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Research

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Novel botanical therapeutic NB-02 effectively treats Alzheimer’s neuropathophysiology in an animal model APP/PS1 mouse model

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Abstract

Background

Alzheimer’s disease (AD) is an incurable neurodegenerative disorder and a major cause of dementia. Some of the hallmarks of AD include presence of amyloid plaques in brain parenchyma, calcium dysregulation within individual neurons, and neuroinflammation. A promising therapeutic would reverse or stymie these pathophysiologies in an animal model of AD.

Methods

We tested the effect of NB-02, previously known as DA-9803, a novel multimodal therapeutic, on amyloid deposition, neuronal calcium regulation and neuroinflammation in 8-10 month old APP mice, an animal model of AD.

Results

In vivo multiphoton microscopy revealed that 2 month-long administration of NB-02 halted amyloid plaque deposition and cleared amyloid in the cortex. Post-mortem analysis verified NB-02-dependent decrease in plaque deposition in the cortex as well as hippocampus. Furthermore, drug treatment reversed neuronal calcium elevations, thus restoring neuronal function. Finally, NB-02 transformed the morphology of astrocytes and microglia to a more phagocytic state, affecting neuroinflammation.

Conclusions
NB-02 was effective at reversing AD neuropathophysiology in an animal model. Therefore, in addition to serving as a promising preventative agent, NB-02 holds potential as a treatment for AD in the clinic.

Keywords

Alzheimer’s disease, multiphoton microscopy, therapeutic, in vivo, calcium imaging, DA-9803, NB-02

Background

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder currently without a cure[1,2]. It is characterized by presence of amyloid beta deposits in the brain parenchyma[3,4]. Circuit dysfunctions underlying cognitive and memory deficits may stem from amyloid beta-dependent calcium dyshomeostasis resulting in elevations of baseline calcium within individual neurons[5,6]. In addition to deposition of amyloid plaques and disruption of calcium homeostasis, AD is characterized by substantial inflammatory response[7–9], manifesting in increased expression of inflammatory markers in non-neuronal cells, such as astrocytes and microglia[10]. Restoration of neuronal calcium to control levels and normalization of neuroinflammatory response would indicate treatment efficacy[11,12].

NB-02, previously known as DA-9803, is a novel proprietary botanical cocktail containing extracts from *Morus alba L.* and *Poria cocos*[13]. The extract is reproducibly prepared according to a standardized recipe. Currently in preclinical development by NeuroBo Pharmaceuticals, it is a multimodal therapeutic that has been shown to prevent deposition of amyloid plaques in young APP/PS1[14] mice, an animal model of Alzheimer’s disease[13]. In addition, it was
effective at preventing neuronal calcium elevations, thus maintaining circuit integrity in these mice. Finally, it transformed astrocytes and microglia, allowing these non-neuronal cells to assume more phagocytic morphology. Thus NB-02 is an ideal candidate to enter the clinic as a preventative therapeutic for AD. However, what is its treatment potential?

To address this question, 8 month old APP/PS1 mice were treated with daily gavage of 300 mg/kg NB-02 for 2 months. We tested the therapeutic’s propensity to slow amyloid deposition, restore neuronal calcium homeostasis, and modify neuroinflammation. Interestingly, NB-02 halted amyloid deposition and cleared some amyloid plaques in cortices of drug-treated animals compared to vehicle-treated mice. Furthermore, it restored calcium homeostasis in neurons that started out with elevated baseline calcium levels prior to treatment onset. Moreover, it modified morphology of astrocytes and microglia, thus affecting neuroinflammation. Theretofore, NB-02 had a restorative effect on neuronal and non-neuronal cells in an AD mouse model. Thus, it has great potential to restore healthy circuit function and thereby slow cognitive and memory decline in the clinic.

Methods

Animals and surgery

Transgenic APPswe/PS1dE9 mice were used (10 males, 11 females; B6:C3-Tg(APPswe, PSEN1dE9)85Db0/Mmjax background). These animals overexpress the Swedish mutation in the human APP gene and deltaE9 mutation in the Presenilin gene[14]. All the studies complied with Massachusetts General Hospital Animal Care and Use Committee as well as NIH guidelines for the use of laboratory animals.

APP/PS1 mice underwent intracortical virus injections followed by cranial window installations over the right hemisphere[15] on the same day starting at 7 months of age similar to earlier
Following anesthesia induction, animals were secured in a stereotaxic apparatus. After disinfection of skin, incision was made, skull was cleared and burr holes were drilled through the skull. Using a 10 µl Hamilton syringe, 3 µl of AAV8-YellowCameleon 3.6 (YC3.6) (U.Penn Vector Core) was injected in the cortex with the following coordinates: anterior-posterior -1.5, medial-lateral -2, dorsal-ventral -0.8 at the rate of 130 nl/min. Thus, excitatory neurons in right somatosensory cortex were targeted. YC3.6 is a genetically encoded calcium indicator (GECI) that allows determination of absolute intracellular calcium concentration based on its ratio of YFP/CFP in individual cells[18]. Viral injections were directly followed by craniotomies over the injection sites. Cranial windows with 5 mm diameter were fixed with a mixture of dental cement and crazy glue. Body temperature of each animal was maintained through the entire surgical procedure and during the recovery from anesthesia. YC3.6 was allowed to express for a month prior to imaging with multiphoton microscopy. This also allowed the inflammatory response associated with craniotomies to subside. Drug treatment and multiphoton imaging commenced when animals reached 8 months of age.

**Therapeutic treatment**

APP/PS1 mice were randomly assigned to the vehicle or the NB-02 treatment conditions. Vehicle consisted of hydroxypropyl methyl cellulose (HPMC) dissolved in water. NB-02 drug consisted of NB-02 mixed with vehicle solution. Drug or vehicle treatment was initiated subsequent to the baseline imaging session. Treatment was administered to the animals via daily gavage with 300 µl of 300 mg/kg NB-02 or vehicle for the duration of 2 months. Drug treatment had no significant effect on animal attrition (data not shown). Treatment continued while the animals were imaged at 1 month and 2 month time-points after treatment onset. Subsequent to the last imaging session, animals received their last gavage treatment, after which mice were euthanized and perfused transcardially with PBS. Their brains were isolated
and processed for immunohistochemistry or biochemistry. 10 animals were treated with vehicle, 11 animals were treated with NB-02, totaling 21 mice.

**Imaging using multiphoton microscopy**

Prior to the baseline imaging session, animals received intraperitoneal injections of 4 mg/kg methoxy-XO4, which crosses the blood-brain barrier and binds to amyloid plaques allowing visualization by multiphoton microscopy[19–21]. Animals were anesthetized with 2% isoflurane and secured into a stereotactic stage. Intravenous administration of Texas Red dextran into the retro-orbital sinus provided a fluorescent angiogram. Multiphoton microscopy allowed imaging of the angiogram, amyloid plaques and YC3.6-expressing neurons using an Olympus Fluoview 1000MPE mounted on an Olympus BX61WI upright microscope. Same fields of view were identified during all 3 imaging sessions (baseline, 1 month, 2 months) using the landmarks present in the angiogram collected during the baseline imaging session. Images were acquired using a 25× water immersion objective (NA = 1.05). A mode-locked titanium:sapphire laser (MaiTai; Spectra-Physics, Fremont, CA, USA) generated two-photon fluorescence with either 800 nm or 860 nm excitation. Amyloid plaques were imaged using 800 nm excitation at 1× zoom. Z stacks were acquired at 5 µm step-size. YC3.6 was imaged using 860 nm excitation at 2×, and 5× zoom. These z stacks were acquired at 1 µm step-size. Laser power was limited below 30 mW to prevent phototoxicity. Animals body temperature was maintained with a heating pad throughout imaging. Methoxy-XO4 was re-administered prior to each imaging session to identify newly appeared amyloid plaques.

At the end of each imaging session, animals were allowed to recover from anesthesia while their body temperature was maintained with a heating pad. At the end of the last imaging session, mice received their last gavage treatment and their CSF was collected (see below). Then mice were euthanized using CO₂. Their bodies were perfused with PBS, and brains
isolated. Left hemibrains (that had not contained cranial windows) were fixed with 4% paraformaldehyde and cryoprotected with 15% glycerol overnight. Optimal Cutting Temperature compound (OCT) was used to freeze the hemibrains, which were subsequently cut into 20-μm coronal sections on a cryostat, and mounted onto slides for immunohistochemical processing. Right hemibrains (that had contained cranial windows) were flash frozen in liquid nitrogen and subsequently processed for biochemistry.

Cerebrospinal fluid (CSF) collection

Prior to euthanasia, each animal was anesthetized with 2% Isoflurane and secured into a stereotaxic apparatus. Cisterna magna containing CSF was identified. Dura was punctured with a 30 gauge needle. CSF was collected using P20 pipette using dissection microscope. CSF was stored in low protein binding tubes in -80ºC before use.

Data analysis

Analysis was performed using ImageJ software (http://rsbweb.nih.gov/ij/) to determine amyloid plaque numbers, amyloid plaque burden, and resting calcium levels within individual neuronal processes, or neurites. Since the same fields of view were imaged over time, the same amyloid plaques and neuronal processes could be tracked longitudinally to determine amyloid plaque burden and intracellular calcium changes in each individual neuronal process. To determine amyloid plaque numbers and amyloid plaque burden, each z-stack was processed into a maximum intensity projection. Amyloid plaques were counted manually in each projected image to determine the amyloid plaque number. Each projected image was thresholded, segmented, and the percentage area occupied by amyloid was measured to calculate the amyloid burden. The signal from the amyloid present in blood vessels, cerebral amyloid angiopathy, was excluded from analysis.
The images containing neuronal processes expressing YC3.6 were also analyzed using ImageJ. YC3.6 is a FRET probe, where a donor, cyan fluorescent protein (CFP), and an acceptor, yellow fluorescent protein (YFP) are connected by a linker[18]. Intracellular calcium concentrations were determined from the ratio of YFP to CFP. The higher the calcium concentration, the greater the YFP to CFP ratio. The background for each channel was calculated by the mode of the intensities of the last slice of each volume. That value was subtracted from its channel. A median filter with a radius of 2 was applied to the fluorescence intensities. YFP was divided by CFP, thus creating a ratio image that could be converted to absolute calcium concentration. Neurons were identified and their processes selected manually as regions of interest (ROIs) using the ‘free hand’ tool on ImageJ in the YFP images. These neuronal ROIs were then exported to the ratio images and the YFP/CFP ratios calculated. The relative change in YFP/CFP ratio ($\Delta R/R_i$) was calculated by tracking the same neurites throughout all three imaging sessions. YFP/CFP ratios were converted to $[\text{Ca}^{2+}]$ with standard equations using the in-situ $K_d$ and Hill coefficient for YC3.6 determined earlier[16]. Matlab was used to create pseudocolored images based on the calcium concentration using the empirical $R_{\text{min}}$ and $R_{\text{max}}$. The ratio values were used to determine the hue and saturation (color) and the brightness values were used to assign the value (intensity) in the pseudocolored images.

**Cell culture**

KCLB No. 22266 SH-SY5Y cell line (human neuroblastoma cell line, passage no. 64) was purchased from the Korean Cell Line Bank. Cells were maintained in a 5% CO$_2$ incubator at 37°C in DMEM/F12 media (Gibco) containing 10% FBS (fetal bovine serum) and 1% P/S (penicillin/streptomycin). 48 hours after seeding (6-well plate: 2x10^5 cells, 96-well plate: 1x10^4 cells), it was maintained in DMEM/F12 medium containing 3% FBS and retinoic acid (final conc. 10 μM) for 5 days.
**MTT assay**

Cell cultures were exposed to 1 μM oligomeric Aβ1-42 (Sigma-Aldrich) for 24 hours followed by treatment with a single concentration of NB-02 for 48 hours. The following concentrations of NB-02 were used: 0.1, 0.3, 1, 3, 10, 30, 100, 300 μg/ml. Alternatively to NB-02, cell cultures were treated with donepezil (donepezil HCl, Waco) in presence of oligomeric Aβ1-42. The following concentrations of donepezil were used: 0.1, 1, 10 μM. Serotonergic receptor blocker Lu AE58054 (Lu AE58054 Hydrochloride, 1, 10 μM, Chem Scene) was used in presence of oligomeric Aβ1-42 and NB-02. Then cell cultures were treated with 50 μl of MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma Aldrich) for 4 hours. After removing the supernatant, 200 μl of DMSO (Sigma-Aldrich) was added and shaken lightly. The absorbance was measured with a microplate spectrophotometer at 595 nm.

**Acetylcholinesterase activity assay**

Acetylcholinesterase activity was determined using the Ellman’s method [22]. Subsequent to 24 hour pretreatment with Aβ1-42 followed by 48 hour treatment with each concentration of NB-02, cells were harvested in RIPA buffer. Cells were centrifuged at 13,000 rpm for 15 minutes to obtain the supernatant. Proteins were quantified using the Bio-rad protein assay kit (Bio-rad Labs). Acetylcholinesterase activity was measured using the Abcam assay kit (Abcam). Each sample was mixed with DTNB, acetylthiocholine and assay buffer in a 96-well microplate, followed by incubation for 10 minutes at 37˚C. Acetylcholinesterase activity was measured using the microplate spectrophotometer at 405 nm absorbance. The enzyme activation was presented in mU/mg protein.

**NGF assay**
Cells were treated with NB-02 for 48 hours. Then cells were incubated with lysis buffer (100 mM of Tris/HCl pH 7.0 containing 2% bovine serum albumin, 1 M NaCl, 4 mM EDTA.Na2, 2% Triton X-100, 0.1% sodium azide, 5 μg/ml of aprotinin protease inhibitor, 0.5 μg/ml of antipain, 157 μg/ml of benzamide, 0.1 μg/ml of pepstatin A and 17 μg/ml of phenylmethyl-sulphonyl fluoride) and harvested. Samples were centrifuged at 13,000 rpm for 15 minutes to obtain the supernatant. Proteins were quantified using the Bio-rad protein assay kit (Bio-rad Labs). Protein NGF levels were measured using NGF ELISA kit (-Abcam). The microplate spectrophotometer absorbance was measured at 450 nm-. The quantity of NGF was presented in pg/mg protein.

Immunohistochemistry and image analysis

20-μm coronal sections of mouse brains were subjected to antigen retrieval in citrate buffer. The sections were then permeabilized using Triton X-100, blocked with normal goat serum (NGS), and incubated with the following primary antibodies: 6E10 (monoclonal 6E10, 1:500; Covance), glial fibrillary acidic protein (GFAP) (mouse monoclonal anti-GFAP, 1:200; Thermo Scientific), or Iba-1 (rabbit monoclonal anti-Iba1, 1:200; ab178846) at 4°C overnight. This was followed by incubation with the respective secondary antibodies (1:500) for 1 hour at room temperature. The slides were then mounted with Vectashield antifade mounting media (Vector Laboratories) either with or without DAPI.

For amyloid plaque burden analysis, 20-μm sections with methoxy-XO4-labeled plaques and ex vivo 6E10 immunostaining were imaged using an inverted Zeiss microscope with a 20x objective. Images were thresholded and amyloid burden was calculated as a percentage of the cortical or hippocampal area.

Coronal hemibrain sections stained with GFAP or Iba-1 were imaged using an inverted Zeiss microscope at 10x for the purpose of glial cell counts. Regions of interest (ROIs) were drawn of
the whole hippocampus or a randomly selected section of cortex using the ImageJ "free hand"
or "rectangle" tools respectively. Cells within the ROI were manually counted while blinded to
c Condition to minimize bias. Representative images were taken with 20x objective.

GFAP and Iba-1 stained sections were also imaged using an inverted Olympus confocal
microscope with a 40x objective for the purpose of morphology analysis. ImageJ was used to
analyze markers of cell morphology such as process length, cell body diameter, soma area, and
shape. Process length was measured as distance from the edge of the soma to the end of the
process. Cell body diameter was measured using the Feret’s diameter, the maximum caliper of
the cell.

**Amyloid-beta ELISAs**

Total soluble amyloid beta 40 and 42 levels were measured using the amyloid beta 40 and 42
sandwich ELISA kits (Wako). Each flash-frozen hemibrain was homogenized in ice-cold tris-
buffered saline (TBS) in presence of phosphatase inhibitor and centrifuged at 25500 rpm for 30
min at 4 °C. Supernatants containing soluble amyloid beta were collected and subjected to
ELISAs using the manufacturer’s instructions. Amyloid beta 40 and 42 levels were determined
using Amyloid beta 40 and 42 standard calibration curves. Protein levels were normalized to wet
brain weights. Similarly, Amyloid beta 40 and 42 levels were determined in CSF samples.

**Statistics**

GraphPad 5.0 was used to run statistical analyses. Data was represented as mean ± SEM.
Datasets were tested for normality using the Shapiro-Wilk normality test or Kolmogorov-Smirnov
test. Normally distributed datasets were subjected to parametric tests, such as t-tests or
ANOVAs. Nonparametric datasets were compared using nonparametric tests, such as Mann
Whitney or Kruskal-Wallis tests. Repeated measures datasets were compared using
Results

**NB-02 is effective at halting amyloid plaque deposition in old APP/PS1 mice**

NB-02 is a multimodal botanical cocktail containing extracts from *Morus alba* L. and *Poria cocos*. To test its propensity for treatment of AD pathology in vivo, 8 month old APP/PS1 mice were subjected to daily gavage treatments with 300 mg/kg NB-02 dose or vehicle cocktail (Fig. 1a). Methoxy-X04[19,23] was administered intraperitoneally, crossed blood-brain barrier, and bound to amyloid deposits to allow visualization of amyloid plaques within the same fields of view of cranial windows using high-resolution multiphoton microscopy over the course of treatment[23–25]. A fluorescent angiogram was acquired during baseline imaging session after injection of Texas Red Dextran to allow visualization of the field of view and increase reliability of finding the same fields of view during subsequent imaging sessions (Fig. 1b, e). Daily gavage treatment was initiated on the same day subsequent to baseline imaging session, starting at 8 months of age, and multiphoton imaging was repeated 1 month and 2 months after treatment onset.

Animals randomly assigned to vehicle and drug treatment conditions started out with similar numbers of plaques in somatosensory cortex (154±9 plaques/mm³ across 62 z-stacks in 10 mice treated with vehicle, 159±11 plaques/mm³ across 63 z-stacks in 11 mice treated with NB-02, mean±SEM, Mann Whitney test, n.s.). Vehicle-treated animals exhibited increases in the number of amyloid deposits within the cortical fields of view over time (Fig. 1d compared to 1c, yellow arrow). Amyloid plaque numbers increased to 200±12 plaques/mm³ in vehicle-treated animals 1 month after treatment onset, significantly different from baseline (Fig. 1h, mean±SEM,
Wilcoxon matched-pairs signed rank test p<0.001). Plaque deposition again increased to 243±16 plaques/mm³ 2 months after treatment onset, significantly different from baseline (Fig. 1h, mean±SEM, Wilcoxon matched-pairs signed rank test p<0.001). Interestingly, NB-02 halted amyloid depositions and resulted in clearance of amyloid plaques in APP/PS1 mice (Fig. 1g compared to 1f). NB-02-treated animals experienced a decrease in plaque numbers per cortical volume at 1 month after treatment onset (Fig. 1h, 146±11 plaques/mm³ mean±SEM, Wilcoxon matched-pairs signed rank test p<0.05). By 2 months after treatment onset, the number of amyloid plaques decreased to 139±14 plaques/mm³ (Wilcoxon matched-pairs signed rank test p<0.01). At the end of treatment period, average plaque number was significantly lower in NB-02-treated animals compared to those treated with vehicle (Fig. 1i, 243±16 plaques/mm³ for vehicle, 139±14 plaques/mm³ for NB-02, mean±SEM, Mann Whitney test p<0.001).

Amyloid plaque burden analysis was performed, taking into account the number and size of plaques. At baseline, vehicle-treated animals exhibited 0.98±0.05% burden (mean±SEM), while NB-02 treated mice had 1.04±0.06% burden (mean±SEM, Mann Whitney test, n.s.). Amyloid plaque burden increased to 1.33±0.07% in vehicle-treated animals 1 month after treatment onset, significantly higher compared to baseline (Fig. 1l, mean±SEM, Wilcoxon matched-pairs signed rank test p<0.001). By 2 months after treatment onset, amyloid plaque burden increased higher to 1.69±0.11%, significantly different from baseline (Fig. 1l, mean±SEM, Wilcoxon matched-pairs signed rank test p<0.001). Complementary to plaque number findings, amyloid plaque burden decreased to 1.0±0.05% in NB-02-treated mice after 1 month (Fig. 1l, mean±SEM, Wilcoxon matched-pairs signed rank test p<0.01) and then decreased further to 0.82±0.06% after 2 months of treatment with NB-02 (Fig. 1l, mean±SEM, Wilcoxon matched-pairs signed rank test p<0.001). Thus, by the end of treatment, vehicle-treated mice had substantially higher amyloid burden (1.69±0.11%) compared to NB-02 treated mice (0.82±0.06%) (Fig. 1m, mean±SEM, Mann Whitney test p<0.001).
These findings suggest that while vehicle treatment failed to prevent deposition of new plaques, NB-02 treatment led to plaque clearance as imaged in vivo (Fig. 1j, Mann Whitney test, p<0.001, n=46 comparisons in 10 mice for vehicle, n=48 comparisons in 11 mice for NB-02). Amyloid plaque burden analysis reflected this conclusion (Fig. 1n, Mann Whitney test, p<0.001). Even though the plaque size was comparable in vehicle and drug conditions (Fig. 1k), treatment with NB-02 led to decreases in intensity of individual plaques (Fig. 1o, Mann Whitney test, p<0.001). Since we had verified that neither NB-02 nor vehicle interfered with methoxy-X04 binding to individual plaques[13], decreases in plaque intensity signaled bona fide plaque clearance in NB-02 condition.

As multiphoton microscopy allowed imaging of small cortical regions, we set out to verify the above findings in larger cortical regions using coronal cross-sections of hemibrains processed post-mortem at the end of treatment. Brain sections were immunostained with anti-amyloid beta antibody 6E10. While methoxy-X04 labelled dense cores, 6E10 decorated the periphery of amyloid plaques as well (Fig. 2a-f). Vehicle-treated brains exhibited high amyloid plaque load, evident with 6E10 immunoreactivity and methoxy-X04 labeling (Fig. 2a, b, c). Intriguingly, NB-02 treatment resulted in decreased amyloid deposition (Fig. 2d, e, f). 6E10 immunoreactivity suggested that amyloid plaque burden was significantly lower as a result of NB-02 treatment compared to vehicle (Fig. 2g, Mann Whitney test, p<0.01, n=21 sections in 8 mice/vehicle; n=25 sections in 8 mice/NB-02). Similarly, methoxy-X04 labeling indicated lower plaque burden at the end of NB-02 treatment (Fig. 2h, Mann Whitney test, p<0.01).

Amyloid plaque burden was also determined in hippocampi of treated mice post-mortem (Fig. 3). There, 6E10 detected amyloid on the periphery of plaques in addition to their centers, while methoxy-X04 identified dense cores exclusively (Fig. 3a-f). Similar to the data in the cortex, NB-02 treatment resulted in decreased amyloid plaque burden as assessed by 6E10 immunoreactivity (Fig. 3g Mann Whitney test, p<0.05, n=24 sections in 8 mice/vehicle; n=23
sections in 8 mice/NB-02) or methoxy-X04 labeling (Fig. 3h Mann Whitney test, p<0.01). We
had previously verified that neither NB-02 nor vehicle interfered with methoxy-X04 binding to
amyloid plaques[13]. Thus, amyloid plaque burden analysis performed post-mortem agreed with
the in vivo data (Fig. 1), where treatment with NB-02 resulted in amyloid plaque clearance in an
animal model of amyloidosis.

NB-02 treatment does not alter soluble amyloid beta levels measured with ELISAs in CSF
or TBS-soluble brain fractions

To determine whether treatment with NB-02 altered soluble amyloid beta levels, cerebrospinal
fluid (CSF) samples were collected from cisterna magna of APP/PS1 mice at the end of the
treatment with the drug or the vehicle. ELISAs specific for amyloid beta 40 or 42 were used to
measure protein levels. Interestingly, CSF amyloid beta 40 levels showed a trend toward an
increase in animals treated with NB-02, however failed to reach statistical significance (Fig.
S1a). Similarly, CSF amyloid beta 42 levels measured after NB-02 treatment also tended to
increase, however these levels were not statistically different from those after vehicle treatment
(Fig. S1b).

We also measured TBS-soluble amyloid beta 40 and 42 levels in brains of APP/PS1 mice
treated with NB-02 or vehicle. Amyloid beta 40 levels in TBS soluble fractions trended up in NB-
02 condition compared to vehicle (Fig. S1c), not reaching significance. Furthermore, TBS-
soluble amyloid beta 42 levels were not significantly different between the two conditions, yet
further supported the trend up in NB-02-treated brains (Fig. S1d). Thus, the ELISA results
suggest that neither soluble amyloid beta 40 nor 42 levels were significantly altered by NB-02
treatment, albeit showing an upward trend.
NB-02 restores neuronal calcium homeostasis

To determine whether NB-02 treatment has a direct effect on neuronal health, we assessed NB-02’s propensity to restore calcium homeostasis within neuronal processes, neurites. Animal models of amyloidosis contain a small cortical neuronal population that is vulnerable to amyloid beta-dependent calcium dysregulation. This results in baseline calcium elevations within these neurons[26]. Therefore, restoration of neuronal calcium to control levels would serve as a functional indicator of treatment efficacy. Hence, we determined whether gavage treatment with NB-02 would alter neuronal calcium. A genetically encoded calcium indicator, YellowCameleon 3.6[18] was virally expressed in cortical neurons (Fig. 1a) to calculate absolute baseline calcium concentration within each neuron expressing the calcium reporter. Healthy neuronal function is maintained at ~100 nM intracellular calcium. Amyloid beta present in animal models of amyloidosis, such as APP/PS1 mice used here, leads to calcium elevations within a fraction of cortical neurons (Fig. 4a). Calcium overload was defined as YFP/CFP ratio that was 2 standard deviations above the mean in healthy wildtype mice, and calculated to be >1.79. As anticipated, we saw 11% of neurons exhibiting calcium overload prior to vehicle treatment onset (Fig. 4c blue bar in red rectangle) and 9% of neurons exhibiting calcium overload before DA-02 treatment commenced (Fig. 4d blue bar in red rectangle), while the majority of neurons exhibited healthy calcium levels, YFP/CFP<1.79. As vehicle treatment progressed the percentage of neurons exhibiting calcium overload increased, reaching 30% by the end of treatment (Fig. 4c). Surprisingly, there was a substantial decrease in the percentage of neurons with calcium overload 1 month after NB-02 treatment onset (Fig. 4d purple bar in red rectangle). The low percentage of neurons with calcium overload was maintained at 2 months of treatment (Fig. 4b, d red bar in red rectangle). At the end of treatment, the percentage of neurites with calcium overload was substantially lower in the NB-02-treated cortices (3.32±3%) compared to those treated with vehicle cocktail (38±10%) (Fig. 4e, Mann Whitney test, p<0.05, N=up to 272
neurites in 4 mice/vehicle; N=up to 368 neurites in 6 mice/NB-02). Thus, NB-02 treatment was effective at restoring calcium homeostasis and maintaining optimal intracellular calcium levels for healthy neuronal function.

**NB-02 treatment transforms morphology of glial cells**

Neuroinflammation is one of the major hallmarks of AD[27–29]. Thus, the effects of NB-02 on non-neuronal cells such as astrocytes and microglia were analyzed using immunohistochemistry post mortem. The number of cells positive for glial fibrillary acidic protein (GFAP)[30,31] or ionized calcium binding adaptor molecule 1 (Iba-1) were counted in the cortices and hippocampi to determine differences in relative cell numbers at the end of treatment. In addition, morphological analyses were performed by measuring the length of processes and the size of somas. Upon analyzing cell counts, no significant differences were found in the number of GFAP positive astrocytes between vehicle and NB-02 treated animals in either cortex (Fig. 5a-c, Mann-Whitney test, n.s; N=8 sections in 8 mice/vehicle, N=7 sections in 7 mice/NB-02) or hippocampus (Fig. 5d-f, Mann-Whitney test, n.s; N=8 sections in 8 mice/vehicle, N=7 sections in 7 mice/NB-02). Similarly, there were no significant differences in Iba-1 positive cell numbers across condition for either cortex (Fig. 6a-c, Mann-Whitney test, n.s; N=8 sections in 8 mice/vehicle, N=8 sections in 8 mice/NB-02) or hippocampus (Fig. 6d-f, Mann-Whitney test, n.s; N=8 sections in mice/vehicle, N=5 sections in 5 mice/NB-02).

While no significant differences were found in the numbers of reactive cells, morphological analyses revealed differences across conditions for both astrocytes and microglia. Astrocytic morphology was altered as a result of NB-02 treatment, as evidenced by shorter processes and larger cell bodies (Fig. 5h, i, j; t-tests, p<0.0001 for processes and cell body diameter; N=50 cells, 100 processes in 5 mice/NB-02). In contrast vehicle-treated astrocytes maintained their
long processes and small cell bodies (Fig. 5g, i, j; N=50 cells, 100 processes in 5 mice/vehicle).

Similarly, morphology of microglia was changed after NB-02 treatment also evidenced by shorter processes and larger cell bodies, both by diameter and area (Fig. 6h-l; Mann-Whitney test, p<0.0001 for all comparisons; N=414 cells, 1080 processes in 8 mice/NB-02). Microglia in animals treated with vehicle possessed long processes and small cell bodies (Fig. 6g, i-l; N=475 cells, 1040 processes in 8 mice/vehicle). To confirm that the significant differences across condition were not simply due to the large numbers of cells being analyzed, the same statistical analyses were performed with cells grouped by mouse, and the results were verified (Fig. S2a-e). Thus, in addition to having an effect on neuronal cells, NB-02 had an effect on non-neuronal cells such as astrocytes and microglia.

**NB-02 is a multimodal therapeutic with multiple mechanisms of action**

To gain insight into the mechanism(s) of action of NB-02, we assessed the effects of NB-02 in cell culture. Alzheimer’s disease is a neurodegenerative disorder, thus we tested the neuroprotective effects of NB-02. Nerve growth factor (NGF) is a neurotrophin that plays a neuroprotective role by promoting maintenance, survival and plasticity of neurons. NGF deficits induce neuronal apoptosis, death and dysfunction as a result of Aβ-induced toxicity in the brain[32]. Through neurotrophic tyrosine receptor kinases (NTRKs), NGF has also been shown to prevent amyloid precursor protein (APP) cleavage by β-secretase 1 (BACE1)[33]. Finally, current AD symptomatic therapies target acetylcholine system, by inhibiting acetylcholinesterase [34]. Thus, we used human neuroblastoma SH-SY5Y cells to assess the effects of NB-02 on cell viability, NGF production and acetylcholinesterase activity.

Cell viability was assessed using MTT assay. Exposure of SH-SY5Y cells to oligomeric Aβ42 resulted in decreased cell survival (Fig. S3a). Treatment with donepezil, acetylcholinesterase inhibitor, restored cell survival in a dose dependent manner. Interestingly, treatment with NB-02 restored cell survival in a dose dependent manner as well. NB-02 mediated its neuroprotective
effects via serotonin receptors, since serotonin receptor 6 antagonist Lu AE58054 blocked the neuroprotective effect (Fig. S3a).

Acetylcholinesterase activity was assessed using acetylcholinesterase activity assay. Exposure of SH-SY5Y cells to oligomeric Aβ42 resulted in increased acetylcholinesterase activity (Fig. S3b). Donepezil treatment of cultures restored acetylcholinesterase activity. Also, treatment with NB-02 restored acetylcholinesterase activity in a dose dependent manner. Its effect was mediated via serotonin receptors, since Lu AE58054 blocked the effect (Fig. S3b).

Finally, NGF production was assessed in healthy SH-SY5Y cells, not treated with Aβ42. NB-02 increased NGF production in a dose dependent manner, while donepezil failed to do so (Fig. S3c). Thus NB-02 is a multimodal therapeutic, which increased cell viability, inhibited acetylcholinesterase and increased NGF production.

Discussion

NB-02, previously known as DA-9803, is an all-natural botanical drug prepared by mixing natural substances that is prepared according to a standardized recipe. It has multiple modes of action and has shown great promise as a preventative agent in animal models of AD[13]. Thus, in this study we assessed its potential as a therapeutic agent.

Starting at 8 months of age, APP mice were treated with the therapeutic or vehicle via daily gavage for two months. Amyloid plaque deposition was monitored with multiphoton microscopy before, during and after treatment. As early as 1 month after treatment onset, amyloid deposition was halted and some amyloid cleared in NB-02-treated mice. Animals treated with vehicle continued accumulating amyloid over time. This resulted in a substantially higher number of amyloid plaques in vehicle-treated animals compared to drug-treated mice at the end of treatment. Amyloid burden, which considers the size of plaques as well as their number, was also significantly higher in vehicle-treated mice. Interestingly, amyloid plaques appeared dimmer
in NB-02 treated animals, however this was not due to NB-02 interference of methoxy-X04 binding to the plaques as we have reported previously[13]. Post-mortem analysis confirmed elevated amyloid plaque burden in the cortices of vehicle-treated animals as well as their hippocampi. This was important, since multiphoton microscopy allows assessment of amyloid in a small cortical volume. Both mechanisms, decreased plaque deposition and increased plaque clearance, were accountable for the difference in amyloid plaque burden between NB-02- and vehicle-treated brains post-treatment. Additionally, we detected a trend toward increased amyloid beta 40 and 42 levels in CSF as well as TBS-soluble fraction of the brains. However, this trend failed to reach significance, possibly due to limited sensitivity of the biochemical procedure. Thus, NB-02 affects extracellular plaques by preventing their deposition and aiding their clearance, possibly by making amyloid more soluble and easier to clear.

APP mice contain a population of cortical neurons that is highly vulnerable to amyloid beta[26]. Not being able to maintain calcium homeostasis, these neurons exhibit calcium elevations or calcium overload. As such, restoration of baseline neuronal calcium levels would serve as a functional indicator of treatment efficacy[12,13,35]. Interestingly, NB-02 treatment restored neuronal calcium thus leading to fewer neurons exhibiting calcium overload. Since intracellular calcium supports a myriad of neuronal functions, calcium normalization would signify restoration of these functions, including those supporting cognitive and memory processes of AD patients[5].

Neuroinflammation is an integral process in AD progression[7,8]. Thus, we assessed the effect of NB-02 treatment on neuroinflammation. The number of reactive astrocytes and microglia were compared between conditions. However, the number of GFAP-positive astrocytes did not differ significantly in vehicle vs. drug-treated mice. Similarly, IBA-1-positive microglia numbers failed to reach significant difference. Analyses of morphometric changes revealed that, while astrocytes maintained the small cell body and long processes in vehicle-treated animals, NB-02
treatment transformed the morphology of astrocytes, allowing them to assume larger cell bodies and shorter processes. Similarly, microglia morphology also changed, assuming shorter processes and larger cell bodies in the NB-02 treatment condition. These morphological transformations have been associated with amyloid beta phagocytosis and clearance[36].

In vitro studies revealed that NB-02, a botanical therapeutic mixture, is multimodal. It increased cell survival and NGF production possibly by neutralizing amyloid-dependent toxicity and restoring neuronal calcium homeostasis, thus restoring neuronal function. NB-02 was an effective acetylcholinesterase inhibitor. And finally, it upregulated neuroinflammation, further potentiating amyloid clearance. Altogether, this suggests that NB-02 has great potential in the clinic as a treatment for Alzheimer’s disease.

Limitations

The AD animal model used here recapitulates some aspects of human disorder. Hence future clinical trials of NB-02 will elucidate true clinical potential of this therapeutic.

Conclusions

2-month treatment with NB-02 halts plaque deposition and clears amyloid in cortex as well as hippocampus. It normalizes neuronal calcium homeostasis, thus restoring neuronal function, and upregulates neuroinflammation. NB-02 is a multimodal botanical therapeutic with several mechanisms of action. It increases cell viability and NGF production. It also inhibits acetylcholinesterase activity. The exact nature of those mechanisms will be assessed in future studies. Since NB-02 was effective in slowing pathophysiology in an animal model of amyloidosis, it holds great promise as a therapeutic approach to treat Alzheimer’s disease.
Declarations

Ethics approval and consent to participate

All the studies complied with Massachusetts General Hospital Animal Care and Use Committee as well as NIH guidelines for the use of laboratory animals.

Consent for publication

Not applicable

Availability of data and materials

Data and materials are available upon request.

Competing interests:

The study was funded by NeuroBo Pharmaceuticals and Dong-A ST

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Author contributions

YFL, AL, S-Z C and ANR conducted the study, analyzed the data. ANR, BJB and KVK interpreted the data. KVK wrote the manuscript. YFL, ANR and BJB edited the manuscript.

Figure Legends

Fig. 1 Chronic treatment with NB-02 halts deposition of cortical amyloid plaques in vivo. a

Experimental schematic showing the time-points of viral injections, craniotomies, multiphoton
imaging sessions and gavage treatments with NB-02 or vehicle of APP mice. b, e Multiphoton microscopy images of red dextran angiograms in APP mice prior to treatments with vehicle (b) or NB-02 (e). c, f Multiphoton microscopy images of methoxy-X04 positive amyloid plaques in cortices of APP mice prior to treatments. Note b and c were taken during the same imaging session and constitute the same field of view. Also, e and f were taken during the same imaging session and constitute the same field of view. d, g Images of amyloid plaques taken after treatments with vehicle or NB-02. Note c and d were taken during different imaging sessions and constitute the same field of view. Similarly, f and g were taken during different imaging sessions and constitute the same field of view. h Plaque numbers per cubic millimeter of cortex across the two conditions over time. Statistical comparisons are made to baseline (0 months). i Cortical plaque densities at the end of treatment across conditions. j Amyloid plaque number changes at the end of treatment compared to baseline across conditions. Vehicle treatment resulted in addition of new plaques, while NB-02 treatment resulted in mild clearance. k Size of amyloid plaques in the course of treatment across conditions. l Amyloid plaque burden, which takes into account plaque number and size, over time across conditions. m Plaque burden at the end of treatment with vehicle or NB-02. n Amyloid plaque burden change at the end of treatment compared to baseline. o Methoxy-X04 intensity change at the end of treatment. Scale bar, 100 µm. Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. n.s. not significant.

Fig. 2 NB-02 treatment results in lower cortical amyloid load compared to vehicle when assessed postmortem. a, d 6E10 immunoreactivity against amyloid in brain sections obtained post-mortem after treatment with vehicle or NB-02. b, e Methoxy-X04 positive amyloid plaques. Images in a, b were acquired from the same field of view. Similarly, images in d and e were acquired from the same field of view. c, f Colocalization of 6E10 and methoxy-X04. g, h Cortical amyloid plaque burden as assessed by 6E10 immunoreactivity and methoxy-X04 signal. Each dot represents amyloid burden in a hemibrain. Scale bar, 75 µm. Mean ± SEM, **p<0.01.
Fig. 3 NB-02 treatment results in lower hippocampal amyloid load compared to vehicle when assessed postmortem. \textbf{a, d} 6E10 immunoreactivity against amyloid in hippocampi obtained post-mortem after treatment with vehicle or NB-02. \textbf{b, e} Methoxy-X04 positive amyloid plaques. Images in \textbf{a, b} were taken from the same field of view. Similarly, images in \textbf{d} and \textbf{e} were taken from the same field of view. \textbf{c, f} Colocalization of 6E10 and methoxy-X04. \textbf{g, h} Cortical amyloid plaque burden as assessed by 6E10 immunoreactivity and methoxy-X04 signal. Each dot represents amyloid burden in a hemibrain. Scale bar, 50 µm. Mean ± SEM, *p<0.05, **p<0.01.

Fig. 4 NB-02 restores neuronal calcium overload in vivo. \textbf{a, b} Multiphoton microscopy images of calcium reporter YC2 expressed in neurons pseudocolored according to intracellular calcium concentrations in APP brains treated with vehicle (\textbf{a}) or NB-02 (\textbf{b}). Yellow arrows point to neuronal cell bodies exhibiting calcium overload, while yellow arrowhead points to neuronal process, neurite, exhibiting calcium overload. \textbf{c, d} Histograms showing percentages of neurites binned into categories according to the YFP/CFP ratios over the course of treatment with vehicle (\textbf{c}) or NB-02 (\textbf{d}). Percentage of neurites with calcium overload, YFP/CFP ratio>1.79, are shaded in red. \textbf{e} Bar graph showing percentages of neurites exhibiting calcium overload at the end of treatment. Scale bar, 50 µm. Mean ± SEM, *p<0.05.

Fig. 5 NB-02 transforms morphology of reactive astrocytes. \textbf{a, b} GFAP immunoreactivity in cortical sections obtained from vehicle (\textbf{a}) and NB-02 (\textbf{b}) treated APP mice. Scale bar, 0.1 mm. \textbf{c} GFAP positive cell counts in cortex across conditions. \textbf{d, e} GFAP immunoreactivity in hippocampal sections obtained from vehicle (\textbf{d}) and NB-02 (\textbf{e}) treated APP mice. Scale bar, 0.1 mm. \textbf{f} GFAP positive cell counts in hippocampus across conditions. \textbf{g, h} Higher magnification images of a single astrocyte in the brain of an APP mouse treated with vehicle (\textbf{g}) or NB-02 (\textbf{h}). Scale bar, 10 µm. \textbf{i} Astrocyte process length across conditions. \textbf{j} Astrocyte cell body diameter across conditions. n.s, not significant, ***p<0.001.
Fig. 6 NB-02 transforms morphology of reactive microglia. a, b Iba-1 immunoreactivity in cortical sections obtained from vehicle (a) and NB-02 (b) treated APP mice. Scale bar, 0.1 mm. c Iba-1 positive cell counts in cortex across conditions. d, e Iba-1 immunoreactivity in hippocampal sections obtained from vehicle (d) and NB-02 (e) treated APP mice. Scale bar, 0.1 mm. f Iba-1 positive cell counts in hippocampus across conditions. g, h Higher magnification images of a single microglia in the brain of an APP mouse treated with vehicle (g) or NB-02 (h). Scale bar, 10 µm. i Microglia process length across conditions. j Microglia cell body diameter across conditions. k Microglia soma area across conditions. l Microglia cell roundness across conditions. n.s, not significant, **** p<0.0001.

Fig. S1 NB-02 does not alter soluble amyloid levels. a, b Human amyloid beta (a: 40, b:42) levels in cerebral spinal fluid (CSF) of APP mice treated with vehicle or NB-02. c, d Human amyloid beta (c: 40, d:42) levels in TBS-soluble brain fractions of APP mice treated with vehicle or NB-02. Each dot represents amyloid measurement in CSF or brain fraction obtained from individual mice. n.s. not significant.

Fig. S2 Cell morphology data analyzed by mouse. a Microglia process length across conditions. b Microglia soma area across conditions. c Microglia cell body diameter across conditions. d Astrocyte process length across conditions. e Astrocyte cell body diameter across conditions. ** p<0.01, *** p<0.001.

Fig. S3 NB-02 has multiple mechanisms of action. a, The effect of NB-02 on cell viability of SH-SY5Y under normal conditions (Nor), after application of 1 µM oligomeric Aβ42, after application of oligomeric Aβ42 and various doses of NB-02, after application of oligomeric Aβ42 and various doses of donepezil, after application of oligomeric Aβ42 and various doses of NB-02 and serotonin receptor 6 blocker AE. b, The effect of NB-02 on acetylcholinesterase activity in SH-SY5Y cells under normal conditions (Nor), after application of 1 µM oligomeric Aβ42, after application of oligomeric Aβ42 and various doses of NB-02, after application of oligomeric Aβ42 and various doses of NB-02.
and various doses of donepezil, after application of oligomeric Aβ42 and various doses of NB-02 and serotonin receptor 6 blocker AE. c, The effect of NB-02 on NGF expression in SH-SY5Y cells treated with vehicle (Veh), various doses of NB-02, or donepezil. *p<0.05, ** p<0.01, *** p<0.001.

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Figure 1

Chronic treatment with NB-02 halts deposition of cortical amyloid plaques in vivo. a Experimental schematic showing the time-points of viral injections, craniotomies, multiphoton imaging sessions and gavage treatments with NB-02 or vehicle of APP mice. b, e Multiphoton microscopy images of red dextran angiograms in APP mice prior to treatments with vehicle (b) or NB-02 (e). c, f Multiphoton microscopy
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Figure 3

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Figure 5

NB-02 transforms morphology of reactive astrocytes. a, b GFAP immunoreactivity in cortical sections obtained from vehicle (a) and NB-02 (b) treated APP mice. Scale bar, 0.1 mm. c GFAP positive cell counts in cortex across conditions. d, e GFAP immunoreactivity in hippocampal sections obtained from vehicle (d) and NB-02 (e) treated APP mice. Scale bar, 0.1 mm. f GFAP positive cell counts in hippocampus across conditions. g, h Higher magnification images of a single astrocyte in the brain of an APP mouse treated with vehicle (g) or NB-02 (h). Scale bar, 10 μm. i Astrocyte process length across conditions. j Astrocyte cell body diameter across conditions. n.s, not significant, ***p<0.001.
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