The Prevention of Fatal Tauopathy in a Mouse Model of Alzheimer Disease by Blocking BCL2

Gerard J. Nuovo, MD, Madison Rice, BA,* Nicola Zanesi, PhD,† Dwitiya Sawant, PhD,* Candice Crilly, PhD,* and Esmerina Tili, PhD‡*

Abstract: A major goal in Alzheimer disease (AD) research is the reduction of the abnormal tau burden. Using multispectral analyses on brain tissues from humans who died of AD it was documented that neurons with hyperphosphorylated tau protein accumulate many proteins of the BCL2 family, including those that block cell turnover (eg, BCL2, MCL1, BCLXL) and those that promote cell turnover (eg, NOXA, PUMA, BAK, BAX). A mouse model of AD with the humanized hyperphosphorylated tau protein was used to test the hypothesis that shifting this balance to a pro-cell turnover milieu would reduce the tau burden with concomitant clinical improvement. Here, we show that a mouse model of AD with death at 11 to 15 months due to CNS tauopathy had a marked reduction in the tau burden after treatment with the FDA-approved drug venetoclax, which blocks BCL2. The reduction of the number of target neurons positive for hyperphosphorylated tau protein after venetoclax treatment in the brain and spinal cord neurons was 94.5% as determined by immunohistochemistry and 98.1% as documented with the modified Bielchowsky stain. The venetoclax treatment began after documented neurofibrillary tangles (NFTs) were evident and there was a concomitant reduction in neuroinflammation. The treated mice were robust until sacrificed at 13 months as compared with the untreated mice that showed unequivocal evidence of brain and spinal cord damage both clinically and at autopsy. We conclude that otherwise inexorable abnormal tau protein deposition, even after initiation, can be prevented by a drug that blocks one anti-cell turnover protein abundant in the NFTs of human AD.

Key Words: BCL2 family, Alzheimer disease, BCL2, venetoclax, tauopathy

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From the *GNOME Diagnostics, Powell; Departments of †Cancer Biology and Genetics; and ‡Anesthesiology, Ohio State University College of Medicine, Columbus, OH.

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Reprints: Gerard J. Nuovo, MD, GNOME DX, 1476 Manning Parkway, Powell 43065, OH (e-mail: jerrynuovo@yahoo.com).

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Multiple recent investigations have suggested that hyperphosphorylated tau protein may be a clinically more relevant marker of Alzheimer disease (AD) than Aβ-42 in humans and mouse models. The abnormal tau protein is associated with significantly more neuroinflammation than Aβ-42,[1–3] whose importance is underscored by the role inflammation plays in AD and other neurodegenerative diseases.[1–13] SARS-CoV2 worsens AD in part due to the diffuse neuroinflammation it induces.[14,15] Several studies have indicated that senescent cells may play a role in AD. Tissues with the abnormal tau protein, but not the Aβ-42 protein, display multiple senescence factors, including the cell cycle regulators $p16^{Ink4a}$, $p19^{Arf}$, $p21^{Cip1/Waf1}$, and the pro-inflammatory genes Pai1, IL-6, and IL-1β which have been localized to astrocytes, microglial cells, oligodendroglial progenitor cells, and neurons.[2,16–21]

The BCL2 family are involved in cell turnover that has been best characterized in leukocytes and epidermal cells.[22–24] The BCL2 family includes proteins that promote cell survival (eg, BCL2, BCLW, BCLXL, MCL1) as well as others that promote cell removal (eg, PUMA, NOXA, BAD, BAK, BAX).[22–24] The overexpression of BCL2 is common in lymphomas and is effectively treated with an FDA-approved small molecule called venetoclax with concomitant elimination of tumor cells.[22]

Neuronal turnover is well documented to be robust in parts of the adult brain in humans and mice, such as in the hippocampus, where one-third of the neurons in humans are subject to exchange.[25,26] By subdividing human AD samples into those with abundant hyperphosphorylated tau protein and adjacent sections with no detectable abnormal tau from the same patient, we noted that the neurons with the abnormal tau had very high levels of BCL2 family proteins including BCL2, BCLW, BCLXL, MCL1 as well as PUMA, NOXA, BAK, BAX, BAD with P-values < 0.00001.[27–29] Co-expression analyses documented that it was the hyperphosphorylated tau protein positive neurons, and not glial cells, that predominantly expressed these BCL2 family proteins.[27–29]

Several groups have successfully reduced the tau burden in mouse models of AD using either the tyrosine kinase inhibitor dasatinib and the drug quercetin or the anticancer drug navitoclax.[2,16,19,20] Some of these works theorized that these drugs removed the senescent microglia and astrocytes which attenuated the neuroinflammation

that, in turn, induced the accumulation of the abnormal tau protein. However, current senolytic drugs have drawbacks since dasatinib is commonly associated with bone marrow suppression, quercetin has poor bioavailability, and navitoclax has a dose-limiting side effect of bleeding since it directly interferes with platelet function.[2,16,19,20] None of these studies have focused on the BCL2 family.

Here, we address the hypothesis that the balance of procell and anticell survival BCL2-family proteins in neurons may be a factor in the pathogenesis of AD. We show that blocking just one of these proteins, BCL2, with the drug venetoclax, reduced the number of target neurons positive for hyperphosphorylated tau protein in the brain and spinal cord neurons by 94.5% in the B6.Cg-Tg(Prnp-MAPT*P301S)PS19Vle/J mouse model of AD with concomitant marked clinical improvement.

MATERIALS AND METHODS

Clinical Samples and Controls

The patient samples (formalin-fixed paraffin-embedded autopsy material) originated from the files of Folio Biosciences. Clinical information included the diagnosis, age/sex of the patient, and the Braak stage. For the multispectral analyses, we accessed a previously published series of 29 tissues of AD (19 cortex and 10 hippocampus) from 13 patients (mean age 77.2 y) in which these in situ-based multispectral studies were not previously done.[1,27–29] We also studied the cortex and hippocampal sections from 4 patients who died of corticobasal degeneration (CBD) with 8 age-matched controls. There was a total of 20 brain sections from the 4 CBD patients and another 10 brain sections from the 8 age-matched controls. Immunohistochemistry for the hyperphosphorylated tau protein documented its presence in each of the CBD blocks and none of the controls.

Mouse Models of AD

The mouse models of AD used in this study were previously described.[1] In brief, C57BL/6 wild-type (WT) mice and 5xFAD mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The 5XFAD (AD) (B6SJL-Tg (APPSwFlLon, PSEN1* M146L* L286V) 6799Vas/Mmjax) mouse is a double transgenic APP/PS1 mouse model that co-expresses 5 AD mutations leading to senile plaque formation and the concomitant Aβ42 production.

Also studied were the mouse models Tau4RΔK (CamKII-tTA;TetO-TauRDΔK) and APP/PS1 (APP^{swe}; PS1ΔE9). The APP^{swe};PS1ΔE9 are double transgenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9).[1] The Tau4RΔK mice express a transgene encoding a mutant 4-repeat domain of human microtubule-associated tau protein (Q244-E372). We have recently reported extensive neuroinflammation data on each of these 4 mouse groups.[1] For treatment with venetoclax, we obtained from Jackson Laboratory the mouse strain #024841 [B6.Cg-Tg(Prnp-MAPT*P301S) PS19Vle/J]. These PS19 transgenic mice (P301S Tg mice) express the P301S mutant form of the human microtubule-associated protein tau (MAPT). Hyperphosphorylated, insoluble mutant human MAPT protein in the brain and spinal cord accumulates with age, resulting in motor weakness at 6 months and death from tauopathy between 11 and 15 months.

Venetoclax (ABT-199) Catalog No. S8048 was purchased from Selleck Chemicals (Houston, TX) and 5 mg were diluted in 10 mL of 2.5% DMSO. The vehicle alone (2.5% DMSO in PBS solution) was used as the sham control. Either 200 μL/mouse (average weight about 20 g) of venetoclax or the sham solution were injected intraperitoneally 3 times/week. These animal experiments were performed according to protocols approved by the Animal Care and Use Committee (IACUC) of the Ohio State University College of Medicine.

Immunohistochemistry and the Bielchowsky Stain

Immunohistochemistry was done as previously reported.[1,27–29] The specific antibodies used (source and catalogue numbers; Ab = ABCAM, Enzo = Enzo Life Sciences, Prot = Proteintech) were as follows with the dilutions in paratheses: hyperphosphorylated tau protein (Ab136407)/1:1000, Aβ-42 (Ab201060)/1:1000, IL6 (Abcam Ab 6672)/1:3000, IL1β (Prot 16806-1)/1:1000, CXCL10 (Prot 10937)/1:2000), TNFα (Pro 26405)/1:3000, BAK (Enzo 210-002)/1:2000), BAX (Ab32503)/1:3000), pyruvate dehydrogenase (Ab168379)/1:1000, neurofilament, heavy (Ab207176)/1:500, PUMA (Enzo 905-237)/1:2000), MCL1 (Enzo AAP-240)/1:6000, NOXA (Enzo 804 408)/1:2,500), cFLIP (Prot 10394)/1:1,500), BCLW (Prot 16026)/1:800), BAD (Prot 10435)/1:666), BCL2 (Ab182858)/1:6000, BCLXL (Ab32370)/1:800), BIM (Ab32158)/1:300), doublecortin (Prot 13925)/1:333), and nestin (Prot 19483)/1:1,500). Each antibody required antigen retrieval that was done for 30 minutes at 95 °C using an EDTA solution (pH 9.0) with the exception of hyperphosphorylated tau protein and Aβ-42 each of which required protease digestion pretreatment for 3 minutes (Enzo Life Sciences, Farmingdale NY, proteinase K, 1 mg/mL). The immunohistochemistry protocol used the Leica Bond Max (Buffalo Grove, IL) automated platform; the Fast Red (DS 9820) and the DAB (DS 9800) detection kits were used and gave equivalent results.

The modified Bielchowsky stain, which detects both neurofibrillary tangles and senile plaques, was used as per the manufacturer's protocol (StatLab, McKinney, Texas).

qRTPCR

This method was done as previously described.[27–29] Briefly, RNA (425 ng) was retrotranscripted using the High Capacity cDNA Reverse Transcription kit (Life Technologies) according to the manufacturer's protocol. The comparative real-time PCR was performed in triplicate and analyzed using QuantStudio 12K Flex Real-Time PCR System. The Ct average of each triplicate was used to perform the relative quantification analysis.

Multispectral and Coexpression Testing and Statistical Analyses

As previously described,[1,27–29] coexpression experiments were performed by analyzing a given tissue section for one protein using 3,3′-Diaminobenzidine (DAB) chromogen and analyzing the other protein with Fast Red chromogen. For 3 to 5 target colocalization, HIGHDEF yellow IHC chromogen HRP (ADI-950-170), HIGHDEF blue IHC chromogen AP (ADI-950-150), and HIGHDEF green AP chromogen (Enz-ACC130), each from Enzo Life Sciences, were used. Coexpression analyses were done using the Nuance software. Quantification for the signal with either single immunohistochemistry or multi-labeled immunohistochemistry was done using either the InForm software or manual counting, which yielded equivalent results. Statistical analysis was done using the InStat Statistical Analysis Software (version 3.36) and a paired *t*-test (also referred to as a "repeated measure *t*-test"). The null hypothesis was rejected if the significance level was below 5%.

Immunohistochemistry Scoring

Immunohistochemistry scoring was done blinded to the genetic background or treatment regimens and varied according to the targets. For scoring of BCL2 family protein and cFLIP data, the standard pathology system of 0, 1+, 2+, and 3+ was used with 0.5+ intervals. Six 200× fields are scored for a given tissue based on both signal intensity and the percentage of target cells positive for a given protein that yields an average value for each data point. For the spinal cord samples, these analyses were done for each spinal cord section (cervical, thoracic, lumbar, and sacral). Each sample (control, test cases) were tested at the same time and/or with the same conditions to limit test data variability. For astrocyte density analyses the number of astrocyte processes as defined by the GFAP immunohistochemistry per 100× field was tallied. For each cytokine assayed (IL6, TNFα, IL1β, and CXCL10), the number of positive cells/200× field was tabulated for the untreated mice at 13 months. This data was normalized to reflect the extensive degeneration. For hyperphosphorylated tau protein, the number of spinal cord neurons positive for this protein by immunohistochemistry at 13 months in the untreated mice was scored as 100% and all other values scored relative to these data. For neuronal markers, the number of spinal cord neurons positive for pyruvate dehydrogenase and heavy chain neurofilament in the WT and 6-week spinal cord of strain # 024841 (which gave equivalent results) was scored as 100%, and all other values scored relative to these data. For both the tau and neuronal quantification, 3 different data sets were generated by obtaining sections 150 μm apart to reduce sampling bias.

Genotyping

The genotyping protocol was as outlined on the Jackson Laboratory website for strain #024841 (https://www.jax.org/Protocol?stockNumber=024841&protocolID=25393) in which the primers for the transgene (GGT ATT AGC CTA TGG GGG ACA C and GGC ATC TCA GCA ATG TCT CC) yield an amplicon by standard PCR of 450 bp and the primers for the internal positive control (AA ATG TTG CTT GTC TGG TG and GTC AGT CGA GTG CAC AGT TT) yield a band at 200 bp.

RESULTS

Distribution of the Bcl2 Family and cFLIP in Mouse Models of AD

We have previously studied several mouse models of AD where it was documented that neuroinflammation was significantly greater in those models that expressed the human abnormal tau protein as compared with the Aβ-42 protein or wild-type control.[1] In the current study, we expanded the molecular analyses of these brains by interrogating each for Bcl2-family proteins that promote cell survival (Bcl2, BclW, BclXL, Mcl1 as well as cFLIP) and Bcl-2 family proteins that can facilitate cell turnover (Noxa, Puma, Bak, Bax, Bim, and Bad) blinded to the genetic information. As previously reported,[1] the abnormal tau protein was highly concentrated in the hippocampus and limbic system areas and was rarely evident in the thalamus/midbrain areas. The Aβ-42 was strongly concentrated in both the hippocampus/limbic areas and the thalamus/midbrain regions.[1] Thus, we segregated the data for the Bcl2 family proteins as well as cFLIP into these different areas of the brain. As seen in Figures 1A–D, although there was a significant increase in each of the Bcl2 family proteins as well as cFLIP in the hippocampus/limbic regions of the Aβ-42+ mice versus controls (P range 0.0030 to 0.0010) there was a dramatic increased expression of each of the proteins in the hippocampus/limbic regions in the hyperphosphorylated tau protein mice versus the Aβ-42+ mice ($P < 0.0001$). Also note in the thalamus/midbrain region of the Aβ-42 mice, where there was little abnormal tau protein but substantial Aβ-42, the global increase in the Bcl2 family proteins versus controls was no longer evident, indicating that the correlation of increased Bcl2 family proteins was with hyperphosphorylated tau protein.

Having established that the abnormal human tau protein in the Tau4RΔK mice model of AD strongly correlated with the increased expression of multiple Bcl-2 family proteins, we studied another mouse model of AD with human hyperphosphorylated tau protein. This model was strain #024841 [B6.Cg-Tg(Prnp-MAPT*P301S) PS19Vle/J; abbreviated name P301S Tg] (n = 14). These mice die of their tauopathy between 11 and 15 months, which involves both their brain and spinal cords; hence, limb weakness is a major sign evident by 6 months of age. As will be described in more detail below, at 13 months there was much greater mutant human hyperphosphorylated tau protein in the spinal cords of these mice relative to their brains. Thus, the spinal cords of these mice at 6, 12, and 24 weeks (each set n = 2) and wild type (n = 4) were tested for the prosurvival Bcl2 family proteins Bcl2, BclW, BclXL, Mcl1 as well as cFLIP plus the proturnover Bcl2 family proteins Puma, Noxa, Bad, Bak, and Bax blinded to the clinical information. As evident in

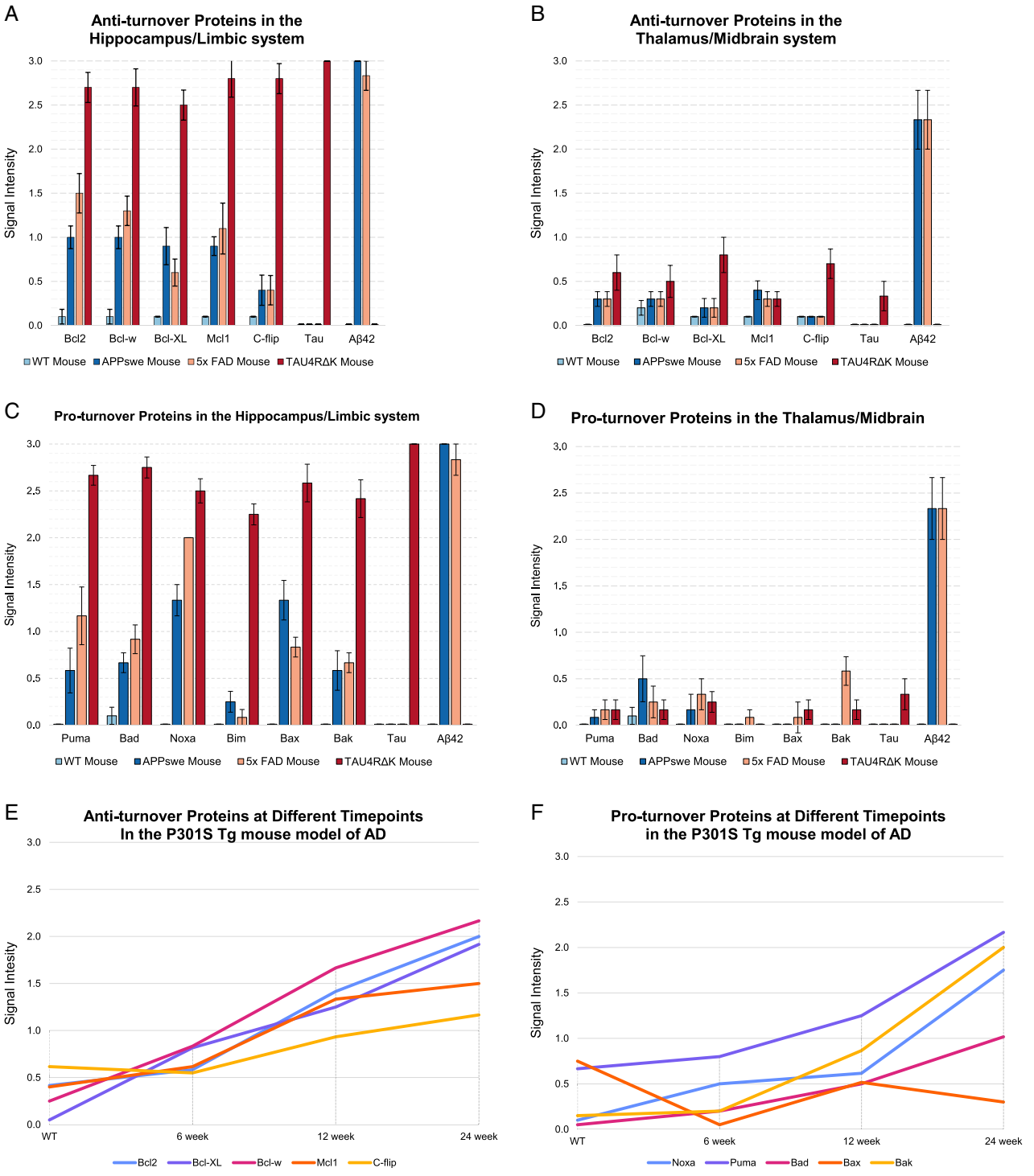

Figure 1E, there was a steady increase in each of the anticell turnover proteins over the 24-week time course relative to the wild type (each *P*-value <0.001 comparing wild type to 24 wk). Figure 1F shows that, whereas Puma, Noxa, and Bax showed equivalent significant increases over the 24 weeks (each *P*-value <0.001); Bak and Bad did not.

qRTPCR was done on the corresponding frozen brain tissues for Bcl2, cFLIP, Mcl1, BclW, BclXL, Noxa, Puma, and Bak; frozen tissues from the spinal cord were not available. Three to 10-fold increases in the mRNA levels were seen every 12 and 24 weeks for Bcl2, cFLIP, and Noxa relative to wild type, with no changes noted for the other mRNAs tested (data not shown), which may

FIGURE 1. Distribution of the bcl2 family proteins in the CNS of different mouse models of AD. (A and B) The distribution of the anti-cell turnover Bcl2 family proteins Bcl2, BclW, BclXL, Mcl1, as well as cFLIP in 4 genotypically different mice: wild type, APP (Aβ-42 only), 5xFAD (Aβ-42 only), and Tau4RΔK (abnormal tau only) with the signal intensity scored as 0 to 3+ blinded to the genotype information. Note in (A) that in the hippocampus/limbic system, where there is abundant hyperphosphorylated tau protein as well as Aβ-42 in the relevant strains, the expression of each protein is markedly higher in the Tau4RΔK strain. B, Shows the area of the thalamus/midbrain in the same mice where the abnormal tau protein is scant but Aβ-42 still abundant. Note the marked decrease in the expression of each protein. Similar data and conclusions were evident for the pro-turnover proteins of the Bcl2 family when comparing the hippocampus/limbic system region (C) and the thalamus/midbrain (D); each data point is mean ± SEM. E and F, Depict the expression of the anti-turnover proteins Bcl2, BclXL, BclW, Mcl1, and cFLIP (E) and the pro-turnover proteins Noxa, Puma, Bad, Bax, and Bak in the spinal cord motor neurons of the humanized hyperphosphorylated tau protein P301S Tg/strain #024841 from 6 to 24 weeks of age versus the wild type mice.

reflect that the abnormal tau burden in the brain neurons was low compared with the spinal cord.

Colocalization of the BCL2 Family Proteins and cFLIP With Hyperphosphorylated Tau Protein in Both Human AD Brain Tissues and in the Tau Mouse Models of AD

We next did multispectral analyses in both the human and murine samples. Multispectral coexpression analyses confirmed that a given neuron with hyperphosphorylated tau protein in human AD also commonly expressed BCL2, MCL1, BCLXL, BCLW, NOXA, PUMA, BAK, BAD, and BAX (Fig. 2); cFLIP was also expressed by these same neurons (data not shown). These multispectral analyses also included the astrocyte marker GFAP, the microglial marker TMEM 119, and the neuron marker pyruvate dehydrogenase plus the heavy chain of neurofilament. For each biomarker, over 97% of the cells in human AD that contained each of these BCL2 family proteins as well as hyperphosphorylated tau protein colocalized with the neuronal markers and $<1\%$ with either the astrocyte or microglial marker (data not shown). Similarly, multispectral analyses showed a strong correlation between the abnormal human tau protein and each of the Bcl2 family proteins in the 2 different mouse models of AD (Fig. 3).

Clinical and Pathology Correlates of Venetoclax Prevention of a Fatal Tauopathy in a Mouse Model of AD With Humanized Mutant Tau Protein

The strong association of BCL2 family proteins, both pro-cell survival, and anti-cell survival, in neurons with hyperphosphorylated tau protein in both the mouse models of AD and in human AD brain samples suggested that these proteins may be playing a role in the pathophysiology of the disease. To test this hypothesis, we treated 5 mice with the drug venetoclax, which pharmacologically is a specific blocker of BCL2. The venetoclax was administered IP 3 times a week (100 μg/ms/dose) beginning at 14 weeks and continuing until sacrifice at age 13 months. Treatment started at 14 weeks because the abnormal tau protein was first evident at 12 weeks, and thus, the model would approximate giving a similar medication to people with early AD. The treated mice were randomly selected, and 3 litter mates of the same sex (all females) and age received only the vehicle in the same schedule. Clinically, 3 differences were noted between the untreated and treated mice over the treatment period: (1) the untreated mice showed much less investigational behavior in their cages compared with the treated mice that were much more mobile and curious [Supplemental Video 1, Supplemental Digital Content 1, http://links.lww.com/AIMM/A513 (untreated) and video 2, Supplemental Digital Content 2, http://links.lww.com/AIMM/A514 (treated)]. To quantify the latter, 5-minute videos of the untreated versus treated mice side-by-side at 12 months were taken, with the number of times a given mouse stopped at least for 1 second, raised its head, and sniffed the air scored. The untreated mice showed 9.0 (SD = 2.7) such behaviors/mouse versus 19.6 (SD = 3.5) for the treated mice ($P < 0.0001$). (2) The untreated mice, but not the treated mice, showed muscle atrophy around the girdle and shoulder regions with a concomitant waddling gait. The rotarod test was done at 8 months and did show differences. A successful test was scored as the mice maintained their position for 291 to 300 seconds, and it occurred in 1/9 tests (11%) for the untreated mice and 7/15 tests (47%) for the treated mice. A failed test was scored as the mice were not able to hold their position for at least 180 seconds, and it occurred in 4/9 tests (44%) for the untreated mice and 3/15 tests (20%) for the treated mice. The spinal cord and concomitant skeletal muscle damage were overt at autopsy as measured by a significant reduction in myocyte diameter of the biceps femoris [28.2 μm (SEM = 3.2) untreated versus 43.7 (SEM = 3.2) treated, $P = 0.005$] and a reduction in the capillary density of 57.3% when compared with treated mice ($P < 0.0001$). (3) The untreated mice looked older, and each showed barbering (focal loss of hair), especially around the head and belly, and dull hair, whereas the treated mice each had intact, shiny fur with a white patch at the site of the venetoclax injection. On histologic exam of the skin at autopsy, the untreated mice showed focal terminal hair follicle atrophy with concomitant prominence of the sebaceous glands, whereas the treated mice had normal-sized hair follicles.

Changes in the Tau Burden, Neuroinflammation, and Neurodegeneration in A Mouse Model of AD With Humanized Mutant Tau Protein After Treatment With Venetoclax

Having established that the untreated humanized tau mice P301S Tg/strain #024841 showed the expected

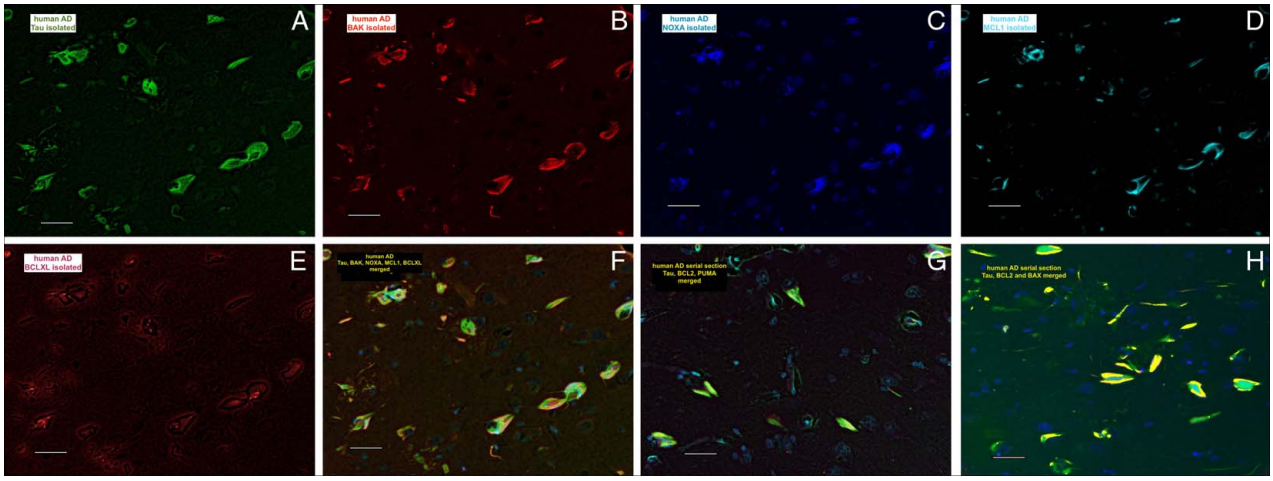

FIGURE 2. Multispectral documentation that neurons with hyperphosphorylated tau protein in human AD express many members of the BCL2 family of proteins. A–E, The same section from the hippocampus of a person who died of AD tested for the abnormal tau protein (A; fluorescent green), BAK (B, fluorescent red), NOXA (C, fluorescent blue), MCL1 (D, fluorescent turquoise) and BCLXL (E, fluorescent brown). Note that each target shows the same pattern and when the channels are merged, extensive co-localization of the 5 targets is evident as fluorescent yellow (F). Serial section coexpression analyses demonstrated that the same set of neurons with NFTs also expressed BCL2 and PUMA (G) as well as BCL2 and its direct target BAX (H) (each scale bar at 50 μm). full color online

marked neuronal degeneration with concomitant limb skeletal muscle atrophy at age 13 months, as well as behavioral changes and that these signs of their tauopathy were markedly reversed by the drug venetoclax, we next sought to correlate these clinical/pathological data with the levels of hyperphosphorylated tau protein by immunohistochemistry as well as the modified Bielchowsky stain which decorates NFTs, the Bcl2 family proteins, and the markers of neuroinflammation at autopsy. Before doing these analyses, we used the genotype analysis recommended by Jackson Laboratory to confirm the presence of the 450 bp sequence in each mouse (treated and untreated) that corresponds to the human abnormal tau gene (data not shown).

The detection of hyperphosphorylated tau protein and NFTs with the Bielchowsky stain was each evident at 12 weeks when 14.3% of the neurons had the abnormal tau protein. The reduction of the number of target neurons positive for hyperphosphorylated tau protein after venetoclax treatment in the brain and spinal cord neurons was 94.5% as determined by immunohistochemistry and 98.1% as documented with the modified Bielchowsky stain in the venetoclax treated mice versus the untreated mice at 13 months (Figs. 4A–D). The following venetoclax-related changes were also recorded between the spinal cords of the mice untreated versus the treated mice at autopsy at 13 months: (1) the astrocyte density in the untreated mice was 317.4/100× field (SEM = 9.5) as compared with 103.9/100× (SEM = 14.4) after venetoclax (reduction significant at $P < 0.0001$); (2) the neuronal count in the untreated mice spinal cord was 13.3/200× (SEM = 4.5) versus 69.4/200× (SEM = 6.8) for the treated mice (increase significantly at $P < 0.0001$) (Fig. 4E–I; (3) the density of the 4 cytokines tested (TNFα, IL6, IL-1β, and CxXL10) ranged from 61.2 to 90.5/200× (SEM range was 5.5 to 8.1) for the untreated mice versus 4.1 to 12.3/200× (SEM range was 2.3 to 3.3) for the treated mice spinal cord (reduction significant at $P < 0.0001$). As previously reported,[1] co-expression analyses documented that these 4 cytokines were mostly expressed (over 90%) by CD11b+ microglia. Histologic review of the heart, lungs, liver, lymph node, and kidneys showed no pathologic changes in either the treated or untreated mice (data not shown). Since venetoclax can cause neutropenia in humans, the bone marrow of the mice was assessed and there was no difference in cell density or lineage maturation in the untreated versus treated mice (data not shown).

Changes in the Expression of the Bcl2 Family of Proteins in a Mouse Model of AD With Humanized Mutant Tau Protein After Treatment With Venetoclax

With regards to the Bcl2 family proteins in the untreated versus treated spinal cord motor neurons, there was a major shift when compared with the levels of these proteins at 24 weeks which was used as the reference point. Specifically, several of the antiturnover proteins (BclW, Mcl1, as well as cFLIP) each were reduced in the neurons of the treated mice spinal cord as compared with the levels in the untreated mice at 24 weeks by 51.2% to 98.3%; there was no such change in the levels of Bcl2 or BclXL. However, the levels of the proturnover proteins (Bad, Noxa, Puma, Bak, Bax) did not decrease in the neurons in the spinal cord after treatment and remained at the levels of the untreated mice at 24 weeks (data not shown). Thus, overall, the ratio of antisurvival versus prosurvival proteins in the neurons of the spinal cord of the treated mice favored cell turnover.

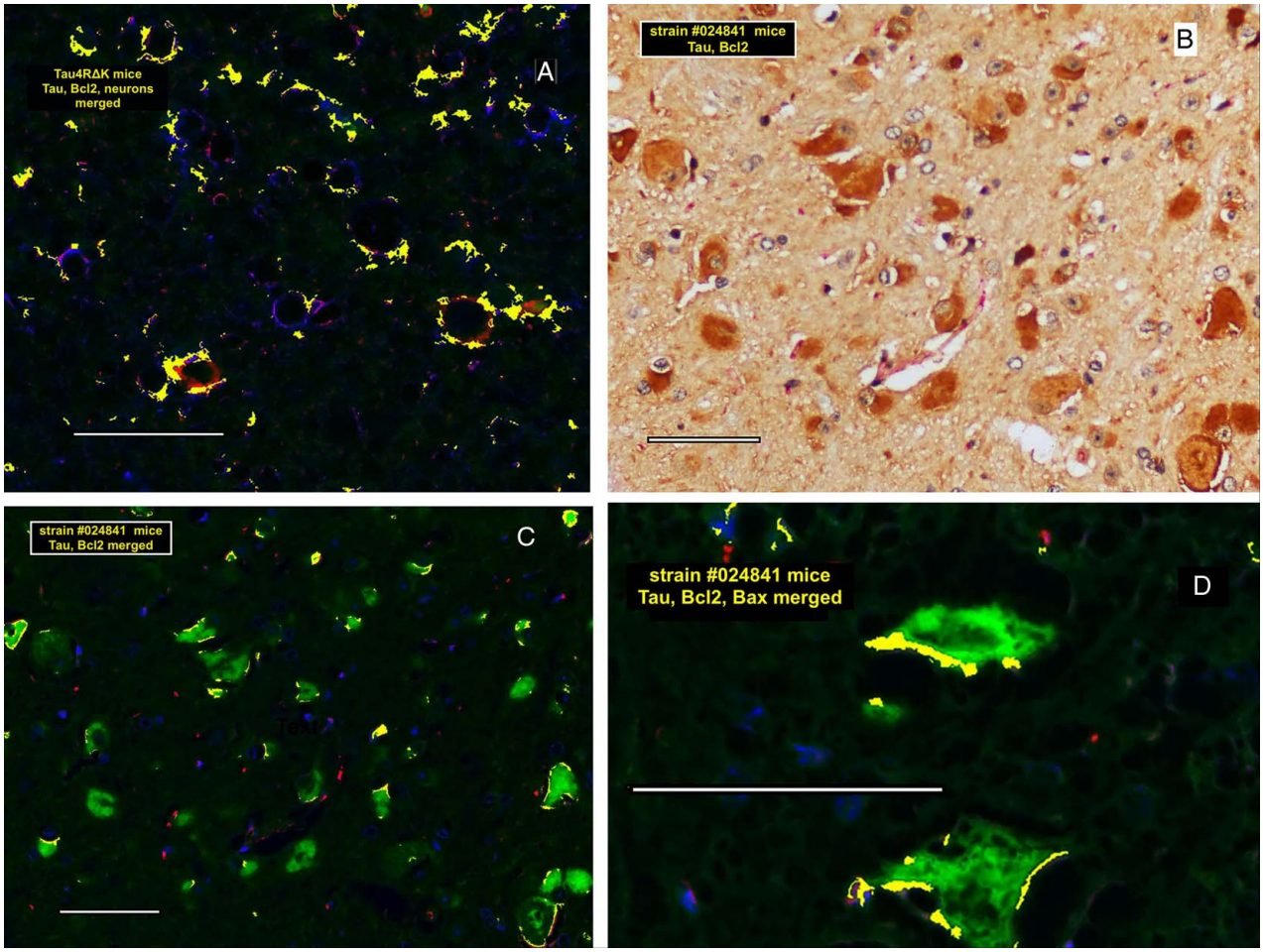

FIGURE 3. Multispectral documentation that neurons with hyperphosphorylated tau protein in the mouse models of AD express many members of the Bcl2 family of proteins. Coexpression analyses of the mouse AD model Tau4RΔK demonstrated that the abnormal human tau protein (fluorescent green), Bcl2 (fluorescent red), and the neuronal marker pyruvate dehydrogenase/neurofilament (fluorescent blue) showed extensive co-localization in the hippocampus (colocalization seen as fluorescent yellow, A). In the untreated mouse AD mouse P301S Tg/strain # 024841 there is coexpression of the abnormal tau protein (fluorescent green) and Bcl2 (fluorescent red with colocalization as fluorescent yellow; C), (B) shows the corresponding RGB image in spinal cord neurons. In the same mouse strain, there is strong co-localization of tau (fluorescent green), bcl2 (fluorescent red), and its direct target BAX (fluorescent turquoise, with coexpression as fluorescent yellow) in the spinal cord neurons (D) (each scale bar at 60 μm; in each image blue is the counterstain hematoxylin). full color online

Evidence of Increased Neuronal Turnover in a Mouse Model of AD With Humanized Mutant Tau Protein After Treatment With Venetoclax

Neuronal turnover can be inferred by the presence of several proteins including nestin and doublecortin.[30,31] These proteins are found in neural progenitor cells and persist for several weeks after these cells differentiate into mature neurons.[30,31] Several groups have shown these 2 markers of neuronal turnover are abundant in the hippocampus of normal adults and are decreased in this area in AD.[30,31] We thus interrogated the brain and spinal cords of wild-type mice and compared these data to the same specimens of the humanized tau mice P301S Tg/strain #024841 untreated at 6 weeks, 12 weeks, 24 weeks, and at 13 months as well as the venetoclax treated mice at 13 months. Doublecortin expression in the hippocampus was strong at 6 weeks and steadily decreased through 24 weeks. In the spinal cord neurons, doublecortin expression maximized at 12 weeks and was not present at 24 weeks. Doublecortin expression was not evident in the neurons of the spinal cord of the untreated mice at 13 months and was 53.2% greater in these neurons of the venetoclax-treated mice at this time point when compared with the maximum expression in the untreated mice at 12 weeks ($P < 0.001$).

Similar data was evident for nestin. Nestin expression in the spinal cord neurons maximized in the untreated mice at 12 weeks and was not evident in these cells in the untreated mice sacrificed at 13 months. Nestin expression in the spinal cord neurons of the treated mice was 61.2% greater in the spinal cord neurons when compared with the maximum value of the untreated mice at 12

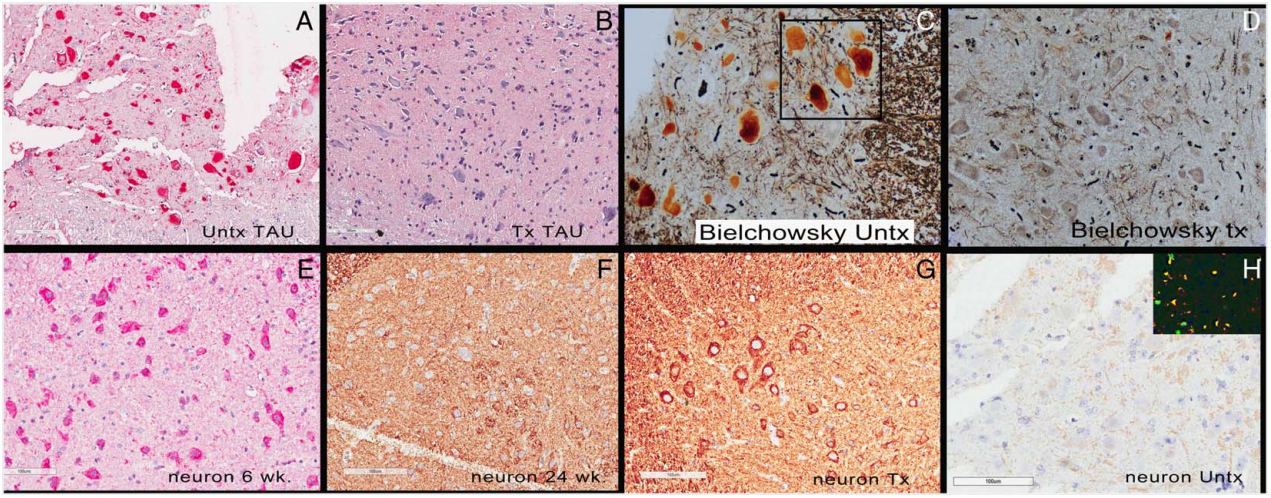

FIGURE 4. Changes in the density of hyperphosphorylated tau protein as well as neuronal degeneration in a mouse model of AD after treatment with venetoclax. A, The large number of spinal cord neurons with a strong signal for the human hyperphosphorylated tau protein in the untreated mice at autopsy at 13 months of age. B, The near total elimination of the abnormal tau protein in the same area of the spinal cord in the treated mice. (C) The many NFTs (rectangle) in the untreated spinal cord, as seen with the modified Bielchowsky stain, were shown, whereas (D) shows their near absence after venetoclax treatment. The high density of spinal cord neurons, as seen by immunohistochemistry for neuronal markers, was evident in both the wild type (not shown) and at 6 weeks in the P301S Tg stain (E, fast red stain). Note the marked reduction in the neuronal signal at 24 weeks in this same strain (F, DAB stain). In comparison, the neurons on the spinal cord after venetoclax treatment showed strong signals (G, DAB stain), whereas the signal was nearly eliminated in the same spinal cord sections in the untreated mice (H, DAB stain); the insert of (H) shows co-expression of hyperphosphorylated tau protein and a neuronal marker in a case of Braak 6 human AD indicative of neurons that were not markedly degenerated. full color online

weeks. Thus, the doublecortin and nestin data each suggested that neuronal turnover maximized at about 12 weeks in the untreated mice, which was also the time period where hyperphosphorylated tau protein was first evident (Fig. 4), then decreased to undetectable levels in the untreated mice at 13 months and was markedly increased at 13 months in the mice treated with venetoclax.

Evidence of a Strong Correlation With the BCL2 Family of Proteins With Hyperphosphorylated Tau Protein in Human CBD

Next, we analyzed 20 tissues from 4 cases of CBD in humans versus 10 aged-match controls using in situ-based serial section and coexpression analyses to determine if the abnormal tau protein in this disease is correlated with the BCL2 family proteins. The in situ-based analyses did show strong coexpression between hyperphosphorylated tau protein in CBD with BCL2, BCLW, BCLXL, MCL1, BAD, BIM, and BAX as noted in this study with both human AD and a mouse model of the disease (data not shown).

DISCUSSION

These data suggest that there may be a heretofore unrecognized strong correlation of BCL2 family proteins as well as cFLIP with the hyperphosphorylated tau protein that is the hallmark of AD. The abnormal tau protein in human AD strongly coexpressed with many members of the BCL2 family, including both procell and anticell survival proteins. This same association was evident in 2 different mouse models of AD that contain the human abnormal tau protein but not in mouse models with Aβ-42. Importantly, by blocking only Bcl2 in the P301S Tg mouse model of AD that contains a mutant form of the human tau protein, the tau burden was reduced by >90% as documented by 2 different assays with a concomitant marked improvement in clinical outcome and reduction in neuroinflammation and neuronal degeneration. Given the strong association of BCL2 family proteins in cell turnover, well documented in leukocytes and squamous cells of the epidermis,[22–24] and the fact that neuronal turnover has been documented in the hippocampus which is a key area for many neurodegenerative diseases including AD,[25,26] we speculate that the common denominator may be abrogated neuronal turnover. It has been documented that in the adult human hippocampus there is an annual turnover of 1.75% of the neurons within the renewing fraction[25] that, over the course of 30 years, would involve the majority of hippocampal neurons which is consistent with the slow preclinical accumulation of the hyperphosphorylated tau protein.[27,28,32] It is important to stress that this study was designed to determine if one could prevent the tau burden by treating the mice soon after the pathologic appearance of hyperphosphorylated tau protein and before clinical symptoms. It was not designed to address the obvious next question: will such drugs work when the tau burden is clinically apparent? This awaits further research. It is also important to stress that this hypothesis that BCL2 may be acting to prevent neuronal turnover in AD is likely incomplete. That is, BCL2 may have other effects

on neuronal function completely apart from neuronal turnover that may be a factor in understanding our data. BCL2 may possibly be affecting neuroinflammation, which is a central component of neurodegenerative diseases, including AD. Nonetheless, the fact that blocking Bcl2 was able to prevent the formation of the mutant form of human tau, which is associated with a more aggressive disease than the much more common late-onset AD, and that the neurons with the abnormal tau typically are not degenerated in the human disease,[27–29,32,33] suggests that treatment of the more common tauopathy in AD may be successful even after a clinically apparent tau burden which clearly awaits further study. Our data with another tauopathy, CBD, suggested that this abnormal BCL2 family protein expression pattern in neurons may be a generalized feature of tauopathies. A limitation of this current study is that we did not actually measure neuronal turnover, although doublecourtin and nestin are well-recognized biomarkers of neuronal turnover. Additional studies that typically use radioisotopes[25,26] will be needed to map neuronal turnover as it relates to drugs such as venetoclax.

Other investigators have reduced the tau burden in mouse models of AD using either anti-inflammatories such as tacrolimus or drugs that remove senescent cells where the authors theorized that either astrocytes, microglia, oligodendroglial precursors, and/or neurons are senescent.[16,19,20] These drugs (dasantinib, navitoclax) block multiple proteins, including several tyrosine kinase inhibitors, BCL2, BCLXL, and BCLW and, thus, are associated with many side effects, including bone marrow suppression and bleeding. This study demonstrated that blocking only one protein, BCL2, was capable of reducing the tau burden in a more dramatic manner than previously reported. Neurons with hyperphosphorylated tau protein in human AD rarely show degenerative changes and, thus, we hypothesize, are not end-stage senescent but rather display a dysfunctional cell metabolism that can be altered by drugs such as venetoclax and, thus, could be restored as functional neurons. Although there was hyperphosphorylated tau protein evident in the brains of these mice, over 90% of the neurons with the abnormal tau protein were in the spinal cord which made this area the focus of our study. Dysregulation of the BCL2 family of proteins has been described in leukemias in tumor cells and has been related to normal brain development in the rat but its role in the human brain is poorly characterized.[34,35] Whatever the mechanism, the ability to use highly targeted small molecule drugs that readily cross the blood-brain barrier[36] unlike monoclonal antibodies, and have few side effects may have important advantages in treating AD given the age and comorbidities of the 55 million people worldwide who have the clinically evident disease.

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