Combined Liver X Receptor/Peroxisome Proliferator-Activated Receptor γ Agonist Treatment Reduces Amyloid-β Levels and Improves Behavior in Amyloid Precursor Protein/Presenilin 1 Mice*

Rebecca Skerrett1, Mateus P. Pellegrino§, Brad T. Casali1, Laura Taraboanta1, & Gary E. Landreth1

From the 1Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio USA 44106
§School of Medicine, University of Campinas, Campinas, Sao Paulo Brazil 13083-887

*Running title: Nuclear receptor combination therapy benefits AD mice.

To whom correspondence should be addressed: Gary E. Landreth, Dept. of Neurosciences, School of Medicine, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106. Tel.: 216-368-3435; E-mail: gel2@case.edu.

Keywords: Alzheimer disease; amyloid beta (Aβ); peroxisome proliferator-activated receptor (PPAR); liver x receptor (LXR); microglia; apolipoprotein E (ApoE)

Background: In the brain, type II nuclear receptors LXR and PPARγ control cholesterol efflux and inflammation, key processes in Alzheimer’s disease pathology.

Results: Combining LXR and PPARγ agonists decreases levels of Aβ and inflammation, resulting in improved cognition.

Conclusion: LXR and PPARγ agonists complement each other, possibly by modulating microglial function.

Significance: Targeting multiple nuclear receptors expands therapeutic opportunities for AD treatment.

Abstract

Alzheimer’s disease (AD) is characterized by the extracellular accumulation of amyloid β (Aβ) that is accompanied by a robust inflammatory response in the brain. Both of these pathogenic processes are regulated by nuclear receptors, including the liver X receptors (LXRs) and peroxisome-proliferator receptor γ (PPARγ). Agonists of LXRs have been previously demonstrated to reduce Aβ levels and improve cognitive deficits in AD mouse models by inducing the transcription and lipidation of apolipoprotein E (apoE). Agonists targeting PPARγ reduce microglial expression of pro-inflammatory genes, and have also been shown to modulate apoE expression. Here we investigate whether a combination therapy with both LXR and PPARγ agonists results in increased benefits in an AD mouse model. We found that the LXR agonist GW3965 and the PPARγ agonist pioglitazone were individually able to increase levels of apoE and related genes, decrease expression of pro-inflammatory genes, and facilitate Aβ decreases in the hippocampus. Combined treatment with both agonists provoked a further increase in expression of apoE and decrease of soluble and deposited forms of Aβ. The decrease in plaques was associated with increased co-localization between microglia and plaques. In addition, the PPARγ agonist in the combined treatment paradigm was able to counteract the elevation in plasma triglycerides that is a side effect of LXR agonist treatment. These results suggest that a combined LXR/PPARγ agonist treatment merits further investigation for the treatment of AD.

Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of amyloid beta (Aβ) peptides in the brain. In cases of late-onset AD, which is the predominant form of the disease, the elevation of soluble forms of Aβ and their subsequent deposition into plaques arises principally from a deficiency in Aβ clearance (1,2). Apolipoprotein E (apoE) modulates Aβ deposition and clearance in an isoform-dependent manner (3,4,5). Possession of an apoE4 allele, the strongest genetic risk factor for late-onset AD (6,7), has been linked to decreased efficiency of Aβ clearance (3,4,5).
ApoE is the principal apolipoprotein in the brain, where it acts as a scaffold for HDL-like particles and facilitates the trafficking of lipids throughout the CNS. Cholesterol and phospholipids are loaded onto nascent apoE primarily by the lipid transporter ATP-binding cassette A1 (ABCA1) and additionally by ATP-binding cassette G1 (ABCG1) (8). Lipidated apoE is produced in the brain by astrocytes, and to a lesser extent, microglia. We have previously shown that lipidated apoE facilitates the proteolytic clearance of soluble forms of Aβ (9), which in microglia is effected by increasing the rate of Aβ trafficking to lysosomes for degradation (10). The ability of apoE to facilitate soluble Aβ clearance is strongly dependent on the apoE isoform (3,4,5) and its lipidation state, with ABCA1-dependent lipidation playing an important role (11,12,13).

The production of apoE is transcriptionally regulated by liver X receptors (LXRs) α and β (14,15). LXRs are type II nuclear receptors that are activated by oxysterol ligands and function as cholesterol sensors. Upon ligand binding, LXRs form obligate heterodimers with retinoid X receptors (RXRs) and promote transcription of apoE, ABCA1, and ABCG1, among other genes involved in cholesterol metabolism (16). Treatment of murine models of AD with synthetic LXR ligands such as GW3965 or TO901317 has been previously shown to result in increased apoE expression and lipidation in the brain, memory improvements, and reduced levels of Aβ (9,11,13,17,18,19,20,21,22,23,24,25). Ligands that target peroxisome proliferator-activated receptor γ (PPARγ) also produce increases in LXR target genes and the associated benefits, likely by inducing the expression of LXRα as well as interaction with enhancer elements (26,27,28). A large body of literature indicates that activation of LXR, PPARγ, or their heterodimeric partner RXR is able to ameliorate Aβ pathology and mediate behavioral improvements in mouse models of AD (29, 30).

Deposition of Aβ induces a robust inflammatory response in microglia, resulting in their migration and subsequent association with the Aβ deposits and increased cytokine and chemokine production (31,32,33). PPARγ activates a program of gene expression that promotes phagocytosis and tissue repair in macrophages (34,35), and has been shown to mediate a similar phenotypic conversion in microglia (27,36,37). LXR agonists also modulate microglial function and act as regulators of phagocytosis in AD models (23,38), partially through transcriptional control of phagocytic proteins such as MerTK and Axl (39). PPARγ and LXR agonists have been reported to mitigate neuroinflammation in AD models by exerting robust anti-inflammatory activity (37,40). Additionally, ligand activation of PPARγ or LXR results in their sumoylation and sumoylated PPARγ or LXR interacts with corepressor complexes at NFκB and AP1 promoters to prevent their clearance (37,40), suppressing proinflammatory gene expression. LXR and PPARγ each act independently to transrepress an overlapping but distinct subset of proinflammatory genes (37,40,41).

In this study, we evaluated the therapeutic potential of combining LXR and PPAR agonists in treating APPswe/PSEN1ΔE9 (APP/PS1) mice, which express familial human mutations in both APP and presenilin. We reproduce previous findings that LXR and PPAR agonists individually are able to decrease Aβ plaques in the hippocampi of AD mice, produce anti-inflammatory effects, and mediate behavioral improvements. Additionally, we demonstrate that a combination of LXR and PPARγ agonists elicits biochemical and behavioral improvements while ameliorating LXR-mediated plasma hypertriglyceridemia. Combination treatment was able to effect a further increase in LXR target gene production and effectively reduced inflammatory markers. Importantly, combination treatment was more effective than individual agonist treatments at stimulating the colocalization of microglia and plaques in APP/PS1 mice and promoting Aβ intracellular degradation. These data provide a rationale for further investigation of combination therapies utilizing LXR and PPARγ agonists in AD.

**Experimental Procedures**

*Animals and treatment*

APPswe/PS1Δε9 (APP/PS1) mice (Jackson Laboratories) co-express a chimeric human-mouse amyloid precursor protein containing the APPswe mutations (K595N/M596L) and human presenilin 1 with an exon 9 deletion (deltaE9) from the...
Nuclear receptor combination therapy benefits AD mice

Male 6-month-old APP/PS1 mice or non-transgenic (NonTg) littermates were orally gavaged daily for 9 days with 50 mg/kg/day GW3965, 80 mg/kg/day pioglitazone, both 50mg/kg/day GW3965 and 80mg/kg/day pioglitazone, or vehicle control (0.5% DMSO in sesame oil). Behavioral analysis was performed during the last two days of treatment. The animals were then sacrificed, and one hemisphere was fixed in 4% PFA and processed for immunohistochemistry. The hippocampus and cortex were removed from the other hemisphere and snap-frozen until subject to RNA and protein extraction. For analysis of plasma triglycerides, male 2-month-old C57Bl/6 mice were treated with GW3965, pioglitazone, both, or vehicle as above, with plasma collected on day 9 of treatment within 3 hours of the last dose. All experiments involving animals followed protocols approved by the Case Western Reserve University School of Medicine’s Institutional Animal Care and Use Committee.

**Behavioral testing**

**Contextual Fear Conditioning** - Freezing behavior was monitored by automated tracking system (Coulbourn Instruments, USA). On day 8 of drug treatment mice underwent training, which consisted of 2 minutes of free exploration in the shock chamber followed by 30 sec of the conditioned stimulus (CS: an 85dB sound at 2800Hz). After a 2 second delay, the unconditioned stimulus (US: 0.56mA) was delivered and the freezing response was measured for 30 sec. The training paradigm was repeated 4 times. Twenty four hours later the retention test was performed, during which mice were returned to the same shock chamber for 5 min for contextual freezing measurement in the absence of CS and US. The percent of time frozen and number of freezes was recorded.

**Protein extraction**

Cortex and hippocampus dissected from hemibrains were homogenized in 800μL of tissue homogenization buffer (250mM sucrose, 20mM Tris pH7.4, 1mM EDTA, 1mM EGTA in diethylpyrocarbonate-treated water). Homogenates were centrifuged at 5000xg for 10 minutes at 4°C, and supernatants were stored at -80°C for Western analysis. For extraction of soluble Aβ species, 250μL of homogenate was added to an equal volume 0.4% diethylamine in 100mM NaCl, and the samples were mechanically homogenized again. Samples were then centrifuged at 135,000xg for 1 hour at 4°C. 0.5M Tris-HCl pH6.8 was added to the supernatant, which was stored at -80°C for analysis of soluble Aβ species by ELISA. The remaining pellet was sonicated in cold 70% formic acid and centrifuged at 109,000xg for 1 hour at 4°C. The supernatant was neutralized and the samples stored at -80°C for analysis of insoluble Aβ species by ELISA.

**Cell culture**

Primary microglia and astrocytes were prepared from P0-P3 mice as previously described (9). Purified microglia and astrocytes were maintained in DMEM/F12 (Invitrogen) containing 5% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin for 3 days. Twenty four hours before treatment, media was changed to serum free DMEM/F12 containing 1% penicillin/streptomycin. For Western blot and qPCR analysis, cells were plated in 6 well plates at 1x10⁶ cells/well and treated for 24 hours with GW3965, pioglitazone, or GW3965 and pioglitazone or vehicle (DMSO) at the indicated concentrations.

**Intracellular Aβ Degradation Assay** – Soluble Aβ was prepared by dissolving lyophilized Aβ1-42 in DMSO to a final concentration of 1mg/ml to create a solution of mostly monomeric Aβ species with very few oligomers (42). Primary microglia were plated in 12-well plates at a density of 4 × 10⁵ cells/well. Microglia were then pre-treated for 24 hours with GW3965, pioglitazone, or GW3965 and pioglitazone or vehicle (DMSO) at the indicated concentrations, and then incubated with 2 μg/ml Aβ1-42 (American Peptide Company) for 18 h. Plates were washed with PBS and cells were lysed in 1% SDS with protease inhibitor cocktail (PIC) (Roche). Remaining intracellular Aβ was measured by ELISA.

**Aβ ELISA**

For the intracellular Aβ degradation assay, ELISAs were performed using 6E10 as the capture antibody and 4G8-HRP as the detection antibody (Covance). To analyze levels of soluble and insoluble Aβ in brain homogenates, ELISAs
were performed using 6E10 as the capture antibody and Aβ1-40-HRP or Aβ1-42-HRP (Covance) for detection. The results were read using a Spectramax colorimetric plate reader (Molecular Devices) and normalized to the total protein.

**Western Blot Analysis**

Cell lysates or brain homogenates were resolved on Bis-Tris 4-12% gels (Invitrogen), transferred to PVDF membranes, and immunodetected using anti-ABCA1 (Novus Biologicals), anti-ABCG1 (Novus Biologicals), anti-apoE (Santa Cruz Biotechnology), and anti-β-actin (Santa Cruz Biotechnology). Band intensities were quantified using NIH Image J software.

**Native PAGE**

Cell lysates or brain homogenates were resolved on Tris-Glycine 4-12% gels (Invitrogen), transferred to PVDF membranes, and immunodetected using an anti-ApoE antibody (Santa Cruz Biotechnology). Native high molecular weight standards (GE High Molecular Weight Native Marker Kit 17044501) were run on each gel and used to determine the Stokes diameter of samples. Intensity of the bands above 8nm in size was quantified using NIH Image J software to determine the apoE lipidation index.

**RNA extraction, reverse transcription, and quantitative PCR**

Quantification of pro- or anti-inflammatory gene expression was performed as previously described (27). For qPCR analysis of cells, RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. For qPCR analysis of brain homogenate, 200μL homogenate was combined with an equal volume of RNA-Bee (Tel-Test), chloroform was added, and samples were shaken vigorously. Samples were incubated on ice for 15 minutes followed by centrifugation at 13,000xg for 15 minutes at 4°C. The aqueous phase was collected, and RNA extraction was performed as above. RNA samples (0.5μg) were reverse transcribed using QuantiTect Reverse Transcription kit (Quiagen). The Taqman PreAmp Master Mix (Life Technologies) was then used to preamplify cDNA for 14 cycles according to the manufacturer’s instructions. Pre-amplified cDNA was run in a 10μL reaction for 40 cycles on

the StepOne Plus Real Time PCR system (Applied Biosystems) using the TaqMan Gene Expression Master Mix (Life Technologies). Primers labeled with FAM probes were from Life Technologies and included Iba1 (Mm00479862_g1), CD45 (Mm01293575_m1), Tnfα (Mm99999068_m1), II-1β (Mm01336189_m1), Nos2 (Mm01309902_m1), IL6 (Mm00446190_m1), and GAPDH (4352339E-0904021). The comparative CT method (ΔΔC_{T}) was used to analyze gene expression.

**Immunohistochemistry and image analysis**

Coronal sections (30μm) were taken from post-fixed hemispheres using a cryostat. Alternate sections were immunostained for analysis of plaque area, microglial area, and microglia/plaque colocalization. Antigen retrieval was performed with 20μL/mL Proteinase K in TE buffer (50mM Tris,1mM EDTA, 0.5% Triton X-100 pH 8) and slides were blocked in 5% normal goat serum in PBS 0.1% Triton X-100. Primary antibodies (6E10 1:1000, Covance; Iba1 1:500, Wako) were incubated overnight at 4°C. Analysis was performed on 2 sections per slide on 3 slides spaced evenly throughout the hippocampus. Images were analyzed by a blinded observer using Image Pro-Plus software (Media Cybernetics) for percentage area of 6E10-positive plaques in the cortex or hippocampus and percentage area occupied by Iba1-positive microglia. The percentage of 6E10 positive area also positive for Iba1 immunostaining was determined for every individual plaque in each section, and the results normalized to Iba1 intensity in a non-plaque area in each image to determine Iba1 enrichment at plaques.

**Triglyceride Assay**

Plasma was collected using 3.8% sodium citrate in water as an anticoagulant. Blood was centrifuged at 1,000 x g for 10 minutes at 4°C. The plasma was removed and stored at -80°C for further analysis. Levels of plasma triglycerides were determined using the Triglyceride Colorimetric Assay Kit (Cayman Chemical Company) according to the manufacturer’s instructions. The assays were read using a Spectramax colorimetric plate reader (Molecular Devices).
Statistics
All statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Two-tailed Student’s t test, one-way analysis of variance (ANOVA) with a Tukey post-test, or two-way ANOVA was used to determine p values.

Results
Enhanced effects of combined activation of PPARγ and LXR in vitro

Because PPARγ and LXR agonists have been independently reported to increase expression of LXR target genes (16,27), we analyzed whether a combined activation of PPARγ and LXR would lead to a greater increase in LXR target gene expression. We cultured primary astrocytes and quantified target gene expression after a 24-hour treatment with an LXR agonist (GW3965), a PPARγ agonist (pioglitazone) or a combined treatment with both. Consistent with our previous findings, we observed that LXR and PPARγ agonists were able to increase protein expression of apoE and its lipidating proteins ABCA1 and ABCG1 in cultured astrocytes (Fig 1A) (9,27). Combined treatment with GW3965 and pioglitazone did not produce an additive effect in the expression of apoE protein, but significantly increased the expression of its lipid transporter ABCA1 over pioglitazone alone (Fig 1A). To determine how this upregulation in ABCA1 affected apoE, we assessed the quantity of large-diameter apoE particles secreted by astrocytes into the extracellular media by native-PAGE (Fig 1B). Detection of large-diameter apoE particles by native-PAGE has been shown to correlate with increases in apoE lipidation (43), but we are unable to exclude the possibility that native-PAGE detects some amount of apoE aggregation. As expected, GW3965 and pioglitazone independently were able to increase the amount of large-diameter apoE secreted, and a combination treatment induced a further increase in large-diameter apoE particles (Fig 1B).

Microglia internalize soluble Aβ from the media and degrade it intracellularly, and this process is facilitated by agonists of LXR, PPARγ and RXR. (9,10,27,29). Microglia were incubated with Aβ for 18 hours resulting in the uptake and degradation of Aβ. The amount of intracellular Aβ remaining after that time was measured as an indicator of Aβ degradation efficiency. Pretreatment with GW3965 or pioglitazone for 24 hours enhanced the degradation efficiency in microglia, and pretreatment with a combination of both drugs further enhanced this effect (Fig 1C).

LXR and PPARγ are also known to have anti-inflammatory effects in microglia due to transrepression of NFκB at the promoters of inflammatory genes (37). To determine whether combination therapy targeting both receptors could enhance these anti-inflammatory effects, we pretreated primary cultured microglia for 24 hours with DMSO, GW3965, pioglitazone, or both nuclear receptor agonists, then induced an inflammatory response with LPS. Induction of transcription for pro-inflammatory cytokines TNFα and IL1β was less in microglia pretreated with either GW3965 or pioglitazone (Fig 1D). Microglia pretreated with a combination therapy exhibited enhanced suppression of these pro-inflammatory genes (Fig 1D). Pretreatment with GW3965 or pioglitazone alone did not decrease transcript levels of iNOS, but the combination pretreatment produced a significant decrease in iNOS transcript (Fig 1D).

Combination therapy increases apoE particle size in APP/PS1 mice

Previously published data indicates that increasing apoE protein levels and lipidation was associated with reversal of the behavioral deficits and some aspects of pathology in AD mouse models (30). Based on our in vitro data indicating that combination therapy enhances the transcriptional effects of LXR and PPARγ agonists and their facilitation of Aβ degradation (Fig 1), we chose to treat APP/PS1 mice with the combination therapy. Four treatment groups of age-matched transgenic male six month old animals were generated and treated for 9 days by oral gavage. The first group was treated with 50mg/kg/day GW3965, the second with 80mg/kg/day pioglitazone, the third with 50mg/kg/day GW3965 and 80mg/kg/day pioglitazone in the same volume, and the fourth with a vehicle of DMSO in sesame oil. Western blot analysis on hemibrain cortical/hippocampal
homogenate indicated that GW3965 and pioglitazone increased transcription of LXR target genes ABCA1, ABCG1, and apoE, with combination therapy providing a significant increase in apoE over GW3965 alone (Fig 2, A and B). Non-denaturing gel electrophoresis was performed on cortical/hippocampal homogenates and detected with apoE antibody to determine the size distribution of apoE. GW3965 and pioglitazone were both able to increase the proportion of large-diameter apoE particles, and combination therapy further increased this effect (Fig 2C).

Pioglitazone treatment reduces GW3965-mediated elevation of plasma triglycerides

LXRs agonists have been reported to upregulate the synthesis of fatty acids in the liver due to LXR-mediated stimulation of a gene expression program which includes SREBP-1c and fatty acid synthase, among other lipogenic enzymes (41). In a preliminary study in a cohort of WT mice, we observed a significant increase in plasma triglycerides after 9 days of treatment with GW3965 compared to vehicle, which was not observable after 9 days of treatment with pioglitazone or with both GW3965 and pioglitazone (data not shown). In 6 month old APP/PS1 mice, we observed a strong trend for GW3965 to increase plasma triglycerides, which was significantly reduced by the addition of pioglitazone in our combination treatment (Fig 2D). Treatment with either GW3965, pioglitazone, or both agonists did not correspond to an increased liver weight (Fig 2E).

Combination therapy reduces inflammatory markers in APP/PS1 mice

APP/PS1 animals have increased expression of Iba1 and CD45, microglial markers which are reflective of a proinflammatory phenotype (Fig 3, A-D). GW3965 and pioglitazone were able to reduce expression of Iba1, as assessed by qPCR on cortical/hippocampal homogenate (Fig 3A) and immunostaining on hemicortical sections, significantly reducing Iba1 immunostaining compared to vehicle or GW3965 alone (Fig 3, B and C). Interestingly, combination therapy effected the largest decrease in Iba1, reducing Iba1 levels to a point below baseline wild-type levels (Fig 3A). The levels of CD45 were also decreased by combination therapy (Fig 3D).

Our in vitro data indicate that treating microglia with GW3965 and pioglitazone together decreases the inflammatory response they exhibit upon challenge with LPS (Fig 1D). In the APP/PS1 mouse model, mice treated with GW3965, pioglitazone, or both, all exhibited different levels of pro-inflammatory gene expression as measured by qPCR (Fig 3, E-G). IL6 was the only cytokine significantly increased in APP/PS1 animals at 6 months of age, and pioglitazone and GW+pio were able to significantly decrease IL6 gene expression (Fig 3E). IL1β was significantly decreased by GW3965 and GW+pio (Fig 3F), and TNFα was decreased by pioglitazone but increased by GW+pio treatment (Fig 3G), suggesting that GW3965, pioglitazone, and GW+pio act on microglia to differently regulate their inflammatory state.

Combination therapy reduces Aβ deposition by increasing co-localization between microglia and plaques

To evaluate the ability of nuclear receptor agonists to reduce Aβ species in 6 month old APP/PS1 mice, we sequentially extracted soluble and insoluble Aβ species from cortical and hippocampal homogenates. Treatment with GW3965 alone did not significantly reduce soluble or insoluble Aβ levels, while pioglitazone treatment significantly decreased only levels of soluble Aβ40 by about 25% (Fig 4, A and B). However, when we evaluated Aβ levels in treated mice by IHC (Fig 4, C-E) we were able to observe significant decreases in the amount of hippocampal (Fig 4E) but not cortical (Fig 4D) 6E10 staining, indicating that GW3965 and pioglitazone were able to facilitate the clearance of deposited Aβ in the hippocampus.

In contrast, mice treated with combination therapy exhibited decreased levels of both soluble Aβ40 and 42 as measured by ELISA (Fig 4A). Insoluble Aβ40 levels were also decreased by about 50%, but the decreases in insoluble Aβ42 were not significant (Fig 4B). These findings correlated with an approximate 60% decrease in cortical plaques.
and a 50% decrease in hippocampal plaques as quantified by IHC (Fig 4, C-E).

Decreases in insoluble species of Aβ and plaque are likely mediated principally by microglia, which are capable of taking up and degrading fibrillar forms of Aβ. We found that the overall Iba1+ area in the brain is decreased following nuclear receptor agonist treatments (Fig 3C), and thus follows overall plaque burden. However, we observed there are more microglia at sites of plaque deposition (Fig 5). To determine the amount of co-localization between microglia and plaques, we quantified the amount of 6E10/Iba1 double positive area in the cortex (Fig 5B) and hippocampus (Fig 5C) of treated and control mice by IHC. GW3965, pio, and GW+pio treatments significantly increased the amount of microglia/plaque co-localization in the cortex, and GW3965 and GW+pio were able to significantly increase co-localization in the hippocampus. These observations suggest that nuclear receptor agonists mediate reductions in insoluble Aβ by altering the interaction between microglia and plaques.

Treatment with nuclear receptor agonists reverses the cognitive deficits in APP/PS1 mice

To determine whether treatment with nuclear receptor agonists is able to improve behavioral impairments in APP/PS1 animals, we evaluated hippocampal-dependent memory in the contextual fear conditioning test. APP/PS1 animals exhibited a decreased freezing response as compared to WT animals during the training period of the task, but drug-treated animals did not exhibit this impairment in learning during the training period (Fig 6A). As expected, WT age-matched littermate controls froze for about 25% of the testing period, while at 6 months of age APP/PS1 mice exhibited deficits in freezing behavior, freezing about 15% less than WT littermate animals (Fig 6B). Consistent with previously published studies, 9 day treatments with GW3965 and pioglitazone were able to restore freezing behavior in APP/PS1 animals to WT levels. A 9 day treatment with GW+pio was able to achieve an equivalent cognitive improvement (Fig 6B).

Discussion

LXR and PPAR agonists have previously been demonstrated to have beneficial effects in AD mouse models. The first study utilizing nuclear receptor agonists in AD was published in 2003 by Yan et al. (44), and reported that long-term treatment of Tg2576 mice with pioglitazone was able to decrease levels of soluble Aβ. However, subsequent studies using PPAR agonists in several AD mouse models had variable success in observing changes in Aβ levels. Several studies using various treatment paradigms with PPARγ agonists observed decreases in soluble Aβ species (45,46), plaques (47,48) or both (27,36,49,50), and others reported decreased intracellular Aβ (51,52). However, there were also cases where PPARγ agonists exhibited no measurable effect on Aβ pathology (53,54,55). LXR agonists have had a similarly mixed success since 2005, when Koldanova et al. reported that TO901317 was able to reduce soluble Aβ levels in APP23 mice (11). Since then, LXR agonists have been reported to decrease soluble Aβ (9,18,20,22,23,25,56), decrease insoluble Aβ or plaques (9,19,20,22,23,24,56), or in one case decrease plaques while increasing soluble Aβ species (13).

One study observed no biochemical changes in Aβ pathology in aged animals (21), but reported behavioral improvements. While the majority of studies report that PPARγ and LXR agonists are able to reduce Aβ pathology, the variability in the clearance of soluble Aβ species and plaques remains unexplained. The genotype, age of the mice, differences in diet, and drug formulation are all likely to influence drug efficacy, but these factors have not been systematically explored.

We found that treatment with GW3965 did not significantly stimulate clearance of soluble and insoluble species of Aβ, while pioglitazone was only able to stimulate clearance of soluble Aβ40 in measured in whole brain homogenates. We also visualized plaques using IHC to evaluate regional effects on Aβ deposition. Interestingly, although plaques did not significantly decrease in the cortices of pioglitazone or GW3965 treated animals, both drugs were able to induce significant decreases in hippocampal plaque load in the same animals. Our results are similar to those of Riddel et al. (18), who reported that an LXR agonist selectively induced apoE and ABCA1 and reduced Aβ42 in the hippocampus of Tg2576 mice. Donkin
et al. (13) also reported that GW3965 mediated a hippocampal-specific reduction in dense-core plaques in APP/PS1 mice, although when Aβ deposition across the whole brain was quantified no significant reduction was observed.

Combination therapy with GW+pio significantly reduced overall levels of soluble Aβ40 and Aβ42. It is likely that the reduction in soluble Aβ we observe in GW+pio treatment is due in part to the enhanced transcription of apoE and its lipid transporters, as levels of lipidated apoE have been previously shown to increase the rate of degradation of Aβ by microglia (9,10). However, we also observe an acute reduction of insoluble Aβ40 and deposited Aβ plaques in both the cortex and hippocampus over the course of our 9 day treatment with GW+pio, which is more likely due to active phagocytic clearance by microglia (57). We postulate that the reductions in Aβ we observe are due mainly to modulation of Aβ clearance rather than Aβ production because previous studies using GW3965 (9) and pioglitazone (27) have established that these drugs do not affect the production or processing of APP in transgenic mouse models of AD. This agrees with our in vitro observations that GW+pio treatment is able to enhance microglial degradation of Aβ to a greater degree than either agonist alone. The mechanistic basis of the enhanced effectiveness of the combined drug treatment may be the ability of the drugs to target independent enhancer sequences to which the nuclear receptors bind and act combinatorially to promote gene expression (7). Future studies will be able to determine whether the Aβ clearance resulting from engaging PPARγ and LXR together via a combination therapy will be more reproducible than the effects of individual PPARγ and LXR agonists.

It is possible that the facilitation of microglial phenotypic conversion to an anti-inflammatory, phagocytic phenotype contributes to the increased benefits of GW+pio treatment. APP/PS1 mice at 6 months of age already exhibit increased microgliosis and astrogliosis as well as pro-inflammatory alterations in their brain cytokine profile (27). Importantly, PPARγ agonists have been shown not only to reduce inflammation, but also to increase phagocytosis of Aβ in vitro and in vivo (27,36). The effects of LXR agonists on inflammation in AD models have not been studied as thoroughly, but treatment with LXR agonists has been reported to be anti-inflammatory (19,24), increase co-localization between glia and plaques (23), and regulate transcription of phagocytic genes (39). Together with evidence that, in macrophages, PPARγ and LXR are able to transrepress overlapping but distinct sets of pro-inflammatory genes (37,38,41), this body of evidence led us to hypothesize that co-stimulation with LXR and PPARγ agonists would have a greater anti-inflammatory effect than either agonist alone. We found that GW+pio treatment effectively decreased pro-inflammatory markers in vitro and in vivo with an interesting exception – GW+pio reduced TNFα transcript levels in cultured primary microglia but not in whole brains in APP/PS1 mice. This could indicate that GW+pio treatment acts differently on non-microglial brain cells that produce TNFα, such as astrocytes, or imply that environment is an important factor for determining the effects of these drugs on microglia. Importantly, GW+pio also increased the co-localization between plaques and microglia. We propose that this enhanced co-localization facilitates improved phagocytosis and together with the increased degradation of Aβ by microglia accounts for the reduction of Aβ species we observe with GW+pio treatment.

Our investigation also reports behavioral improvements in APP/PS1 mice after LXR or PPARγ agonist treatment, which is consistent with the vast majority of previous studies. LXR (9,13,18,20,21,22,23,24) and PPARγ (27,36,46,48,50,52,55,59,60) agonists have been reported to ameliorate memory deficits using a variety of tests in a range of mouse models. It should be noted, however, that Nicolakakis et al. (53), Masciopinto et al. (59), and Papadopoulos et al. (54) reported no behavioral improvements after treating mice with pioglitazone. We find that APP/PS1 mice at 6 months of age are impaired in performance of the contextual fear conditioning task, and that this impairment is rectified by treatment with GW, pio, or both agonists. Our dosage paradigm did not allow for closer examination of this effect, since we utilized high doses of GW+pio that have been previously shown to restore freezing behavior to wild-type levels. It will be important in future studies to treat at lower
doses of GW+pio that are less individually effective to determine if combination treatment is able to further enhance memory improvements.

Importantly, although pioglitazone has entered phase III clinical trials for the treatment of AD, no LXR agonists have reached clinical trials due to an unfavorable side-effect profile that includes hypertriglyceridemia (61,62). This is likely due to LXR-dependent upregulation of the sterol response element binding protein-1c (SREBP1C) pathway in the liver. PPARα agonists have been shown to decrease hypertriglyceridemia, possibly through stimulating β-oxidation of fatty acids (42), and a PPARα agonist was shown to counteract LXR agonist-induced hypertriglyceridemia in mice (63). There is evidence that PPARγ activation could also normalize LXR-mediated hypertriglyceridemia, possibly by mediating the redistribution of triglycerides from plasma to storage in fatty tissue by inducing, genes involved in lipoprotein uptake and hydrolysis (64,65). Our finding that the addition of a PPARγ agonist is able to normalize the hypertriglyceridemia observed in LXR agonist treated mice renews the potential for LXR agonists to enter clinical trials for AD. Additionally, we did not observe changes in liver weight in mice treated with GW3965, pioglitazone, or GW+pio, which is consistent with previously reported work (66,67,68,69,70).

In this study we provide support for the beneficial actions of LXR and PPARγ agonists in the treatment of amyloid pathology, inflammation, and behavioral impairments in AD mouse models. LXR and PPARγ activation in combination more effectively improves several biochemical markers and alleviates cognitive impairments in AD mice, with an additional reduction in side effects. Our results demonstrate potential for therapies targeting multiple heterodimer partners of RXR for the treatment of AD.

Acknowledgments- We thank J. Colleen Karlo for invaluable technical assistance.

Conflict of interest- The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions- RRS and GEL designed the study and wrote the paper. MPP performed and analyzed the experiments shown in Figures 3 and 4 and contributed to the preparation of the figures. BTC designed, performed and analyzed the serum triglyceride assays and provided technical assistance. LT provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

Nuclear receptor combination therapy benefits AD mice


FOOTNOTES

*This work was supported by NIH grant R01 AG030482 (to G.E.L.), NIH Grant 5T32NS067431-13 (to R.R.S.), the Gail & Elliott Schlang Philanthropic Fund, and CAPES Foundation Grant 5758/12-2 (to M.P.P.).

1 To whom correspondence should be addressed: Gary E. Landreth, Dept. of Neurosciences, Case Western Reserve University, School of Medicine,10900 Euclid Ave., Cleveland, OH 44106. Tel.: 216-368-3435; E-mail: gel2@case.edu.

2 The abbreviations used are: AD, Alzheimer disease; Aβ, β-amyloid; APP, amyloid precursor protein; PS1, presenilin 1; LXR, liver X receptor; PPARγ, peroxisome proliferator-activated receptor γ; apoE, apolipoprotein E.

FIGURE LEGENDS

Figure 1. Combination therapy increases target gene expression and Aβ degradation and decreases inflammatory markers in vitro.
(A) Cultured primary astrocytes were incubated for 24 hours with DMSO (uM), 500nM GW3965, 100nM pioglitazone, or both doses combined, then LXR target genes were quantified by immunoblotting and normalized to actin. Representative blots are shown on right. (B) Conditioned medium from the same treated primary astrocytes was collected and separated by native PAGE, then immunoblotted for apoE. (C) Primary microglia were treated for 24 hours with GW, pio, or GW+pio, followed by the addition of 2μg/mL Aβ1-42 for 18 hours. Remaining intracellular Aβ was quantified using ELISA and normalized to total protein. (D) Cultured primary microglia were incubated for 24 hours with the indicated concentrations of drug or DMSO for controls, then 100ng/mL LPS was introduced to the media for 12 hours. RNA was extracted and expression of pro-inflammatory genes examined by qPCR analysis. The dotted line indicates baseline transcript levels in DMSO only control. The LPS-treated control was pre-incubated with DMSO for 24 hours then 100ng/mL LPS for 12 hours. n=4. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the vehicle-treated control; ##p < 0.01, ###p < 0.001 compared with the LPS-treated control; student’s t test. Abbreviations: nm, nanometers.

Figure 2. Nuclear receptor agonists stimulate transcription of LXR target genes in AD mice
(A) 6 month old transgenic APPsw/PSENΔE9 mice were orally gavaged with 50 mg/kg/day GW3965, 80 mg/kg/day pioglitazone, or both for 9 days. Cortical/hippocampal homogenate was immunoblotted for ABCA1, ABCG1, and apoE. Representative blots are shown. (B) Each sample was normalized to actin, and results are expressed as fold difference compared to vehicle controls. (C) Equal volumes whole brain homogenate from each treatment group were analyzed by native PAGE and immunoblotted for apoE. n=7-11 animals/group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the vehicle-treated control. (D and E) 6 month old transgenic APPsw/PSENΔE9 or non-Tg littermate control animals were orally gavaged with doses described above for 9 days. (D) Plasma was collected and analyzed for triglyceride content by colorimetric assay. n=6 animals/group. (E) Complete livers were removed from each animal and weighed, and liver weight is normalized to total body weight for each animal. *p < 0.05 compared with vehicle-treated control; #p < 0.05 compared with GW-treated group; student’s t test. Abbreviations: nm, nanometers.

Figure 3. Nuclear receptor agonists reduce the pro-inflammatory environment in APP/PS1 mice.
Transcript levels from RNA isolated from whole-brain homogenate (A) and quantification of immunofluorescence in cortex and hippocampus (B) of the microglia marker Iba1 in 6 month old APP<sub>sw</sub>/PSENΔE9 orally gavaged with the indicated drugs for 9 days. 9 hemicoronal sections per animal were immunostained for Iba1 and Iba1 area analysis was performed as described in “Experimental Procedures.” (C) Representative images of Iba1 immunostaining in the cortex are shown. (D-G) Transcript levels of pro-inflammatory markers CD45 (D) IL6 (E) IL1β (F) and TNFα (G) were analyzed by qPCR on RNA isolated from whole-brain homogenate. n=7-11 animals/group. *p < 0.05, **p < 0.01 compared with vehicle-treated APP/PS1 mice; #p < 0.05, ##p < 0.01, ###p < 0.001 compared with non-Tg littermate control mice. A, D-G: One way ANOVA with Tukey post-test, B: student’s t test.

Figure 4. Combination therapy significantly reduces amyloid burden in AD mice.
Aβ was sequentially extracted using DEA for soluble Aβ (A) and formic acid for insoluble Aβ (B) from half-brain cortex/hippocampal homogenates. Samples were analyzed by ELISA and Aβ values were normalized for total protein loaded and to vehicle treated animals. To quantify plaque load by immunohistochemistry, 9 hemicoronal sections per animal were immunostained for 6E10 and plaque area analysis was performed as described in “Experimental Procedures.” (C) Representative images are shown from the cortex. Aβ plaque area was quantitated in the cortex (D) and hippocampus (E). n=7-11 animals/group. *p < 0.05, **p < 0.01 compared with vehicle-treated APP/PS1 mice; student’s t test.

Figure 5. Nuclear receptor agonists promote microglial colocalization with plaques.
(A) Hemicoronal sections from each treatment group were co-immunostained with Iba1 (green) for microglia and 6E10 (red) for Aβ plaques. Representative images from the cortex are shown. Colocalization between 6E10 and Iba1 was analyzed according to “Experimental Procedures” in the cortex (B) and hippocampus (C). n=7-11 animals/group. *p < 0.05, **p < 0.01 compared with vehicle-treated APP/PS1 mice; student’s t test.

Figure 6. Nuclear receptor agonists ameliorates cognitive deficits in AD mice.
Training for the fear conditioning assay was performed on day 8 of drug treatment and mice were tested on day 9. (A) The number of freezes by each treatment group during training is shown as a function of periods. #p < 0.05, ###p < 0.001 between non-TG control mice and TG Veh mice by two-way ANOVA. (B) The percentage of time spent freezing by each treatment group during the contextual fear conditioning test period. *p < 0.05 compared with vehicle-treated APP/PS1 mice; #p < 0.05 compared with non-Tg littermate control mice by one way ANOVA with Tukey post-test. n=7-11 animals/group.
Figure 1

A

![Graph showing relative density normalized to DMSO treatment for ABCA1, ABCG1, and ApoE. The graph includes data points for DMSO, GW, PIO, and GWPIO.](image)

B

![Graph showing ApoE lipoprotein index (a.u.) for DMSO, GW, PIO, and GWPIO.](image)

C

![Graph showing relative intracellular Aβ42 levels for DMSO, GW, PIO, and GWPIO.](image)

D

![Graph showing fold change in IL1β, TNFα, and iNOS normalized to DMSO treatment.](image)
Figure 2

A

<table>
<thead>
<tr>
<th>Group</th>
<th>ABCA1</th>
<th>ABCG1</th>
<th>ApoE</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG Veh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG GW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG PIO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG GWPIO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Graph and Table]

C

![Graph and Table]

D

![Graph and Table]

E

![Graph and Table]
Figure 3

A) Iba1

B) Iba1 Immunofluorescence

C) Iba1 - Cortex

D) CD45

E) IL6

F) IL1β

G) TNFα
Nuclear receptor combination therapy benefits AD mice

Figure 4

A

![Bar chart showing fold change in soluble 40 and 42 for TG Veh, TG GW, TG PIO, and TG GWPIO treatments.](image)

B

![Bar chart showing fold change in insoluble 40 and 42 for TG Veh, TG GW, TG PIO, and TG GWPIO treatments.](image)

C

![Immunofluorescence images of 6e10 staining in different brain regions.](image)

D

![Histogram showing percentage of area with 6e10 fluorescence in the cortex for TG Veh, TG PIO, TG GW, and TG GWPIO treatments.](image)

E

![Histogram showing percentage of area with 6e10 fluorescence in the hippocampus for TG Veh, TG PIO, TG GW, and TG GWPIO treatments.](image)
Figure 5

Nuclear receptor combination therapy benefits AD mice
Figure 6

A

Number of Freezes

Training 1  Training 2  Training 3  Training 4

WT  TG V  TG GW  TG PIO  TG GWPIO

B

Freeze Time (% Total Time)

WT  TG Veh  TG GW  TG GWPIO  TG PIO

*  *  *
Combined Liver X Receptor/Peroxisome Proliferator-Activated Receptor γ Agonist Treatment Reduces Amyloid-β Levels and Improves Behavior in Amyloid Precursor Protein/Presenilin 1 Mice

Rebecca Skerrett, Mateus P. Pellegrino, Brad T. Casali, Laura Taraboanta and Gary E. Landreth

_J. Biol. Chem. published online July 10, 2015_

Access the most updated version of this article at doi: 10.1074/jbc.M115.652008

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2015/07/10/jbc.M115.652008.full.html#ref-list-1