# GM6 Attenuates Alzheimer's Disease Pathology in APP Mice

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Neuro-immunology: The Impact Of Immune Function On Alzheimer's Disease
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## ABSTRACT

Alzheimer's disease (AD) results in the deposition of amyloid β (Aβ) peptide into amyloid fibrils and tau into neurofibrillary tangles. Regardless of whether these entities are a cause or consequence of the disease process, preventing their accumulation or accelerating their clearance may slow the rate of AD onset.

Motoneuronotrophic factor (MNTF) is an endogenous neurotrophin that is specific for the human nervous system, and some of the observed effects of MNTF include motoneuron differentiation, maintenance, survival, and reinnervation of target muscles and organs. GM6 is a hexapeptide fragment of MNTF that appears to replicate its activity spectrum.

In this study, we investigated the effect of GM6 in a mouse model of AD before the development of amyloid plaques and determined how this treatment affected accumulation of Aβ peptide and related pathologic changes (e.g., inflammation, nerve growth factor (NGF) expression, cathepsin B, and memory impairment).

**Application of GM6 over a 4-month period in young APP/ΔPS1 double-transgenic mice resulted in attenuation in Aβ peptide levels, reduction of inflammation and amyloid load, increased cathepsin B expression, and improved spatial orientation. In addition, treatment with GM6 increased brain NGF levels and tempered memory impairment by ~50% at the highest dose. These data suggest that GM6 may modulate disease-determining pathways at an early stage to slow the histological and clinical progression of AD.**

## INTRODUCTION

Alzheimer's disease (AD) affects more than 35 million people worldwide and is known as the most common form of dementia. The frequency of diagnosis of AD increases to 1 in 3 after the age of 85 and results in death 3-9 years after the onset of symptoms. AD is associated with specific clinical and pathological features such as cognitive impairment and neuropsychiatric disturbances (Li et al., 2010; Sperling et al., 2010). AD is characterized by the aggregation of amyloid-β (Aβ) peptide into neuritic plaques and hyperphosphorylated tau protein accumulating into neurofibrillary tangles (NFTs). Aβ is generated from the sequential cleavage of the amyloid-precursor protein (APP) resulting in the production of $A\beta_{40}$ and $A\beta_{42}$ peptides, which give rise to aggregated fibrils and plaques (Walsh & Selkoe, 2007; Lahri et al., 2008). Aβ is known to be neurotoxic and is thought to play a major role in the pathogenesis of AD.

About 25 years ago, motoneuronotrophic factor 1 (MNTF1) was isolated from rat muscle tissue (Chau, R.M.W., et al., 1992). This factor was capable of supporting in vitro growth and/or regeneration of anterior horn motoneurons and spinal cells of rat lumber spinal cord. Within the MNTF protein is a 6 amino acid peptide referred to as MNTF6mer or GM6 (Chau, 2001), which had similar neuroprotection activity as the parent MNTF molecule (Chau, 2005; 2007).

GM6 was able to penetrate the blood brain barrier. (Yu et al., 2008). These studies suggest that GM6 has significant protective effects in the CNS. Based on this information, we decided to test the efficacy of GM6 in a mouse model of AD.

## OBJECTIVE

- To test the efficacy of GM6 in a mouse model of AD
- To test impact on neurological outcomes
- To test biochemical changes and amyloid pathology
- To test for decrease of inflammation biomarkers
- To test for increase in brain Nerve Growth Factor

## MATERIALS and METHODS

A total of 60, 3-month-old transgenic mice were used for the studies. The mice expressed the mutant form of human presenilin-1 (DeltaE9) and the mutant form of the chimeric mouse/human amyloid precursor protein (APP695) (Jankowsky et al., 2004; Savonenko et al., 2005.) These APP/ΔPS1-Tg mice start developing amyloid plaques at ~3 to 4 months of age. These mice were on a C3H/HeJ*C57BL/6J background. Ten 7-month-old wild-type (C3H/HeJ*C57BL/6) mice were used to obtain baseline levels of the different variables.

Animals were randomly assigned to a vehicle group (n=20) or treatment groups (n=20) treated with daily intravenous injection of a 6-mer active component of MNTF (GM6) at a dose of 1 or 5 mg/kg. Formulation of GM6 was performed by reconstituting GM6 with normal saline solution that was stored at 4°C. Vehicle control received phosphate buffered saline (PBS) solution. The IV injections via tail vein were given. Four months after the treatment, animals were transcardially perfused under deep anesthesia with 1× PBS and their brains removed for further analysis. The right brain hemispheres were immersion-fixed in 4% paraformaldehyde for 24 hours, followed by immersion in 4% paraformaldehyde containing 30% sucrose for 2 to 3 days. After fixation, the right brain hemispheres were frozen in OCT medium and sectioned with a cryostat to obtain 30-µm frozen sections for immunohistochemical analysis. The left brain hemispheres were frozen as quickly as possible and used to quantitate the levels of Aβ peptide ($A\beta_{1-40}$ and $A\beta_{1-42}$), inflammatory markers, NGF and cathepsin B.

**Morris Water Maze Test:** APP/PS-1 vehicle, 1 mg/kg and 5 mg/kg GM6 were examined for memory acquisition. APP/PS-1 mice were trained in the Morris water maze test on each of 4 consecutive days to learn the location of a submerged, invisible platform in a pool of water. The time it took the mice to swim to the platform during training was recorded each day (A), measured as the latency period (seconds), with shorter latency times indicating better memory acquisition. Latency (secs) is shown as x ± s.e.m. The latency period (B) and distance (C) traveled for animals to swim to the submerged, invisible platform assessed 2 days after completion of the training are recorded. The percent time each animal swam in the NE quadrant from which the platform had been removed (Greater memory retention) (D), and the percent time an animal swam in the annulus of the pool were recorded (E). (Fig. 7)

## TISSUE ANALYSIS

**Brian and spinal cord tissue analysis:**

- **Immunohistochemistry Staining with right brain sections for Aβ Peptide (Fig. 1) CTFβ and sAβPPα Analyses by Western blot and ELISA (Fig. 2)**
- **Quantitative analysis of activated microglia and astrocytes in the brain by Immunohistochemistry Staining with right brain sections (Fig. 4)**
- **ELISA Analysis for quantitative analysis of cytokines: tumor necrosis factor-α (TNF-α), interlukin-1 β (IL-1β), or transforming growth factor-β (TGF-β) in brain tissue. (Fig. 6)**
- **Western Blot and ELISA analysis of GM6 effect on cathepsin B, an inflammasome.**
- **Determination of NGF I the brain by Immunohistochemistry Staining with right brain sections (Fig. 3)**

## RESULTS

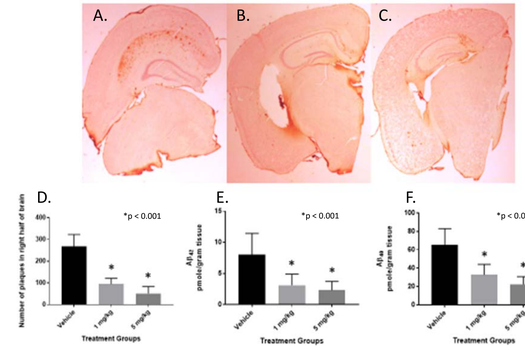


**Figure 1. Level of amyloid load after 4 months of GM6 treatment in APP/ΔPS1-Tg mouse brains.**
Right brain hemisphere sections obtained from control mice (A) GM6 treated with 1 mg/kg GM6 (B) or GM6 treated with 5 mg/kg GM6 (C) were immunostained with mouse anti-human Aβ peptide (clone 10D5) antibody to detect amyloid plaques. The number of amyloid plaques in the brain sections (10 sections per mouse) from each set of control mice or mice treated with GM6 were counted and averaged (D). The left-brain hemisphere was examined for guanidine-extractable $A\beta_{1-40}$ (E), and $A\beta_{1-42}$ peptides (F). Vehicle: 7-month-old APP/ΔPS1-Tg mice that were not treated with GM6 were used as controls (*p < 0.001, n = 20 per group).

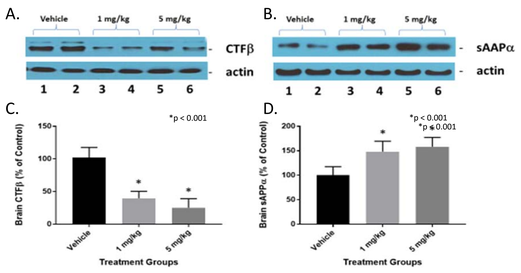


**Figure 2. GM6 lowers and increases brain CTFβ and sAβPPα, respectively.**
A) GM6 caused a reduction in brain CTFβ. CTFβ is a proteolytic product of β-secretase cleavage of AβPP and thus these data are also consistent with GM6 acting through the inhibition of β-secretase activity. B) In contrast, GM6 mediated an increase in brain sAβPPα levels. These data are consistent with GM6 mediating the inhibition of β-secretase activity because sAβPPα is derived from AβPPα-secretase cleavage, which competes with β-secretase cleavage of AβPP, and thus the inhibition of β-secretase activity provides more AβPP for β-secretase to produce more sAβPPα (*p < 0.001; n = 20).

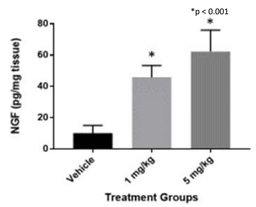


**Figure 3. Concentration of NGF in the brain of APP/PS-1 mice.**
Bar graphs show the concentration of NGF in picograms (pg) per milligram (mg) of brain tissue from APP/PS-1 mice treated with GM6 (1 or 5 mg/kg) versus APP/PS-1 mice untreated. The difference was statistically significant (*p<0.001; n = 20 per group).

## RESULTS

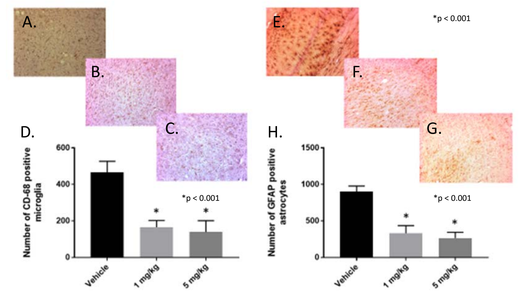


**Figure 4. Quantitative analysis of activated microglia and astrocytes in the brain of APP/PS1 mice after 4 months of GM6 treatment.**
Brain sections from APP/PS1 control mice (A) or mice treated with GM6 1 mg/kg (B) or 5 mg/kg (C) were stained with anti-mouse CD68 antibody to detect the activated microglial cells. Five sections of each mouse were counted and averaged for the number of CD68-activated microglia in the brain (*p<0.001, n=20). Brain sections from APP/PS1 control mice (E) or mice treated with GM6 1 mg/kg (F) or 5 mg/kg (G) were immunostained with anti-mouse GFAP antibody to detect the activated astrocytes. Five sections of each mouse were counted and averaged for the number of GFAP-activated astrocytes in the brain (*p<0.001; n=20). D and H: quantification of data from the section for CD-68 and GFAP, respectively.

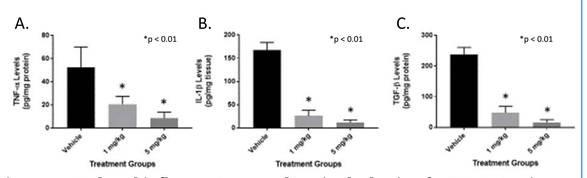


**Figure 5. Reduced inflammatory markers in the brain of APP/PS-1 mice.**
Mice were treated with vehicle (Vehicle) or GM6 for 4 months at 1 mg/kg or 5 mg/kg. Quantitative analysis of TNF-α (A), IL-1β (B), and TGF-β (C) in the APP/PS-1 brains was determined by enzyme-linked immunosorbent assay (ELISA). n = 20 per group. *p < 0.01 (compared with vehicle).

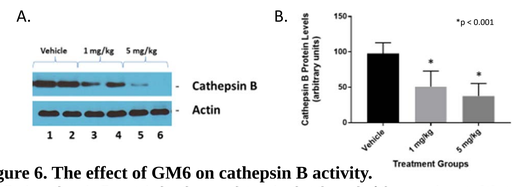


**Figure 6. The effect of GM6 on cathepsin B activity.**
(A) Brain cathepsin B protein levels were determined at the end of the experiment. Western blot analysis of the cathepsin B levels in the brains of vehicle, 1 mg/kg, and 5 mg/kg GM6 treated mice. (B) Quantitative analysis of cathepsin B protein levels of the mice in A. The results are expressed as mean +/- SD (n=20; *p<0.001 compared to the vehicle group).

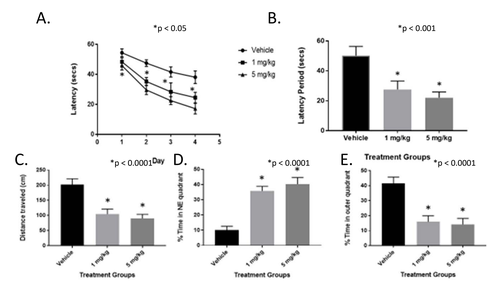


**Figure 7. Behavioral analysis of mice treated with GM6 in the Morris water maze.**
(A) APP/PS-1 vehicle, 1 mg/kg and 5 mg/kg GM6 were examined for memory acquisition in 4 consecutive days of training. (*Statistically significant, p < 0.05, n = 20 per group). Mean distances traveled are 201, 109, and 95 cm, respectively. n = 10 per group. *Statistically significant (p<0.001). Two days after training animals swim to the submerged platform, for GM6 1 mg/kg, and GM 6 5 mg/kg mice (B) latency period are 49, 26, and 21 seconds, respectively, and (C ) distance traveled are 201, 109, and 95 cm, respectively. n = 10 per group. *Statistically significant (p<0.001). (D) Vehicle, GM6 1 mg/kg, and GM6 5 mg/kg mice had percent times in the NE quadrant of 10%, 36%, and 41%, (E) in the annulus are 42%, 17%, and 15%. n = 20 per group. *Statistically significant with p<0.001

## DISCUSSION

### Efficacy:

In this study, in the APP/ΔPS1 mouse model, we demonstrated IV administration of GM6 at 1 and 5 mg/kg daily dosages revealed a dose-dependent effect to:

1. attenuate the increase in Aβ peptide levels and amyloid plaque deposition in the brain that manifested in a decrease in plaque formation,
2. improve behavioral features,
3. reduce inflammatory conditions, and
4. increase Nerve Growth Factor in brain.

### Hypothesis Of GM6 in AD Mechanisms Of Action:

These results may be due to replication of the endogenous MNTF neurotrophic effects by GM6 (Kindy et al., 2017). MNTF, and specifically GM6, might function as a master regulator of numerous pathways that can alter Aβ production, inflammation and mediate pathways to enhance recovery and repair (Yu et al., 2008; Kindy et al., 2017). As indicated in the present study, the reduced levels of Aβ might be due to the inhibitory effect on β-secretase and/or an increase in a-secretase activities (Hook et al., 2014). In addition, GM6 appears to regulate cathepsin B expression which functions as not only a regulator of the inflammasome, but also as a β-secretase and generating Aβ peptide. We expect that these effects of GM6 involve a number of different pathways rather than limited activation or inhibition of any one pathway individually (Valko et al., 2018 a, b).

### The Impact Of Immune Attack In Alzheimer's Disease:

Attenuation of inflammation can limit the development of the pathology (Yu et al., 2017). Modulation of inflammation has been an approach to treat AD.

Here we show that animals injected with GM6 exhibited a reduction in inflammation. We found that in the AD mouse models, inflammation seems to play a key role in the disease process (Hook et al., 2014). This study has shown GM6 reduced the following inflammation markers:

1. cytokines (TNF-α, IL-1β, TGFβ etc)
2. inflammatory mediators (CD-68 and GFAP) which can contribute to the pathogenesis (Hook et al., 2014).
3. modulation of cathepsin B and cleavage of APP to Aβ.

However, whether this is a direct effect on the enzymes or mediated via upstream pathways that facilitate the expression of the genes/proteins needs to be further developed.

## CONCLUSIONS

**Our data suggest that GM6 (an active site of MNTF) can attenuate disease symptoms and reduction in inflammation in an AD mouse model with corresponding improvements in histological and biochemical features. This may reflect replication of MNTF neurotrophic activity as a consequence of GM6 treatment. These studies establish a framework for clinical trials to clarify effects of GM6 in AD and other neurodegenerative disorders.**

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Competing interest: Dorothy Ko is an employee of Genervon and has interest in the company.