phosphorylation status of GSK-3 α / β using phospho-GSK-3 α (Ser21) [pGSK-3 α (Ser21)], phospho-GSK-3 β (Ser9) [pGSK-3 β (Ser9)], and total GSK-3 α / β antibodies (tGSK-3 α or tGSK-3 β). (b) Densitometry analysis data shows that diosmin significantly increases the band density ratios of pGSK-3 α (Ser21) to tGSK-3 α (58% DLO, 71% DHI) as well as increased the band density ratios of pGSK-3 β (Ser9) to tGSK-3 β (60% DLO, 62% DHI). (c) Representative images of phospho-tau (Thr231) [p-tau (Thr231)] or phospho-tau (Ser202) [p-tau (Ser202)] antibody immunostaining for Ctrl-, DLO-, or DHI-treated coronal brain sections. (d) Image analysis shows that diosmin reduced p-tau (Thr231) positive area/total area (55% DLO, 86% DHI) and reduced p-tau (Ser202) positive area/total area (42% DLO, 75% DHI). All data are presented as mean $\pm$ SEM (N = 6 mice per group).

Fig. 12

Figure 3. Diosmin improves hippocampal- and amygdala-dependent learning and memory in 3xTg-AD and WT mice, as determined using the fear conditioning test

Using the fear conditioning test, cognitive performance was assessed in male (a) and female (b) 3xTg-AD and WT mice after 2, 4 and 6 months of treatment with diosmin-supplemented or control diets. When placed in the context 24 hours following training, 3xTg-AD mice showed a decrease in freezing compared with WT mice. Likewise, 3xTg-AD mice showed a decrease in freezing when placed in the novel environment and presented with the conditioned stimulus (cued test) 24 hours after training, indicating that the genetic impairment in 3xTg-AD mice lead to a limitation in both hippocampal- and amygdala-dependent learning. However, both 3xTg-AD and WT mice showed an increase in freezing in response to either conditioned stimulus or context after treatment with diosmin for 2 – 6 months. All data are presented as mean $\pm$ SEM ($N = 4 - 7$ mice per group for 6-month test ($N = 2 - 3$ mice per group for 2- and 4-month test). * $P < 0.05$ versus corresponding mice treated with control diet.

Figure 4. Diosmetin inhibits A β generation and decreases γ -secretase cleavage activity without inhibition of Notch processing in cultured cells

(a) CHO cells expressing wild-type human APP695 (CHO/APP695) and primary neuronal cells cultured from brain tissues of Tg2576 mouse embryos were treated with diosmetin as indicated for 12 hours followed by analysis of A $\beta_{1-40, 42}$ peptides secreted in the cell culture media by A β ELISA (a & b) and WB analysis (c & d, left). The A β ELISA results are represented as the mean $\pm$ SEM of A β_{1-40} or A β_{1-42} (pg/mg) in cell culture media after diosmetin treatment. These results are representative of three independent experiments with $n = 3$ for each condition. One-way ANOVA followed by *post hoc* comparison revealed significant differences between the 2.5, 5, or 10 μ M and 0 μ M concentrations of diosmetin in terms of A $\beta_{1-40,42}$ reduction. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$). In addition, γ -secretase activity was analyzed in cell lysates following 90 minutes incubation using a secretase cleavage activity assay ($N = 3$ for each condition). Given the obvious implications on γ -secretase activity, we analyzed what effect diosmetin treatment had on both CHO/APP695 and primary neuronal cells using a fluorometric assay for γ -secretase mediated cleavage. In accordance with expectations, diosmetin lowered γ -secretase cleavage activity in both cell cultures dose dependently (c & d, right). These results are presented as the percentage of fluorescence (units/mg of protein) 90 minutes after diosmetin treatment relative to control (ctrl, untreated). A difference was noted between each dose examined ($P < 0.01$).

To examine whether diosmetin can affect Notch processing, APP695/Notch- E CHO cells were plated at 8×10^5 /well in 6-well dishes and treated with diosmetin or DFK-167 (positive control for inhibiting Notch cleavage) at different doses as indicated for 18 hours. The conditioned media and cell lysates were routinely prepared from these cells for A β ELISA and WB analysis. The A β ELISA results are represented as the mean $\pm$ SEM of A β_{40} or A β_{42} (pg/mg) for 3 independent experiments for each condition (e) (* $P < 0.05$, ** $P < 0.01$). WB analysis with both c-Myc antibody (9E10) and cleaved Notch antibody (Val1744) showed that diosmetin treatment did not inhibit Notch cleavage compared to DFK-167 (f). WB analysis with 82E1 A β_{1-17} antibody for the conditioned media. Notably, diosmetin treatment dose-dependently inhibits A β production further confirmed by WB (data not shown).

Figure 5. Diosmetin dose-dependently increases inhibitory GSK-3β (Ser9) phosphorylation and decreases tau phosphorylation

WB with phospho-GSK-3β (Ser9) and total GSK-3β antibodies. (a) Human neuroblastoma (SH-SY5Y) cells and (b) murine primary neuronal cells were treated with diosmetin at the indicated concentrations for 12 hours. Phosphorylated GSK-3β (Ser9) [pGSK-3β (Ser9)] was notably elevated following diosmetin treatment in both SH-SY5Y and murine primary neuronal cells. Densitometry analysis shows the band density ratio of pGSK-3β (Ser9) to total GSK-3β in response to diosmetin below each figure panel. These data are representative of three independent experiments with $N = 3$ for each condition. A t -test revealed significant difference in the ratio of pGSK-3β (Ser9) to total GSK-3β for both SH-SY5Y cells and primary neuronal cells treated with either 5 or 10 μ M diosmetin compared with untreated control ($*P < 0.05$).

Human tau stable transfected HeLa cells (HeLa/tau cells) were treated with diosmetin at the indicated concentrations for 12 hours. WB for both total and phosphorylated levels of GSK-3β and tau. (c) Phosphorylation status of GSK-3β [pGSK-3β (Ser9)] using phospho-GSK-3β (Ser9) antibody. (d) Phosphorylation status of tau using phospho-tau [p-tau (Thr231)] and PHF1 antibodies and of total tau (phosphorylated and non-phosphorylated) using tau-46 antibody. Densitometry data show the band density ratio of pGSK-3β (Ser9) to total GSK-3β as well as p-tau (Thr231) to total tau below each figure panel. Densitometry data are representative of two independent experiments for pGSK-3β (Ser9) to total GSK-3β and three independent experiments for PHF1, p-tau (Thr231) and total tau. A t -test revealed a significant increase in the ratio of pGSK-3β (Ser9) to total GSK-3β and decrease in p-tau to total tau for HeLa/tau cells treated with 10 μ M diosmetin compared with untreated control ($*P < 0.05$, $**P < 0.01$).

Figure 6. Diosmetin inhibits microglial activation-induced by IFN γ and CD40 signaling and enhances microglial phagocytosis of A β

Murine primary microglial cells were treated with diosmin's metabolite, diosmetin, in the presence of IFN γ (100 U/mL) or/and CD40 ligand (CD40L, 1 μ g/mL) for 8 hours and then examined pro-inflammatory microglial activation, as examined by flow cytometric (FACS) analysis and ELISA. **(a)** FACS analysis showed significant dose dependent decreases in IFN γ -induced CD40 expression by diosmetin following 8 hours of co-treatment in primary microglial cells. Data are represented as mean % of CD40 expressing cells ($\pm$ SEM) of two independent experiments. **(b)** Cell culture supernatants were collected and subjected to cytokine ELISA as indicated. Data were represented as mean pg of TNF α or IL-12 (p70) per mg of total cellular protein ($\pm$ SEM) of three independent experiments. **(c)** Primary microglial cells were pre-treated with diosmetin at 10 μ M or vehicle (1% DMSO in medium) for 6 hours and incubated with 1 μ M aged FITC-A β ₁₋₄₂ for 1 hour. Cellular supernatants and lysates were analyzed for extracellular (**upper panel**) and cell-associated (**lower panel**) FITC-A β ₁₋₄₂ using a fluorometer. Data are represented as the relative fold of mean fluorescence change, calculated as the mean fluorescence for each samples at 37°C divided by mean fluorescence at 4°C (N = 4) for each condition presented.

Diagram 1.

Schematic illustration of the structure of γ -secretase complex and the roles of flavones in amyloid precursor protein (APP) processing. Diosmin, together with its analogues (diosmetin and luteolin), exerts glycogen synthase kinase-3 (GSK-3; both α and β isoforms) inhibitory activity. The reduced GSK-3 α protein activity promotes the phosphorylation of presenilin 1-C-terminal fragment (PS1-CTF), which impairs the interactions between APP and γ -secretase, and consequently decreases the amyloidogenic processing of APP. GSK-3 β phosphorylates APP-CTF at its Thr668 residue to promote β -secretase-mediated amyloidogenic processing of APP. In addition, this Thr668 phosphorylation also promotes the binding of FE65 to APP-CTF to form a FE65/APP-CTF complex, which can be translocated to the nucleus to regulate gene transcription. The inactivation of GSK-3 β by flavones will block these bio-functional pathways.