Polytherapy for Alzheimer's Disease: Combining Gamma Secretase Modulation and Corticotrophin-Releasing Factor Receptor 1 Antagonism

Permalink
https://escholarship.org/uc/item/1st610f5

Author
Vo, Uyen

Publication Date
2016

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA, SAN DIEGO

Polytherapy for Alzheimer’s Disease: Combining Gamma Secretase Modulation and Corticotropin-Releasing Factor Receptor 1 Antagonism

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

Uyen Vo

Committee in charge:

Professor Robert Rissman, Chair
Professor Brenda Bloodgood, Co-Chair
Professor Randolph Hampton

2016
The thesis of Uyen Vo is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2016
DEDICATION

I dedicate this thesis to David Truong
and my family for their love and support.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>vii</td>
</tr>
<tr>
<td>Abstract of the Thesis</td>
<td>viii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>5</td>
</tr>
<tr>
<td>Results</td>
<td>10</td>
</tr>
<tr>
<td>Discussion</td>
<td>22</td>
</tr>
<tr>
<td>References</td>
<td>27</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Representative coronal sections of Thioflavin S stained images..................13
Figure 2. Effects of drug treatment on Aβ plaque load.............................................14
Figure 3. Effects of drug treatment on water maze acquisition performance.............15
Figure 4. Effects of drug treatment on water maze spatial memory performance........16
Figure 5. Effects of drug treatment on water maze cognitive flexibility performance.....17
Figure 6. Impact of drug treatment on body weight as a function of age.....................20
Figure 7. Effects of drug treatment on liver protein and substance levels...............21
Supplemental Figure 1. Neuroanatomical structures of vehicle-treated AD mouse......26
ACKNOWLEDGEMENTS

I would first like to express my sincere appreciation to Dr. Robert Rissman for giving me the opportunity to join his lab. His knowledge and down-to-earth mentorship contributed to this outstanding educational experience. The two years that I have spent here in the lab has not only taught me many skills and techniques needed for future endeavors, but they have also been one of the best experiences that I have had. I would also like to acknowledge Drs. Brenda Bloodgood and Randolph Hampton for being members of my committee.

Also, my project would not have been possible without Drs. Steve Wagner, Robert Clark and Kent Osborn, and the animal core associates Ed Rockenstein, Mike Mante, and Jazmin Florio. I thank you kindly for your collaboration and contribution; it was imperative to the success of this research.

Certainly, I am truly grateful for the mentorship and help with the data analyses by Drs. Charisse Winston and Vickie Zhang. I would also like to express my gratitude to Louise Monte for being a great supervisor and for always being available when I felt lost in science. Of course, I shall not forget to thank Shannon Campbell, Phuong Nguyen, Olga Prikhodko, and Debbie Masliah for working alongside with me on many of the experiments.

Finally, I want to thank my family for their love and support. I am extremely grateful for my fiancé David Truong whose support, understanding, and thoughtfulness have been much appreciated throughout my academic journey.
ABSTRACT OF THE THESIS

Polytherapy for Alzheimer’s Disease: Combining Gamma Secretase Modulation and Corticotropin-Releasing Factor Receptor 1 Antagonism

by

Uyen Vo

Master of Science in Biology

University of California, San Diego, 2016

Professor Robert Rissman, Chair
Professor Brenda Bloodgood, Co-Chair

Alzheimer's disease (AD) is the most common neurodegenerative disorder and age-related dementia in the elderly. It affects 5.4 million people in the United States and it is the sixth-highest cause of death. Neuropathologically, AD is characterized by the
accumulation of insoluble extracellular plaques composed of β-amyloid (Aβ) and intracellular tangles consisting of phosphorylated forms of the microtubule-associated protein, tau, in the brain. Current treatments only temporarily and mildly boost cognitive function and as such they are unable to slow or halt the underlying pathophysiological progression of the disease.

Our group has previously shown that two small molecules, CRFR1 antagonist (R121919) and γ-secretase modulator (BPN-15606), were able to attenuate Aβ plaque load in vivo. Here we extend the study by using a polytherapeutic approach. We tested R121919 and BPN-15606 alone as monotherapy and together as polytherapy in an Alzheimer’s disease transgenic mouse model. We hypothesized that polytherapeutic approaches that impact multiple pathways in the AD brain will be efficacious over monotherapy due to the complexity of pathways of neurodegeneration. Their effects alone and in combination was compared on relevant behavioral, pathological, and biochemical endpoints.

Our results indicate that chronic administration of R121919, BPN-15606, or polytherapy present to be safe in terms of liver function. AD mice treated with BPN-15606 or polytherapy significantly ameliorated Aβ, but also showed significant lack of weight gain. No treatment effect was seen in mice receiving R121919. Using water maze to evaluate cognitive function, AD mice receiving any of the treatments did not show improvement in either the acquisition or spatial memory assessment. However, the combo-treated cohort was impaired in latency acquisition compared to vehicle suggesting adverse cognitive function. Our results suggest that polytherapy was not more efficacious
over monotherapy, but BPN-15606 alone demonstrated to be a potential disease-modifying therapeutic approach with limited adverse effects and without liver toxicity.
INTRODUCTION

Alzheimer’s disease (AD) is a progressive and age-related neurodegenerative disorder characterized symptomatically by decline in anterograde episodic memory, thinking, and reasoning skills. While memory impairment is the earliest, key feature [1], AD is also defined by alterations to personality and confusion about events, time, and place. As the disease progresses, decline in visuospatial skills, language, abstraction, planning, attention, and executive functions are observed [2,3].

Estimates from the Alzheimer’s Association 2015 indicate that over 5.4 million people in the United States have AD and 47 million people worldwide are living with dementia. It is the most common cause of dementia, and the number may triple without development of medical breakthroughs to prevent or cure the disease. AD is the sixth-highest cause of death and is the only disease among the top 10 causes of death in America that cannot be prevented, cured, or even slowed. From 2000–2013, AD death increased by 71%, and by 2050, national cost could top $1 trillion [4, 5, 6].

Neuropathologically, both sporadic and familial AD is characterized by the accumulation of insoluble extracellular plaques composed of β-amyloid (Aβ) and intracellular tangles consisting of phosphorylated forms of the microtubule-associated protein, tau, in the brain [7]. These neuropathological changes start in the entorhinal cortex and hippocampus, advancing into other temporal, parietal, and finally frontal association cortices [8].

Dominantly inherited mutations in amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) genes are the primary causes of familial Alzheimer’s disease (FAD), accounting for 1–6% of all AD cases [6]. The amyloid precursor protein
(APP) gene provides instructions to make a single-pass transmembrane protein with large extracellular domains. While its function remains unclear, it is expressed at high levels in the brain and metabolized in a rapid and complex fashion by a series of sequential proteases [9].

The non-amyloidogenic processing of APP involves α-secretase followed by γ-secretase, while the amyloidogenic processing of APP involves β-secretase followed by γ-secretase [10]. While there are more than 200 mutations associated with AD, the most notorious APP mutation (APP-Swedish) causes a change in amino acids adjacent to the β-secretase cleavage site. PSEN1 and PSEN2 genes encode integral components of the multiprotein protease complex, γ-secretase. The PSEN mutations predominantly result in defective presenilin protein, which interferes with the function of the γ-secretase complex. Both APP and PSEN mutations selectively enhance production of a longer, toxic version the amyloid peptide Aβ42 relative to the less amyloidogenic Aβ40 generation [11,12], suggesting a toxic gain-of-function pathogenic mechanism.

Preceding neuronal cell loss, these sticky Aβ peptide fragments accumulate extracellularly to form plaques predominantly in the hippocampus and neocortex. The multiprotein protease complex, γ-secretase, is therefore a recognized therapeutic approach. Previous approaches to therapeutic intervention aimed to lower total Aβ peptide production by inhibiting the catalytic activities of γ-secretase. Besides regulating intramembrane proteolysis of APP, γ-secretase also regulates Notch, a large cytoplasmic intracellular domain that is necessary for proper cellular differentiation and development [13]. Inhibiting γ-secretase therefore leads to potential adverse effects by also preventing Notch intracellular domain (NICD) formation [14, 15, 16]. As an alternative to inhibiting
γ-secretase, γ-secretase modulator (GSM) has been identified, which lowers the levels of the most fibrillogenic Aβ peptide, Aβ42, without affecting NICD [17].

The underlying mechanisms that give rise to the sporadic, far more common form of AD remain unknown. However, it is widely believed that sporadic AD is caused by complex interactions between various genetic influences and environmental factors [18]. Several lines of epidemiological studies indicate that individuals prone to psychological distress as a consequence of stress exposure had higher risk of developing AD than those less prone to distress. Corticotropin-releasing factors (CRFs) and their receptor corticotropin-releasing factor receptors (CRFRs) are greatly distributed throughout the hippocampus and cortex to regulate the hypothalamic-pituitary-adrenal (HPA) axis in response to stress [19], which in turn may affect stress-sensitive processes, such as memory and anxiety [20, 21].

Since the hippocampus and cortex are the earlier sites of Aβ accumulation and hippocampal degeneration is a prominent characteristic of the disease [22], the CRF system has been identified as a potential target for intervention. Previous studies have shown that repeated restraint stress accelerates Aβ pathogenesis in AD mouse models via generation of metabolic oxidative stress, and the effects were reversed by administration of the CRFR antagonist, NBI27914 [23].

Recently, two different pharmaceutical-like small molecules, γ-secretase modulator (BPN-15606) and CRFR1 antagonist (R121919), have been characterized to attenuate Aβ plaque load in animals models. Our group has previously reported that CRFR1 antagonist (R121919) as a monotherapy significantly prevented the onset of
cognitive impairment and reduced Aβ levels in female AD mice [24] whereas BPN-15606 reduced Aβ plaque without inhibiting Notch formation [17].

To extend from our previous findings, the two compounds, BPN-15606 and R121919, in this study will be tested alone as monotherapy and together as polytherapy in an Alzheimer’s disease transgenic mouse model. It is hypothesized that polytherapeutic approaches that impact multiple pathways in the AD brain will be efficacious over monotherapy due to the complexity of pathways of neurodegeneration. We predict that this polytherapy provides greater efficacy than either monotherapy, in terms of biochemical markers, neuropathology, and cognition. Their effects alone and in combination will be compared on relevant behavioral, pathological, and biochemical endpoints.
MATERIALS AND METHODS

Subject

Thirty-five female double transgenic AD mice (model PSAPP) bought from Jackson Laboratory (B6.C3-Tg [APPswe, PSEN1dE9] 85Dbo/Mmjax, stock no. 0034829) and three bred in house were used in the study at 90 days of age. The mice were housed in a temperature-controlled room at a constant 22 °C in a 12:12-h light/dark cycle (lights off at 18:00), with food and water available ad libitum. Age-matched mice were housed by drug group in groups of 2-4 per cage, with the exception of the wild-type mice (C57BL/6J background) added to behavioral experiments. All experimental procedures were reviewed and approved by IACUC at UC San Diego.

Pharmacological Treatment and Administration

Gamma secretase modulator (BPN-15606) and CRFR1 antagonist (R121919) were used independently as a monotherapy and in combination as a polytherapy. Subjects were randomly assigned to one of the drug groups or vehicle, with individual groups ranging from 9-10 mice. Mice were treated for six months with BPN-15606 or R121919 with the same dose of 167 mg/kg/day, or the combination therapy (polytherapy) at the dose of BPN-15606 167 mg/kg/day plus R121919 167 mg/kg/day. For route of administration, the drugs were milled into standard rodent chow, processed by Research Diets, Inc (New Brunswick, NJ). All animals were weighed three times weekly to assess any adverse effects on normal weight gain during the six-month treatment period. Food consumption was determined by weighing the metal cage, including the chow, to the
nearest 0.1 g. For drug level PK analysis, blood samples were obtained monthly from the facial vein and stored at -80°C.

**Water Maze Apparatus**

Water maze testing was conducted in a 1.8-m diameter pool of water rendered opaque by the addition of nonfat powdered milk. The water was maintained between 19-21 °C. The testing room contained a number of constant, high-contrast, and salient extramaze cues (posters, objects, and equipment) on the walls. A video camera was mounted on the ceiling directly above the pool and was used in conjunction with a video-tracking system (San Diego Instruments) to record the swim path of each mouse. We used an Atlantis platform (12.7-cm diameter) with Styrofoam attached on the surface, which could be raised or lowered remotely. When the platform was in the raised position (standard training trials), 1 cm below water surface, it remained invisible to the mouse but provided a means to escape the water. AD mice not enrolled in the study were tested beforehand to ensure that they could climb and would remain on the platform. When the platform was in the lowered position (probe trials), the mouse could neither detect the platform nor escape from the water.

**Water Maze Behavioral Testing**

Water maze paradigm was adapted from Clark and Squire Laboratory, UCSD. All behavioral testing was done two weeks prior to the end of the six-month drug treatment to assess spatial memory. Each day, mice were given an initial probe trial after a 24-hour delay, followed by three standard training trials, and another probe trial, immediately
followed by three additional standard training trials for five consecutive days, during which each mouse was released into the pool at different starting points. After each trial, the mice were placed on a cotton towel to dry and then placed back into their home cages.

For the standard training trials, the mice were permitted 60 sec to find the platform, and if found, they needed to remain on the platform for 30 sec. If the mice failed to find the platform within the allotted time, they were gently guided to the platform, in which case they also needed to remain on the platform for 30 sec. During probe trials, the platform was in its lowered position for 60 sec to assess spatial memory. It was then raised, and mice were given an additional 60 sec to find the raised invisible platform. After five consecutive days of testing, the mice were given a two-day break, and then a reversal task was performed to assess cognitive flexibility. The platform was placed in the opposite quadrant of the pool, and a similar set of tests was performed for additional five days.

Latency to escape the water and distance travelled to the hidden platform during training trials were measured to evaluate learning. Performance on the probe trial, which assessed memory, was calculated by measuring the percentage of time mice spent in the target quadrant of the pool where the platform had been located during training (chance = 25%). Mice with an inability to swim were removed from the experiment and analysis.

**Tissue and Organ collection**

Two to five days after behavioral testing, the mice were sacrificed under deep anesthesia with isoflurane. Urine was collected if possible. After decapitation, enough blood was collected to obtain both serum and plasma. CSF was collected and frozen at -
80°C. Brains and eyes were harvested. The right hemisphere was fixed in 4% PFA for five hours, 30% sucrose overnight, and 30 µm coronal sections were cut with a freeze slide microtome, cryoprotected, and stored at −20°C. The left hemisphere was dissected into four parts: cerebellum, hippocampus, cortex, and the rest of the brain for biochemical assays. The eyes were fixed in eye fixative and sent to Excalibur Pathology Inc. for sectioning. Other organs such as liver, GI tract, heart, thymus, spleen, and spinal cord were fixed in 10% formalin and sent to Dr. Kent Osborn in the Pathology Core of the Animal Care Program Diagnostic Laboratory at UCSD for histopathology screening.

**Thioflavin S Staining and Amyloid Fibril Quantification**

To determine Aβ fibril plaque load, floating coronal sections from AD and wild-type mice, n = 4 for each drug group, were washed in miliQ water and mounted on Fisherbrand Superfrost Plus microscope slides, before being processed for 1% Thioflavin S staining. All images were taken with Leica fluorescence microscope at 5X and with the same exposure across all images. The percent plaque within specified areas of a series of hippocampal and frontal cortex sections was quantified using ImageJ software from NIH. Images were converted to 8-bit gray scale, area of interest was traced and determined. Brightness and B/W threshold were adjusted appropriately and consistently across all images. The area of plaque particles within the area of interest was obtained and the percent plaque was calculated. The series of sections of each animal was averaged and grouped accordingly prior to statistical analysis. The number of series of section per animal ranged from 3-14 sections.
Statistical analysis

All data were analyzed using one-way ANOVA Tukey’s post hoc multiple comparison test.
RESULTS

Amyloid Plaque Load

To determine whether BPN-15606, R121919, or the combination therapy could ameliorate Aβ accumulation, the percentage of area occupied by plaques in AD mice was quantified based on treatment. It is well established that the specific AD mouse model used in the study develops Aβ plaques by 2-3 months of age with reliable onset at six months [26]. Using Thioflavin S stained coronal sections and densitometry, we confirmed that vehicle-treated female AD mice at nine months of age demonstrated accumulation of Aβ plaques in the hippocampus and cortex (Fig. 6B). As regards to the treatment impact, BPN-15606 and the combo-treated cohorts had significantly reduced accumulation of Aβ in both the hippocampus and cortex (Fig. 6), while no effect was observed in AD mice treated with R121919. Comparing these findings to our safety and behavioral data, significant lack of weight gain in AD mice treated with BPN-15606 or polytherapy, and deficit in acquisition performance in AD mice treated with polytherapy appeared to be a consequence of reduced Aβ load.

Water Maze

The Morris water maze test is popularly used to analyze changes in the hippocampal dependent learning and spatial memory abilities in rodents. To examine the extent to which the drug treatment impacted the AD mice, we utilized the water maze test two weeks preceding the drug treatment endpoint. Acquisition of an effective spatial search was determined by the latency to escape the water and swim-path distance to the hidden platform, while spatial memory retention was determined by the performance on
the probe trials. Mice were trained to locate the platform over five days, with eight trials a
day, in which two probe trials took place on the first and fifth trial of each day
interpolated with blocks of three standard training trials. Regardless of the genotype, all
mice were able to learn the water maze task by having significantly reduced latency (Fig.
3A) and swim-path distance to the platform (Fig. 3C) on day five. Wild type control
mice, however, were able to locate the platform most effectively compared to AD mice
(P<0.05, Fig. 3BD). None of the drug-treated AD mice groups showed improvement in
acquisition compared to vehicle-treated AD mice. However, the combo-treated cohort
demonstrated cognitive impairment by having significantly higher latency than vehicle
(P<0.05).

Analyzing spatial memory using one-way ANOVA to compare change in
performance as a function of time (Fig. 4A) and overall performance (Figure 4B) across
the first probe trial of each day in the NE target quadrant (chance = 25%), where spatial
memory was assessed after a 24-hour delay, did not differ based on genotype or
treatment. However, on day five, performance of the wild-type mice showed marginal
effect when comparing to vehicle (P<0.1), while R121919 was the only group who
performed significantly above chance compared to vehicle (P<0.05). Performance on the
second probe trial, which was assessed shortly after one block of standard training trials,
revealed that change in performance as a function of time (Fig. 4C) and overall
performance (Fig. 4D) differ based on genotype but not treatment.

Interestingly, during the second week of testing, in which the platform was
relocated to the SW quadrant to test for cognitive flexibility, change in performance (Fig.
5AC) and overall performance (Fig. 5BD) of all drug treated cohorts was at the same
level as the wild-type mice except for the combo-treated cohort. The combo-treated cohort performed significantly worse than the vehicle-treated on both first and second probe trials, suggesting adverse effects on spatial memory (P<0.05).
Figure 1. Representative coronal sections of Thioflavin S stained images. Three months old AD mice were treated with BPN-15606, R121919, combo, or vehicle for six months. Gray scale images represent AD mice at nine months of age. (A) Representative coronal section of age-matched wild-type negative control. (B) Representative coronal section of vehicle-treated AD mice. (C) Aβ deposits indicated with arrows at 50X magnification. (D) Representative coronal section of BPN-15606-treated AD mice. (E) Representative coronal section of R121919-treated AD mice. (F) Representative coronal section of combo-treated AD mice.
Figure 2. Effects of drug treatment on Aβ plaque load. Aβ accumulation in Thioflavin S stained coronal sections was quantified using densitometry in 9 months old AD mice chronically treated for 6 months. BPN-15606 and the combo-treated cohorts had significantly reduced accumulation of Aβ in both the hippocampus and cortex, while no effect was seen in AD mice treated with R121919 compared to vehicle. Treatment effect: *P<0.05, **P<0.01, ***P<0.001. All values are expressed as mean ± SEM, n = 4 mice per AD mice group, n = 2 WT.
Figure 3. Effects of drug treatment on water maze acquisition performance. Acquisition was determined using standard training trials. (A) Change in latency to escape to the hidden platform of all drug-treated groups and wild-type control. (B) Five-day average escape latency showed all AD mice groups were significantly impaired compared to wild type mice, and the combo-treated cohort significantly performed worse than vehicle-treated, suggesting adverse treatment effects. (C) The change in swim-path distance to locate the hidden platform of all drug treated groups and wild-type control. (D) Five-day average swim-path distance showed that all groups significantly performed worse than wild-type mice and no positive indication of treatment effects compared to vehicle. Treatment effect: *P<0.05. All values are expressed as mean ± SEM, n = 7–11 mice per group.
Figure 4. Effects of drug treatment on water maze spatial memory performance. Spatial memory was evaluated by determining the percentage of time the mice spent in the target quadrant (chance = 25%) during the first (A) and second (C) probe trial of each day over five days. The five-day average spatial memory performance of all groups showed no significant differences in either first or second probe trials (B) and (D). NE = Northeast. P1 = First probe trial assessed after 24-hour delay. P2 = Second probe trial assessed after one block of training trials. All values are expressed as mean ± SEM, n = 7–11 mice per group.
Figure 5. Effects of drug treatment on water maze cognitive flexibility performance. A reversal task was performed in order to assess cognitive flexibility by relocating the platform to the opposite quadrant, and an identical water maze paradigm was given. The percentage of time mice spent in the target quadrant (chance = 25%) during the first (A) and second (C) probe trial of each day over five days. The 5-day average spatial memory performance of all groups showed no significant differences in either first or second probe trials (B) and (D). SW = Southwest. P1 = First probe trial assessed after 24-hour delay. P2 = Second probe trial assessed after one block of training trials.
Safety and Tolerability

Evaluating the tolerability and safety of the drugs is a crucial and necessary practice for any pharmacological study. Here, we evaluate tolerability and safety by inspecting the mice three times weekly by assessing grooming behavior, fur condition, food intake, and body weight (Fig. 1). Grooming behavior and fur condition were assessed by visual inspection. Excessive grooming rendering the mice nearly furless, exclusive of lesions or rashes, was seen in two of the vehicle-treated mice, suggesting social or anxiety-like behavior. All drug-treated cohorts, however, were indistinguishable in terms of grooming and appearance. As for the body weight, the R121919-treated cohort gained weight normally compared to vehicle-treated cohorts (Fig. 1A). Conversely, BPN-15606-treated mice significantly lacked weight gain (P<0.05, Fig. 1B), and the combination of R121919 and BPN-15606 had had a severe impact on body weight (P<0.01) (Fig. 1C). A couple of the combo-treated mice lost up to 2 grams a day (10% of body weight), which was unusual compared to other mice; subsequently, they were under careful observation. They appeared inquisitive and active, and had no signs of hunched posture or distress. The food intake was similar across all animal groups, ranging from 2.8-3.4 g/day/mouse.

In addition to inspecting the mice during the treatment period, blood was drawn monthly for drug-level PK analysis. Sera samples were obtained at the endpoints to screen for liver proteins and substances. Levels of alanine transaminase (ALT), alkaline phosphatase (ALP), albumin, total bilirubin, and blood urea nitrogen (BUN) were
analyzed from all groups (n = 1-5 per group). Determining liver function is fundamental in chronic drug studies in order to screen for any toxicity or side effects.

According to Healthline [25], ALT is an enzyme that plays a crucial role in metabolism and is almost exclusively found in the liver. ALT can be released into the bloodstream if the liver is damaged or inflamed, which causes serum ALT levels to rise. ALP is another critical enzyme, which circulates in the bloodstream and is produced largely by the liver. Abnormal levels of ALP in the blood possibly indicate problems with the liver, gall bladder, or bones. The liver also produces albumin, one of the most abundant proteins in the blood, which helps the blood maintain fluid balance. Abnormal serum albumin levels may indicate that the kidneys or liver are not working properly.

Bilirubin is a yellow pigment made in the body from the breakdown of red blood cells. After circulating in the blood, it flows through the liver's bile ducts and is dissolved in bile. If bilirubin is not being adequately removed from the blood, it suggests damage to the liver. Urea nitrogen is a waste product created in the liver when the body breaks down proteins. Normally, the kidneys filter out this waste, and urinating removes it from the body. Blood urea nitrogen level is used to determine how well the kidneys are working by measuring the amount of urea nitrogen in the blood. No indication of abnormal liver function was observed in any of the groups (Fig. 2) as the levels were within the normal range.
Figure 6. Impact of drug treatment on body weight as a function of age. Three-month old AD female mice were treated with R121919, BPN-15606, the combination of both (combo), or vehicle for six months. Mice were weighed and monitored three times weekly to assess tolerability to the drugs. Mice body weight is shown as a function of age. (A) No aberrant effects were seen due to treatment of R121919 compared to vehicle. (B) BPN-15606-treated mice compared to vehicle revealed significant lack of weight gain. (C) Combo-treated mice compared to vehicle revealed significant lack of weight gain. Treatment effect: *P<0.05, and **P <0.01. All values are expressed as mean ± SEM, n = 9–10 mice per group.
Figure 7. Effects of drug treatment on liver protein and substance levels. Levels of alanine transaminase (A), alkaline phosphatase (B), albumin (C), blood urea nitrogen (D), and total bilirubin (E) obtained from serum samples from female wild type and AD transgenic mice treated with drug or vehicle. There was no indication of liver toxicity as all levels were within the normal range. All values are expressed as mean ± SEM, n = 1–5 mice per group.
DISCUSSION

Current treatments, such as cholinesterase inhibitors and memantine, for AD only temporarily and mildly boost cognitive function. Researchers are actively looking for new treatments that can improve the quality of life for people with dementia. This study investigates the impact of two small molecule drugs in isolation and in combination and assays how these agents alter the course of the disease and affect the cognitive function in an AD mouse model. Due to the complexity of pathways in AD, it is postulated that a polytherapeutic approach using the combination of γ-secretase modulator (BPN-15606) and CRFR1 antagonist (R121919) will be efficacious over the monotherapy as it impacts multiple pathways of neurodegeneration.

Findings of this study showed that chronic administration of CRFR1 antagonist (R121919), γ-secretase modulator (BPN-15606), or the combination therapy present to be safe in terms of liver function. However, the AD mice treated with BPN-15606 or polytherapy showed significant lack of weight gain. As for the cognitive function, AD mice receiving any of the treatments did not show improvement in either the acquisition or spatial memory assessment. In fact, the combo-treated cohort was impaired in latency acquisition compared to vehicle. Pathologically, AD mice receiving BPN-15606 or polytherapy had greatly reduced accumulation of Aβ deposits, while no treatment effect was seen in mice receiving R121919.

This is inconsistent with previous findings that AD mice treated with R121919 significantly prevented the onset of cognitive impairment assessed by the water maze test [24]. It is important to note that the two water-maze paradigms were different, possibly obscuring the results. The major differences in the paradigm entail the size of the pool
Regardless, our new findings clearly showed no indication of cognitive improvement in these drug-treated AD mice, but adverse effect was seen in AD mice treated with polytherapy.

A surprising finding was observed during the cognitive flexibility task, in which AD mice treated with vehicle performed as well as their wild-type counterparts. Most studies, including recent ones [27, 28], have shown that this AD mouse model exhibits cognitive deficits in the water maze task, while selected older studies have shown this same mouse model is not impaired in the water maze task [29]. It is unclear why the discrepancy is seen. One possibility is that one water maze paradigm specifically requires the hippocampus to form and store the essential spatial maps, while the other also taps into other brain regions such as the caudate nucleus for procedural memory. Isolated studies have shown water maze deficits were also found in animals with damage to striatum, basal forebrain, cerebellum, and several neocortical areas [30]. Based on this idea, we propose that the water maze performance appears to depend on coordinated action of different brain regions in order to create an integrated neural network for spatial navigation and memory. Extensive analysis of our Thioflavin S stained sections, the cerebellum, putamen, and caudate nucleus (structures involved in procedural memory) are relatively spared of Aβ deposits regardless of treatment (Supplemental Fig. 1). As such, it supports the idea that this mouse model may not be completely impaired in the water maze task.

Our results from Aβ plaque load determination using densitometry analysis clearly demonstrated that AD mice receiving BPN-15606 or polytherapy, but not
R121919, significantly reduced Aβ accumulation in the hippocampus and cortex. This suggests that polytherapy is more efficacious in terms of preventing or delaying the pathology but consequently resulted in adverse effects in normal weight gain and cognition. R121919 at this orally administered dose does not ameliorate the percent plaque load in AD mice, and when combined with BPN-15606, the effect of polytherapy did not significantly differ from the BPN-15606 monotherapy. Combining the treatments was principally due to the fact that corticosteroid hormones target and worsen the central nervous system, and R121919 would potentially decelerate the pathology when used along with a more direct disease-modifying drug like BPN-15606. Nonetheless, R121919 did not significantly contribute to reduction of Aβ plaque load.

Unlike previous studies, in which female AD mice treated with R121919 significantly reduced accumulation of Aβ in both the hippocampus and cortex [24], we were unable to replicate the same treatment effects as previously mentioned. An important note is that the significance in our Aβ plaque load determination is merely preliminary. We have yet to determine the Aβ plaques by using N-terminal-specific anti-human Aβ monoclonal antibody (82E1) and a larger sample size. We used the Thioflavin S staining procedures to identify neuritic plaques. This homogenous dye mixture non-selectively binds to beta sheet motifs of proteins, such as those in Aβ oligomers. As such, it provided us a quick way to screen for Aβ plaques by fluorescence emission, but this method subsequently lacks specificity as the dye also binds to other proteins constituting of extensive beta sheets [31]. Additionally, the route of administration for R121919 differed in the two studies—orally administered versus subcutaneous injection—which may have contributed to the dissimilar effects.
Taken together, we believe that $\gamma$-secretase modulation monotherapy (BPN-15606) or polytherapy can be used chronically to delay or prevent Aβ pathology, but not cognitive deficits in an AD transgenic mouse model. We also believe that the desired effects due to treatment with BPN-15606 or polytherapy were associated with the lack of weight gain. Our preclinical data suggest that polytherapy was not efficacious over monotherapy. Irrespectively, BPN-15606 demonstrated to be a potential disease-modifying therapeutic approach with limited adverse effects and without liver toxicity, while polytherapy is questionable at the moment. We will perform further analyses, such as obtaining drug-level PK analysis, using specific antibody for immunohistochemistry, quantifying Aβ peptides with MesoScale bioassays, and obtaining histopathology reports, in order to better characterize and evaluate the impacts of these drugs.
Supplemental Figure 1. Neuroanatomical structures of vehicle-treated AD mouse. Visualizing relevant neuroanatomical structures with gray scale images of vehicle-treated AD mice at 9 months of age. Plaque load is not accumulating in the cp. Identifiable brain structures: ctx, cortex; hi, hippocampus; dg, dentate gyrus; cp, caudate/putamen; crbl, cerebellum.
REFERENCES


