

Research Report

The Compound ATH434 Prevents Alpha-Synuclein Toxicity in a Murine Model of Multiple System Atrophy

David I. Finkelstein^{a,*}, Jay J. Shukla^a, Robert A. Cherny^a, Jessica L. Billings, Eiman Saleh^a, Nadia Stefanova^b, Kevin J. Barnham^a and Paul A. Adlard^a

^a*Florey Institute of Neuroscience and Mental Health and the University of Melbourne, Parkville, Victoria, Australia*

^b*Laboratory for Translational Neurodegeneration Research, Division of Neurobiology, Department of Neurology, Medical University of Innsbruck, Innsbruck, Austria*

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Abstract.

Background: An elevation in iron levels, together with an accumulation of α -synuclein within the oligodendrocytes, are features of the rare atypical parkinsonian disorder, Multiple System Atrophy (MSA). We have previously tested the novel compound ATH434 (formally called PBT434) in preclinical models of Parkinson's disease and shown that it is brain-penetrant, reduces iron accumulation and iron-mediated redox activity, provides neuroprotection, inhibits alpha synuclein aggregation and lowers the tissue levels of alpha synuclein. The compound was also well-tolerated in a first-in-human oral dosing study in healthy and older volunteers with a favorable, dose-dependent pharmacokinetic profile.

Objective: To evaluate the efficacy of ATH434 in a mouse MSA model.

Methods: The PLP- α -syn transgenic mouse overexpresses α -synuclein, demonstrates oligodendroglial pathology, and manifests motor and non-motor aspects of MSA. Animals were provided ATH434 (3, 10, or 30 mg/kg/day spiked into their food) or control food for 4 months starting at 12 months of age and were culled at 16 months. Western blot was used to assess oligomeric and urea soluble α -synuclein levels in brain homogenates, whilst stereology was used to quantitate the number of nigral neurons and glial cell inclusions (GCIs) present in the substantia nigra pars compacta.

Results: ATH434 reduced oligomeric and urea soluble α -synuclein aggregation, reduced the number of GCIs, and preserved SNpc neurons. *In vitro* experiments suggest that ATH434 prevents the formation of toxic oligomeric "species of synuclein".

Conclusion: ATH434 is a promising small molecule drug candidate that has potential to move forward to trial for treating MSA.

Keywords: Synuclein, iron, parkinsonism, neuroprotection, drug development

INTRODUCTION

Synucleinopathies are a group of conditions characterized by the pathological accumulation of intracellular deposits of α -synuclein (α -syn) [1]. This includes Parkinson's disease (PD), Multiple System Atrophy (MSA), Dementia with Lewy bodies (DLB),

*Correspondence to: Professor David I. Finkelstein, Florey Institute of Neuroscience and Mental Health, 30 Royal Parade, Parkville, Victoria 3010, Australia. Tel.: +61 3 9035 6680; E-mail: david.finkelstein@florey.edu.au.

and other rarer conditions such as pure autonomic failure [2]. Each of these conditions is distinguished by the anatomical location of the α -syn deposits, which can be within CNS neurons, glial cells or in peripheral nerves. Furthermore, recent evidence has suggested that different “strains” or “types” of α -syn may be involved in different diseases [3].

It has been shown that iron is elevated in synucleinopathies such as PD, MSA, and DLB, and the unique iron profile, more recently magnetic resonance imaging (MRI) is being developed as an aid in distinguishing the different conditions [4–8]. The significance of a localized accumulation of iron has been the subject of much speculation, but iron could initiate and/or potentiate oxidative stress [9, 10], influence the aggregation state and type of α -syn [11–14], and also regulate the amount of α -syn, as the mRNA contains an untranslated iron response element that is involved in the post-transcriptional regulation of the protein level [15, 16].

MSA is a rare, fatal neurodegenerative brain disorder that presents with predominant autonomic dysfunction, parkinsonism, cerebellar impairments, or a variable combination of these manifestations. MSA patients are generally not responsive to typical PD therapies, and there are no disease modifying treatments for this condition. As such, MSA represents an unmet medical need. Pathologically, MSA is characterized by glial cytoplasmic inclusions (GCIs), structures that are composed of aggregated, urea soluble α -syn that are found in the areas of degeneration of affected brain regions including the substantia nigra pars compacta, putamen, pontine nuclei, cerebellum, and spinal cord. Evidence suggests that the deposition of α -syn and the toxic effects of soluble α -syn aggregates in oligodendrocytes are key events in the hypothesized spreading of pathology from the oligodendrocytes to result in neuronal cell death. MSA is also associated with elevated iron concentrations in the pons and putamen, and this may be a critical factor in the pathogenesis of MSA given the potential role of iron in the aggregation of α -syn in GCIs [6].

ATH434 (formally called PBT434), which has successfully completed a Phase 1 study [17], is a brain-penetrant, small molecule inhibitor of α -syn aggregation. In several rodent models of PD, ATH434 reduced iron accumulation, α -syn aggregation, CSF α -syn levels, and oxidative stress, preserved neurons in the SN and improved motor function [18]. Importantly, one of the models in which ATH434 showed neuroprotection was the hA53T mutant α -syn transgenic mouse, which provided a rationale for

exploring the effects of the compound in other synucleinopathies. The PLP- α -syn transgenic mouse is an animal model of MSA in as much as it overexpresses human α -syn in the oligodendrocytes (the synuclein transgene is driven by the myelin proteolipid protein (PLP) promoter), develops GCI pathology and manifests motor and non-motor aspects characteristic of the disease [19–21]. The aim of this study was to determine whether the effects of ATH434 would translate into therapeutic benefits in a genetic model of MSA.

METHODS

Mice

The MSA transgenic mice used in this study were derived from a colony initially established from breeding animals that were a generous gift from the laboratory of Dr Nadia Stefanova, Clinical Department of Neurology, Innsbruck Medical University, Innsbruck, Austria. These mice express human wild-type α -syn within the oligodendrocytes, driven by the proteolipid protein (PLP) promoter, they show that α -syn is localized only within cells immunopositive for cyclic nucleotide phosphodiesterase oligodendroglia [19]. The phenotype of these mice and pathology has been extensively characterized [20, 21]. The PLP- α -syn mice are maintained on a C57BL/6 background (Animal Resources Centre, Western Australia) and littermate wild types were bred from heterozygous mating at the Florey Neuroscience Institute animal facility for more than 10 generations. Mice were housed in Techniplast brand individually ventilated cages. Mice had *ad libitum* access to food and water. All procedures involving mice conformed to the Australian National Health and Medical Research Council Code of Practice for the care and use of animals for scientific purposes and were approved by the Florey Institute Animal Ethics Committee. All experiments were designed to minimize the number of animals used, whilst still providing sufficiently powered group sizes to observe effects. Both male (M) and female (F) mice were used in this study. In the 12 MO treated group and control groups – 6 F and 3 M were used. In the 16 MO group; Control 9 F 6 M; 3 mg/kg 10 F 5 M; 10 mg/kg 9 F 6 M and 30 mg/kg 9 F 6 M.

Treatment

The mice were treated with either standard, non-irradiated rodent food or the same food but with

the addition of ATH434 (provided by Alterity Therapeutics). The compound was incorporated into the food by Specialty Feeds (Glen Forrest, WA) at nominal doses of 0.025 g/kg food (equating to ~3 mg/kg/day of ATH434 in the mice), 0.083 g/kg food (~10 mg/kg/day of ATH434 in the mice), and 0.25 g/kg food (~30 mg/kg/day of ATH434 in the mice). The mice and food were weighed weekly throughout the four months of the experiment. The group that was culled at 12 months of age were treated from 8 months of age; the group culled at 16 months of age were treated from 12 months of age. No mice in any of the treatment groups exhibited any adverse reaction to ATH434. One mouse in the control group was culled for human reasons.

Pole test of motor performance

The pole test was used to assess the speed of a complex motor skill and coordination. In the week before culling, the mice were placed (nose up) on top of a pole (wooden rod; 300 mm long and 5 mm in diameter; attached to a stable weighted base) and the time required for the mouse to rotate 180 degrees (nose down), and to return to the home cage ("Time Complete") were recorded. The tests were performed on 2 different days (habituation day and test day). Each mouse attempted 5 trials and the fastest time was analyzed [18].

Transcardial perfusion

Following the treatments and the pole motor test was performed, the mice were anaesthetized with 80 mg/kg sodium pentobarbital (Lethabarb, Clifford Hallam, Australia) diluted in 0.9% saline. Mice were perfused and exsanguinated with 50 ml 0.1 M phosphate-buffered saline (PBS). The left-hand side of the brains were post-fixed in 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA) in PBS at 4°C overnight and then immersed in 30% sucrose in 0.1 M PBS at 4°C in preparation for histology. The right-hand side was micro-dissected and stored at -80°C for biochemical analysis and Inductively Coupled Plasma Mass Spectrometry (ICPMS).

Tissue preparation

Substantia nigra samples (containing compacta and reticulata) were homogenized (sonication; Branson, CT, USA) according to their wet weight at 1 : 5 dilution in Dulbecco's calcium- and magnesium-free

PBS (Invitrogen, CA, USA) with a protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor: phosStop (Sigma-Aldrich). Protein concentrations were determined with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, MA, USA). One of the duplicate samples was lyophilized for ICPMS analysis, the other frozen for α -syn analysis.

Iron content analysis by inductively coupled plasma mass spectrometry

Samples were weighed, lyophilized, and digested overnight at room temperature in nitric acid (HNO₃) (65% Suprapur, Merck). The samples were further digested by heating at 90°C for 20 min. An equal volume of hydrogen peroxide (H₂O₂) (30% Aristar, BDH) was added to each sample and left for ~30 min and then heated for 15 min at 70°C. Samples were then further diluted with 1% HNO₃. Metal levels were measured using an Agilent 7700 series ICPMS instrument under routine multi-element operating conditions using a helium reaction gas cell. Results are expressed as microgram of metal per gram of protein weight (μ g/g or ng/ μ g).

Western blot

The tissues were homogenized according to their wet weight at 1 : 5 dilution in Dulbecco's calcium- and magnesium-free PBS (Invitrogen, #10010049) with a protease inhibitor (Roche, #05056489001) and phosphatase inhibitor: phosStop (Sigma-Aldrich, 4906837001) using a sonicator (Branson, stepped micro-tip). Protein concentrations were determined with a Protein Assay kit (Pierce, #23225). The fractionation procedure commenced with tissue homogenates of a set concentration (125 μ g in 500 μ l PBS). These were spun using an ultracentrifuge (Beckman Coulter, California, USA) at 100,000 \times g for 30 min at 4°C [22]. The supernatant (S1) comprised the soluble fraction; 10 μ g of protein was loaded on the S1 gels. The remaining pellet was washed 3 times in a PBS buffer and resuspended in 5% SDS (Sigma-Aldrich). The resuspended pellets were homogenized again using sonication and centrifuged at 100,000 \times g, 30 min at 4°C. The resulting supernatant (S2) was stored and not used. The remaining pellet was washed with 5% SDS/PBS solution and resuspended in 10 μ l 8% SDS/8M Urea (Sigma-Aldrich) in PBS to solubilize the fraction not soluble in SDS. This solution, soluble fraction 3 (S3), was

re-homogenized and incubated at room temperature for 2 h. 5 μ l of the S3 fractions were reserved for the S3 gels. 10 μ g of S1 and 5 μ l S3 fractions were loaded into 4–12% Bis-Tris gels (Invitrogen) with 1X NuPAGE™ MES SDS Running Buffer (Invitrogen). The gel was run at 120V for approximately 90 min and transferred onto PVDF membrane (Invitrogen) using a semi-dry system (Iblot2, Invitrogen).

The membrane for the S1 fraction was stained with 0.1% Ponceau S Solution (Sigma-Aldrich) for at least 5 min to measure total protein levels (using digitized images, LAS-4000 FujiFilm). The stain was removed using Tris-Buffered Saline (TBST pH 8.0) and three washes with MilliQ water. Membranes were then blocked with 5% skim milk in TBST (pH 8.0) for 1 h at room temperature. After a rinse with TBST (pH 8.0), the membranes were incubated with primary antibody (1 : 10,000 LB509 Abcam, Cambridge, UK) followed by rocking overnight at 4°C. The membranes were then rinsed 3 times using TBST and then incubated in secondary antibody (1 : 10,000, Rabbit-anti-mouse-HRP; Dako, Glostrup, Denmark), rocking for 1 hour at room temperature. Secondary antibody was then washed off and the membrane incubated with chemiluminescence reagents (1 : 1 ratio, ECL, GE Healthcare, Chicago, IL, USA) for at least 1 minute and imaged and analyzed using a LAS-4000 FujiFilm imager and Multi Gauge Software (GE Healthcare).

Immunohistochemistry

Brains were sectioned with a Leica Biosystem Cryostat at -24°C . The left-hand hemisphere was sectioned at 30 μm through the SN and Pons, the right-hand hemisphere was used for the biochemistry. The sections were mounted to gelatinated microscope slides (0.5% gelatin in dH_2O) (Grale Scientific cat# S21102A). Sections were stored at -80°C until staining was performed. The slides were removed from -80°C then incubated in a blocking solution (containing 10% normal goat serum in 0.1 M PBS pH 7.4 for 30 min). Slides were then incubated in primary antibody overnight at room temperature (GCI: α -syn: MJFR1, Sapphire bioscience, AB138501 at 1 : 1000). The slides were then washed with PBS three times and incubated in secondary antibody (Goat Anti-Rabbit IgG Antibody, biotin-SP conjugate) for 3 h at room temperature. Slides were then washed with PBS three times and incubated in avidin peroxidase for 1 h at room temperature. The slides were washed again and incubated in 0.05% nickel sulphate

and 0.05% cobalt chloride for 20 min and further developed by adding 0.001% H_2O_2 for a maximum of 5 min. Slides were then washed, counterstained with 2% Neutral Red solution for 2 min, dehydrated with increasing concentrations of ethanol (50%, 70%, 90%, and 100%), followed by xylene and mounted with Ultramount No.4 mounting medium (Trajan Scientific, Cat #II 065C). Neutral red is a versatile stain that is used to distinguish neuronal and glial cell membranes, lysosomes, and nuclei.

Stereology of substantia nigra pars compacta

The total number of neurons in the SN was estimated using an optical fractionator sampling design as published previously [23–25] using StereoInvestigator version 11 software (MBF Bioscience, Williston, VT, USA). The regions of interest were traced, and counts were made at regular predetermined grid intervals of x:140 μm and y:140 μm with an unbiased counting frame of 45 $\mu\text{m} \times 45 \mu\text{m}$ and a disector height of 12 μm . Systematic samples of the area occupied by the Neutral Red stained nuclei were made from a random starting point. The average thickness of every section counted was also measured. All stereological analyses were undertaken in a blinded manner, with the data decoded after all the samples had been processed.

Glial cell inclusions stereology

Stereological estimates of GCIs were performed using a fractionator sampling design with the StereoInvestigator version 11 Software (MBF Bioscience, USA). Grid intervals of x: 100 μm and y: 100 μm with a counting frame of 50 \times 50 μm were systemically and randomly positioned by the software using a 63 \times objective lens (NA 1.63). All stereological analyses were undertaken in a blinded manner, with the data decoded after all of the samples had been processed.

In vitro α -syn assay

To test if ATH434 affected the rate of α -syn disaggregation, *in vitro* experiments were performed. Purified and lyophilized recombinant human α -syn (Monash Protein Production Unit, Monash University, Australia) was reconstituted with Tris Buffer Saline (TBS, pH 7.4). Pooled aliquots were spun

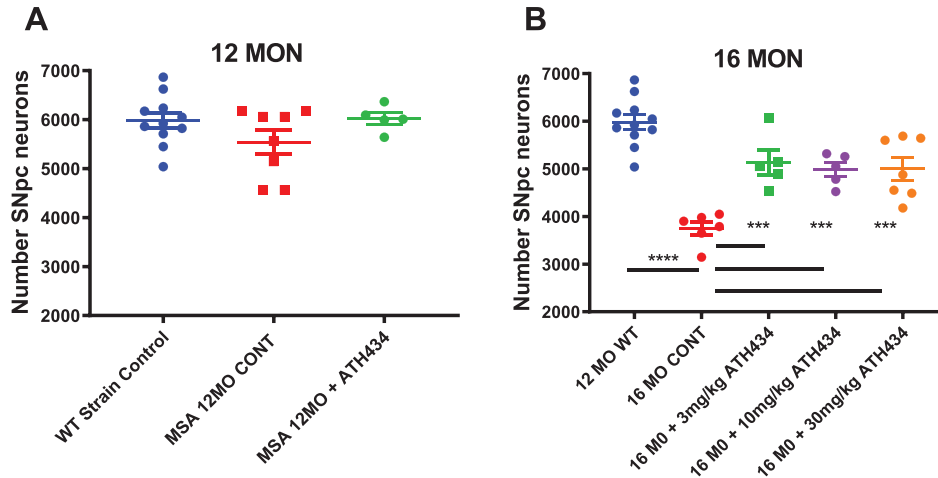


Fig. 1. Total Substantia Nigra pars compacta neuron counts in 12-month (Left) and 16-month (Right) PLP- α -syn mice after approximately 4 months of ATH434 or Control diet (Mean \pm SEM). A) No loss of neurons is observed in the 12-month group. B) At 16 months of age, there was a 37% loss in the number nigral neurons compared to the 12-month-old strain control. Treatment from the age of 12 months with ATH434 reduced the size of this loss to \sim 16% at 16 months. One way ANOVA Dunnett's *Post hoc* test comparing to the 16-month untreated animals *** p < 0.001, **** p < 0.0001. (12 MO WT vs. 16 MO CONT p \leq 0.0001; CONT vs. 3 mg/kg p = 0.0003; CONT vs. 10 mg/kg p = 0.0012; CONT vs. 30 mg/kg p = 0.0004)

at 100,000 \times g for 30 min at 4°C to remove preformed aggregates/seeds. The supernatant containing the monomeric form was collected and used in the assay. The protein concentration was determined using the BCA method. Iron nitrate was weighed and dissolved in TBS solution. ATH434 was dissolved in 100% DMSO, then diluted to a stock solution using MilliQ water. To each tube, TBS, iron, ATH434/vehicle and then α -syn was added in sequence with equal concentrations. The final concentration of α -syn, Fe, and ATH434 was 140.0 μ M. Triplicates of the samples were assayed in the presence of ThT in a Perkin-Elmer Enspire multi-mode plate reader. ThT fluorescence intensity was measured over time at wavelengths of 450 nm emission and 485 nm excitation. The relative fluorescent units (RFU) values were normalized to TBS + ThT control wells and were plotted over time. The method has been previously published [11, 18].

Data analysis

The data were plotted as Standard Error of the Mean (SEM). Data were analyzed with either a one-way ANOVA followed by Dunnett's and Tukey multiple comparisons test, a two-way ANOVA, or unpaired *t*-tests as appropriate. The software used was GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

Neuroprotective effects of ATH434

At 12 months of age the MSA mice did not show a significant decrease in SNpc neuron number compared to littermate controls, and treatment with ATH434 from 8 months of age did not alter nigral neuronal number (Fig. 1). As such, the rest of the studies focused on older mice. In contrast, the 16-month-old MSA mice had a 37% decrease in the number of nigral neurons compared to controls (thus providing the rationale for our selection of this timeframe as our primary analysis endpoint), and four months of treatment with ATH434 resulted in a significant protection against α -syn mediated nigral cell death (p < 0.001, One way ANOVA with Dunnett's *Post hoc* test comparing to the 16-month untreated animals). Each of the doses of ATH434 trialed were equally effective suggesting a threshold effect below 3 mg/kg/day.

Motor performance

To investigate whether ATH434 altered nigral-mediated complex movement, mice were assessed for their performance on the pole test (Fig. 2A). The time to complete the pole test behavioral task was significantly improved (28% faster) in the MSA mice receiving the ATH434 food, as compared to those provided the control food (ANOVA, F = 3.01, p = 0.04). No dose response relationship was observed.

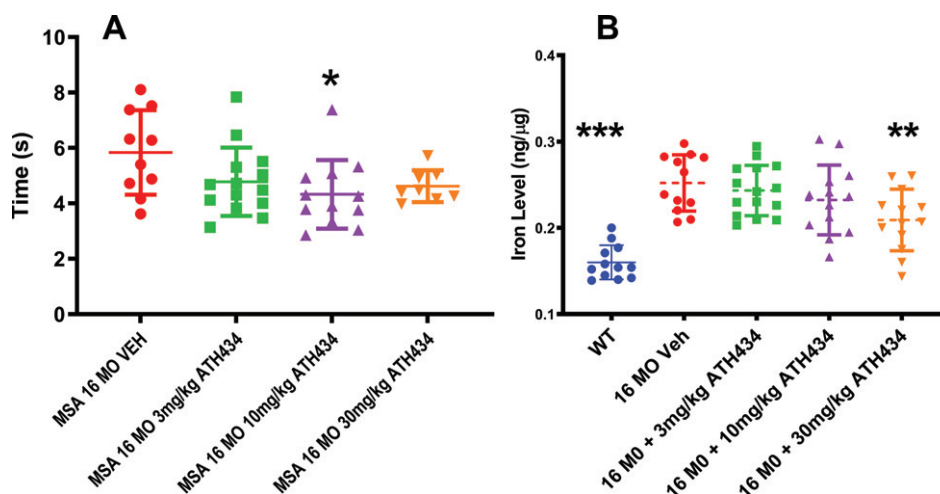


Fig. 2. A) Time to complete the pole test motor task was improved by treatment with ATH434 in the PLP- α -syn mice after approximately 4 months of ATH434 compared to those eating Control diet (Mean \pm SEM, Ordinary ANOVA, $F = 3.01$, $p = 0.04$). One way ANOVA Dunnett's *Post hoc* test comparing to the 16-month untreated animals indicated that the 10 mg/kg reached significance $*p = 0.02$. B) Substantia nigra iron was elevated in the 16-month PLP- α -syn mice compared to wild type litter mate controls. Four months of treatment with ATH434 in various doses reduced the iron within the SN (Mean \pm SEM, Ordinary ANOVA, $F = 16.03$, $p < 0.0001$). One way ANOVA with Dunnett's *Post hoc* test comparing to the 16-month untreated animals indicated that the 30 mg/kg reached significance $**p = 0.006$; WT vs. VEH $***p \leq 0.0001$

Substantia nigra iron levels

Utilizing inductively coupled plasma mass spectrometry, we demonstrated that iron levels were elevated by 57% in the SN of 16-month-old MSA mice, as compared to littermate wildtype controls. It should be noted that the microdissection included both the substantia nigra reticulata and pars compacta within the dissected tissue block. A primary question in this study was whether this iron elevation was tractable to therapy. As we have previously shown that ATH434 has a dissociation constant (K_d) for Fe(III) and Cu(II) of $\sim 10^{-10}$ M, and affinities for Fe(II) and Zn of $\sim 10^{-5}$ M and $\sim 10^{-7}$ M respectively [18], this is a likely candidate to target iron in the MSA mice. Four months of treatment with ATH434 in various doses reduced the iron within the SN (ANOVA, $F = 16.03$, $p < 0.0001$). A Dunnett's *post-hoc* test revealed that only the 30 mg/kg treatment group reached significance ($p = 0.006$, -20%) (Fig. 2B).

Western blot data for α -syn

ATH434 treatment did not alter the levels of soluble monomeric α -syn (14 kDa) but significantly decreased both oligomeric (66 kDa, $p < 0.01$ by 18%, Fig. 3A–C) and insoluble (urea extracted, $p < 0.01$ by 69%) α -syn in the SN (Fig. 3D, E). Long term

treatment of the mice with the compound appeared to either prevent the formation of higher order species or increased α -syn clearance from the nigra. It should be noted that the microdissection included both the substantia nigra reticulata and pars compacta within the dissected tissue block.

Glial cell inclusions in the substantia nigra pars compacta

Four months of treatment with ATH434 significantly reduced the number of GCI within the SN (ANOVA, $F = 19.98$, $p < 0.0001$). A Dunnett's *post-hoc* test demonstrated that each of the treatments significantly reduced the number of GCIs (3 mg/kg (46% $p < 0.0001$), 10 mg/kg (37%, $p < 0.0001$), 30 mg/kg (31%, $p < 0.0001$); Fig. 4).

In vitro α -syn assay

In an *in vitro* assay utilizing iron-mediated aggregation of α -syn, we previously demonstrated that the addition of ATH434 slowed the rate of aggregation [18]. An aim for some drug makers is to find compounds that break apart or disaggregate α -syn aggregates. In this study, we used the ThT fluorescence assay to assess if ATH434 could disaggregate already aggregated α -syn. After the day 3 measurements the samples were divided into two groups,

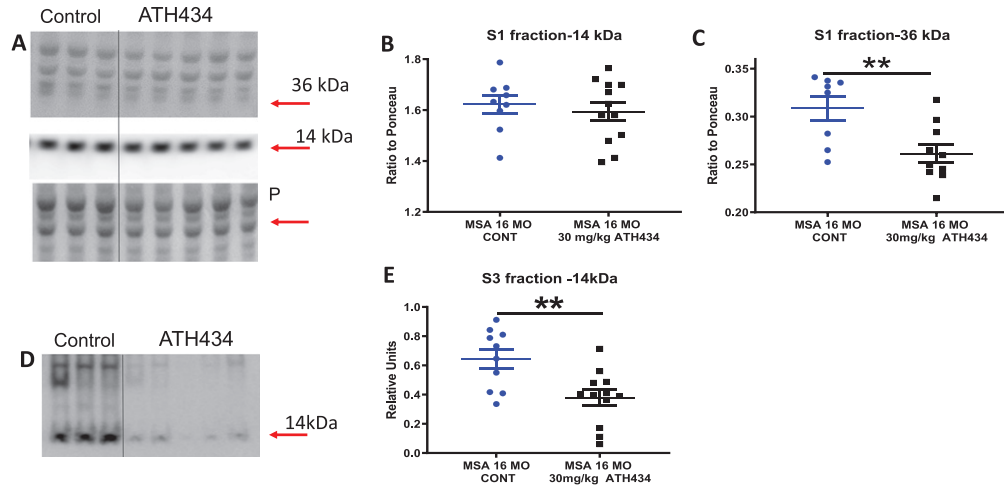


Fig. 3. A) Western blot data for soluble α -syn levels in the Substantia Nigra (SN) in 16-month PLP- α -syn mice after 4 months of ATH434 or Control diet, Soluble (S1) fraction of SN transferred to western blots and stained for α -syn with LB509 antibody. Arrows indicate the α -syn monomer and oligomeric bands. Loading control ponceau stain (P) is show under the labels. B) Plots of density (relative to ponceau) of 14kDa. C) Plots of density of 36 kDa bands (relative to ponceau), Mean \pm SEM, *T* test $**p = 0.0081$. D) Western blot data for aggregated (urea soluble) α -syn levels in the Substantia Nigra in 16-month PLP- α -syn mice after approximately 4 months of ATH434 or Control diet. Urea soluble (S3) fraction of SN transferred to western blots and stained for α -syn with LB509 antibody. The arrow indicates the α -syn urea-soluble band. Laddering of higher order oligomeric bands can be observed in the control 16-month PLP- α -syn mice but are greatly reduced in the animals that have been treated with ATH434. Mean \pm SEM. *T*-test $**p = 0.0049$. The S3 plots were constructed using relative fluorescence units.

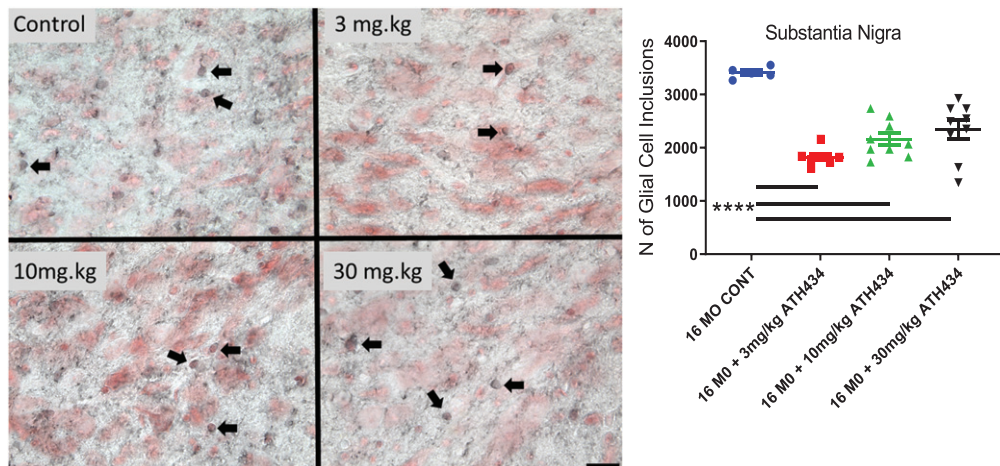


Fig. 4. The number of Glial Cell Inclusions (dark staining, Arrows) in the Substantia Nigra pars compacta (neurons red staining) in 16-month PLP- α -syn mice. Four months of treatment with ATH434 in various doses reduced the number of GCI within the SN (Mean \pm SEM, Ordinary ANOVA, $F = 19.98$, $p < 0.0001$). One way ANOVA with Dunnett's *Post hoc* test comparing to the 16-month untreated animals indicated that 3 mg/kg (46% decrease), 10 mg/kg (37% decrease), 30 mg/kg (31 % decrease) significantly reduced the number of GCI $p < 0.0001$. (16 MO CONT vs. 3 mg/kg $p \leq 0.0001$; 16 MO CONT vs. 10 mg/kg $p \leq 0.0001$; 16 MO CONT vs. 30 mg/kg $p \leq 0.0001$) Panels show photomicrographs showing GCIs within the SN (scalebar = 25 microns).

control (α -syn + iron + water as vehicle control) or ATH434 (α -syn + iron + ATH434). Visual inspection of Fig. 5 suggests that the average rate of aggregation in the control group is the similar to baseline, whereas the addition of ATH434 the mixture did not allow new aggregates to form and importantly did

not disaggregate the already formed fibrils. Analysis of data after treatments were introduced used a two-way ANOVA; samples were paired but the variance component for the pairing was zero. Pair wise multiple comparisons of treatment were performed for each day, as an interaction of treatment and day

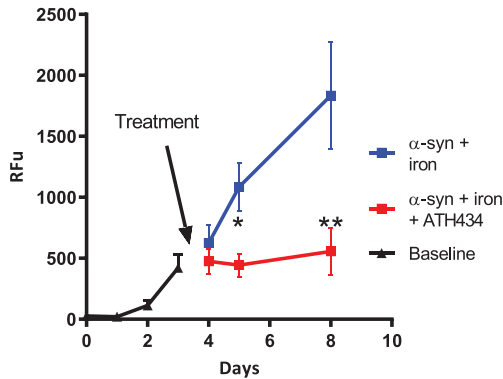


Fig. 5. Inhibition of α -synuclein (α -syn) aggregation. Recombinant α -syn (140.0 μ M) was incubated in the presence of equimolar concentrations of iron nitrate ($\text{Fe} = \text{Fe}(\text{NO}_3)_3$) and or ATH434. The ATH434 was delivered at day 3 after the α -syn aggregation had commenced and prevented further iron-mediated α -syn aggregation. The presence of the ATH434 did not dis-aggregate the α -syn as the signal does not decrease. Aggregation was measured by using ThT fluorescence binding (RFU = relative fluorescent units) $p = 0.013$ (*) and at day 8 $p < 0.001$ (**)

was detected. Pairwise analysis shows that at day 5 the differences reached a $p = 0.013$ (*) and at day 8 $p < 0.001$ (**) (Fig. 5).

DISCUSSION

Multiple System Atrophy is a rare atypical parkinsonian disorder in which affected individuals deteriorate quickly [26–28]. MSA is pathologically characterized both by the accumulation of α -syn within the oligodendrocytes and by an elevation in brain iron levels. As there is a known link between α -syn and iron, with oxidative stress and free iron shown to promote the aggregation of α -syn [11, 29] and to increase the toxicity to cells [30, 31], then there is a strong rationale for the hypothesis that iron-targeting compounds will be therapeutically efficacious in this disease.

To interrogate this hypothesis, we utilized a transgenic mouse model that expresses human wild-type α -syn within the oligodendrocytes, driven by the proteolipid protein (PLP) promoter. These MSA transgenic mice were originally imported from the laboratory of Dr. Nadia Stefanova, after which they were backcrossed onto a C57/BL6 background (Animal Resources Centre, Western Australia) for more than 10 generations. The phenotype of the mice appears to be very similar to the published data [19–21, 32], although as discussed later, there was some minor drift in the timing of the phenotype. To determine if iron was a tractable therapeutic target

in these mice, we utilized an iron-targeting compound, ATH434, that we have previously shown to be efficacious in both PD toxin models and in the transgenic hA53T α -synuclein model of PD [18]. Further, this compound was well-tolerated in a first-in-human oral dosing study in healthy and older volunteers, and demonstrated a favorable, dose-dependent pharmacokinetic profile [17].

Mouse colony

Although originating from the same source, the minor drift in the timing of the phenotype observed in the Australian colony may be attributed to different genetic background of the mice or subtle variations in the experimental methods. Heras-Gavin et al. observed loss of TH immunoreactivity in six-month-old animals, consistent with data from Refollo et al. who also found behavioral differences at this time [20, 32]. The Australian colony showed a small but insignificant decrease in total SNpc neuron at 12 months of age, that became significant at 16 months. This finding of temporal differences in the results is not surprising as differences in colonies is known to occur [33]. Importantly GCIs are present in both colonies from an early age.

Alpha synuclein in MSA

It is believed that the pathogenesis of MSA results from spread of misfolded α -syn from neurons to oligodendroglia [34, 35]. The α -syn then accumulates in the oligodendroglia to form toxic fibrils and GCIs. How this accumulation within the glia subsequently causes death of neurons is not clear. Interestingly, it has been shown that the α -syn aggregates that form in the oligodendroglia micro-environment are more toxic and structurally distinct from the aggregates found within the neurons from individuals with PD [3, 36].

In the PLP promoter α -syn transgenic mice, the production of the α -syn is not under the natural promoter that has the iron response element within mRNA. In humans and wild type rodents the iron response element is within mRNA of α -syn [15, 16]. In this study we found that the amount of monomeric α -syn was not altered by ATH434, which is consistent with the α -syn being controlled by the PLP promoter. ATH434 reduced the amount of soluble aggregates and insoluble (urea-soluble) α -syn aggregates that are formed in the oligodendroglial microenvironment. The *in vitro* study indicated that ATH434 was not

able to dis-aggregate α -syn but was able to prevent further aggregation. Therefore, it is deduced that the effects observed *in vivo* may have been achieved by increasing clearance of α -syn [37, 38] and/or slowing the formation of iron mediated α -syn aggregation. Importantly, this treatment culminated in a reduction in the number of glial cell inclusions which are constituted from aggregated α -syn. Given the data from this manuscript, ATH434 could act on several different pathways to prevent or slow the pathological process. These include potential effects on: 1) levels of synuclein protein, by removing access the iron-response element of the mRNA of α -syn; 2) preventing the formation of extracellular misfolded α -syn; 3) enhancing clearance of the α -syn burden; 4) prevent the formation intracellular α -syn aggregates.

Iron in MSA

Iron accumulates in a specific pattern in MSA[8]. Whilst studies have found that iron deposits in the putamen precede the clinical presentation of MSA by 2 years [39], most find that iron accumulation occurs during atrophy, and this is thought to be a secondary event of the neurodegeneration process [6]. Whilst the precise cellular and subcellular localization of this elevated iron has not been fully established, synchrotron microscopy technology is becoming available to address this question, although it does not yet appear to have been applied to PD or MSA [40].

We have been investigating the normal distribution of iron and the consequences of alterations in the metabolism and homeostasis of iron in parkinsonism [41–45]. We believed that this elevation in iron results in an increase in the weakly bound or labile iron pool [46, 47]. We have shown that this a tractable target and that a reduction in iron is associated with a reduction of damaging radicals which have a number of pathological consequences[18]. In the current MSA study we found that the elevation of iron caused by oligodendroglial α -syn expression could be reduced by the treatment with ATH434 in an apparent dose dependent way.

CONCLUSION

In 16-month-old MSA animals with a degenerating phenotype the compound ATH434; reduced oligomeric, aggregated and urea soluble α -synuclein, reduced the number of GCIs and preserved SN neurons. *In vitro* experiments suggest that ATH434

can prevent the formation of toxic oligomeric species of α -synuclein. The United States Food and Drug Administration and European Commission has granted Orphan drug status for ATH434 (formerly PBT434) as there are no approved treatments for MSA. Given this status, it is concluded that ATH434 is a small molecule drug candidate with potential for treating MSA.

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CONFLICT OF INTEREST

DIF, RAC, KJB, and PAA consulted for Altery\Prana.

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