ATH434 Reduces α-Synuclein-Related Neurodegeneration in a Murine Model of Multiple System Atrophy

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ABSTRACT: Background: Multiple system atrophy (MSA) is a fatal neurodegenerative disorder characterized by aggregated α-synuclein (α-syn) in oligodendrocytes and accompanied by striatonigral and olivopontocerebellar degeneration and motor symptoms. Key features of MSA are replicated in the PLP-α-syn transgenic mouse, including progressive striatonigral degeneration and motor deterioration. There are currently no approved treatments for MSA. ATH434 is a novel, orally bioavailable brain penetrant small molecule inhibitor of α-syn aggregation.

Objectives: To characterize ATH434 for disease modification in a mouse model of MSA.

Methods: Six-month-old PLP-α-syn mice (MSA mice) were ATH434-treated (ATH434 in food) or untreated (normal food) for 6 months. Motor behavior and numbers of nigral and striatal neurons were evaluated. α-syn aggregates and oligomers were quantified by immunohistochemical and western blot analyses. Microglial activation and neuroinflammation were assessed by histological and molecular analyses. Ferric iron in the Substantia nigra was evaluated with the Perls method.

Results: ATH434-treated mice demonstrated preservation of motor performance in MSA mice that was associated with neuroprotection of nigral and striatal neurons. The rescue of the phenotype correlated with the reduction of α-syn inclusions and oligomers in animals receiving ATH434. ATH434-treated mice exhibited significantly increased lysosomal activity of microglia without increased pro-inflammatory markers, suggesting a role in α-syn clearing. ATH434-treatment was associated with lower intracellular nigral iron levels.

Conclusions: Our findings demonstrate the beneficial disease-modifying effect of ATH434 in oligodendroglial α-synucleinopathy on both the motor phenotype and neurodegenerative pathology in the PLP-α-syn transgenic mouse and support the development of ATH434 for MSA. © 2021 The Authors. Movement Disorders published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: multiple system atrophy; ATH434; α-synuclein; Parkinson’s disease; neurodegeneration

Multiple system atrophy (MSA) is a rare neurodegenerative disorder clinically characterized by autonomic failure and progressive motor impairment.1 MSA pathological hallmarks include the abnormal accumulation of α-synuclein (α-syn) mainly in the cytoplasm of oligodendrocytes, forming the so-called glial cytoplasmic inclusions (GCI), accompanied by striatonigral degeneration (SND) in the parkinsonian variant of the disease (MSA-P) and by olivopontocerebellar atrophy (OPCA) in the cerebellar variant (MSA-C).1 MSA is a fatal disorder, leading to death a few years after symptom onset, with no treatment available.2 Similar to

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Parkinson’s disease (PD), the abnormal aggregation and accumulation of α-syn in MSA is associated with glial and neuronal dysfunction, neuroinflammation and neurodegeneration, however the origin of α-syn inclusions in MSA is still unknown. Numerous publications have recently shown the ability of α-syn to be transferred from cell to cell and spread in a prion-like manner and, therefore, the inhibition of α-syn aggregation constitutes a promising therapeutic strategy in α-synucleinopathies. The use of small molecules to target α-syn aggregation has shown promising results in pre-clinical models of PD and MSA, supporting their use in current clinical trials. ATH434, formerly PBT434, is a novel orally bioavailable and brain penetrant small molecule that is able to inhibit α-syn aggregation. ATH434 has an iron binding affinity competitive for α-syn but not for endogenous iron trafficking proteins. In a previous study, the use of this compound prevented loss of neurons in the substantia nigra pars compacta (SNC), lowered nigral α-synuclein accumulation and nigral iron levels, and rescued motor performance in animal models of PD. Therefore, ATH434 could constitute a promising compound to attenuate disease progression in MSA.

The PLP-α-syn transgenic mouse model of MSA recapitulates many of the clinical and pathological features observed in patients by overexpressing human α-syn under the oligodendrocytic PLP (proteolipid protein) gene promoter. These mice develop GCI-like aggregation, autonomic failure, progressive SND and motor impairment, recapitulating the natural history of the human disease. Thus, this animal model constitutes a valuable preclinical tool to evaluate the effect of disease modification therapies for MSA. In the present preclinical study, we evaluated the efficacy of ATH434 in PLP-α-syn mice to alter the performance in motor tasks, to change the progression of SND, the density of GCI-like aggregates and the level of α-syn oligomers.

Material and Methods

Animals and Treatments

PLP-α-syn mice were kept under temperature-controlled pathogen-free conditions with a light/dark 12 hours cycle. All experiments were performed according to the ethical guidelines with the permission of the Austrian Federal Ministry of Science and Research (BMBWF-66.011/0080-V/3b/2019). Six-month-old PLP-α-syn mice were randomized in two groups, one fed with normal food pellets (non-treated group, N = 13), and another fed with pellets containing ATH434 (N = 14). Food pellets were provided by Specialty Feeds, Australia. We used a daily dose of 30 mg/kg as reported in previous experiments in mice. Six-month-old PLP-α-syn mice were used as a baseline group (N = 14). Food pellets were provided ad libitum to the animals throughout the whole experiment. After 6 months of treatment, behavioral analysis was performed followed by the sacrifice of the animals and brain extraction.

Challenging Beam Test

Motor performance and coordination were analyzed with a modified version of the traditional beam test adapted from a previously published method. Behavioral analyses were performed with the analyst blinded to the treatment. Five performances per animal were video recorded at baseline (6 months of age), and every 2 months until the end of the treatment (at 8, 10 and 12 months of age), and the number of slips per step with the hind limbs was measured. The mean per animal was used for statistical analyses.

Pole Test

We assessed the coordination and bradykinesia of each mouse at the end of the treatment period with the pole test as described previously. After a training session the day before testing, each mouse was placed with the head up on the top of a wooden pole. We recorded time to turn downward (T-turn) and the time to climb down the 50 cm-pole (T-total) for each animal. Five trials were performed with each mouse and mean values were calculated for the statistical analysis.

Tissue Processing

After the final motor test, mice were perfused intracardially with phosphate buffered saline (PBS, pH 7.4, Sigma) under deep thiopental anesthesia and brains were extracted. For molecular analyses midbrains were quickly dissected from the right hemisphere, snap frozen in liquid nitrogen and stored at −80°C. For histological analyses, left hemibrains were post-fixed overnight in 4% paraformaldehyde (pH 7.4, Sigma) at 4°C. After fixation, brains were washed in PBS and then cryoprotected in 30% sucrose (in PBS). Finally, brains were frozen using 2-methylbutane (Sigma) and stored at −80°C.

Immunohistological Analyses

Brains were serially cut in 40 μm-thick coronal sections using a freezing microtome (Leica) and stored free-floating in a cryoprotectant buffer at −20°C. One series was directly mounted on slides and stained with cresyl-violet. Free-floating sections were stained following standard protocols. To analyze the number of dopaminergic neurons (TH+ neurons) in the SNC and medium spiny neurons (DARPP32+ neurons) in the striatum (STR), representative brain sections were respectively stained with rabbit anti-tyrosine hydroxylase (TH) antibody (1:1000, Millipore) and mouse anti-DARPP32 antibody (BD Bioscience; 1:2000). In order to evaluate the number of GCIs, microglial activation and microglial phagocytic
activity in the SN, representative sections of SN were respectively stained with rabbit anti-phosphorylated α-synuclein (pS129) antibody (1:1000, Abcam), rat anti-CD11b antibody (1:200, BIORAD) and rat anti-CD68 antibody (1:200, BIORAD). To evaluate the presence of α-syn within CD68+ lysosomes, double immunostaining was performed with anti-aggregated α-syn antibody, clone 5G4 (1:1000, Linaris) and anti-CD68 antibody (1:200, BIORAD). Sections were then incubated with biotinylated secondary antibodies followed by Vectastain ABC reagent.
(Vector Laboratories) and 3,3'-diaminobenzidine (Sigma) to visualize the immunohistochemical binding sites. Stained sections were mounted on slides, dehydrated and coverslipped with Entellan (Merck). For immunofluorescence, suitable IgGs conjugated with Alexa 488 or Alexa 594 (Life Technologies) were applied, followed by nuclear staining with 4',6-Diamidino-2-phenylindol (DAPI, 1:1000, Sigma) and finally coverslipped with mounting medium Fluoromount-G (Southern Biotech).

Detection of Ferric Iron (Fe$^{3+}$) in Cells

We detected and quantified available ferric iron (Fe$^{3+}$) in brain sections containing the SNc of ATH434-treated or untreated MSA mice using a Perls Prussian blue staining protocol as per the kit’s manual (HEMATOGNOST Fe$^{3+}$ kit; Merck, Darmstadt, Germany). Briefly, the sections were incubated in a solution of potassium hexacyanoferrate [K$_4$Fe(CN)$_6$] in HCl which causes tissue Fe$^{3+}$ to precipitate, resulting in the Prussian blue stain (Fe$_4$[Fe(CN)$_6$]$_3$). The sections were counterstained with a Fast Red solution to visualize the cellular nuclei. Slides were coverslipped prior to quantification.

Image Analyses

Neuroanatomy was assessed using a Mouse Brain Atlas. Stereological analysis was performed using the Nikon E-800 microscope equipped with Nikon digital camera DXM1200 and Stereoinvestigator software (Microbrightfield Europe e.K) as described previously. The number of TH+ neurons in the SNc and DARPP-32 + in the STR as well as the total number of neurons in cresyl violet (CV) staining were measured by applying the optical fractionator workflow. The density of GCIs in the SNc was assessed with the meander scan and is expressed in GCI/mm$^2$. For microglial activation and phagocytic activity assessment, SN images were acquired with a fluorescence microscope (Leica DMI4000) and the CD11b and CD68 positive areas were estimated using ImageJ (National Institutes of Health). Results are presented as percentage of CD11b or CD68 area per total section area. To evaluate the presence of α-syn within CD68+ lysosomes, images were acquired with a confocal microscope (Zeiss LSM 700). The density of Prussian blue-positive profiles in the SNc was estimated applying the Nikon E-800 microscope equipped with Nikon digital camera DXM1200 and Stereoinvestigator software (Microbrightfield Europe e.K). The density of
Prussian-blue-positive profiles per area was defined in the SNc at 40x and presented as arbitrary units (AU). All morphometric analyses were done by an observer blinded to the treatment of the animals.

**Western Blot Analyses**

Fresh frozen midbrain samples were homogenized in RIPA extraction buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 1 mM EDTA; Sigma) containing protease and phosphatase inhibitors (Roche). The lysates were centrifuged (16,000g for 15 minutes at 4°C) to remove debris and the supernatant was collected and stored at −80°C. Protein concentrations were determined with BCA Protein Assay Kit (Sigma). Equal amounts of protein (30 μg) per sample were subjected to SDS-PAGE and western blotting following standard procedures. Primary antibodies included anti-human α-synuclein (1:2000, Abcam), anti-phosphorylated α-syn in Ser129 (1:1000, Abcam), anti-TNFα (1:1000, Abcam), anti-CD11B (1:1000, Abcam), anti-GFAP (1:2000, Life Technologies). Antibody against GAPDH (1:5000, Sigma) was used as loading control. Signal detection was performed using HRP-conjugated anti-rabbit (1:20,000, Cell Signaling), anti-rat (1:20,000,
Cell Signaling) and anti-mouse (1:10,000, GE Healthcare) antibodies. Images were acquired using the Fusion FX system (Vilber Lourmat) for Western blot and gel imaging. Relative protein levels were measured by densitometry using FUSION CAPT V16.09b software (Vilber Lourmat). A reference sample was loaded in all gels for gel-to-gel normalization. All gels were run, transferred, incubated and developed in parallel.

**Statistical Analyses**

All statistical analyses were conducted using the software Graph-Pad Prism 8 (Graphpad Software). All data are expressed as mean value ± SD, if not indicated otherwise. For all statistical tests performed, a probability level of 5% (P < 0.05) was considered significant. One-way ANOVA with post hoc correction for multiple comparisons was used for multi-group comparisons.

**Results**

**ATH434 Preserves Motor Function and Halts SND in the PLP-α-Syn Mice**

PLP-α-Syn mice fed with ATH434 exhibited no motor dysfunction at the end of the treatment compared to mice fed with placebo pellets (normal food, “non-treated”), with a significant reduction in the number of slips per step in the challenging beam test (Fig. 1A). At the end of the treatment period, we confirmed the improved motor performance of PLP-α-Syn mice fed with ATH434 as compared to non-treated animals with the pole test (Fig. 1B). Associated with the motor improvement,

![Graphical abstract showing statistical analyses and results](image-url)

**FIG. 4.** ATH434-treated mice show increase of microglial phagocytic activation. (A) Representative immunofluorescence images of SN sections stained against CD68. Scale bar, 150 μm. (B) CD68 positive (CD68+) area in the different groups expressed as the % of the total area of the SN. N = 12–14. Error bars indicate SD. Unpaired t test, ***,P < 0.001. (C) Representative confocal microcopy of the co-localization of α-syn (green) and CD68 (red) in microglial lysosomes (dot-like structures within the white box). Scale bar, 5 μm. (D) Representative immunofluorescence images of SN sections stained against CD11b. Scale bar, 150 μm. (E) CD11B positive (CD11b+) area in the different groups expressed as the % of the total area of the SN. N = 12–14. Error bars indicate SD. (F) Representative blots showing levels of GFAP, TNFα and GAPDH in midbrains of non-treated and ATH434-treated mice. GAPDH was used as loading control. Individual lanes represent brain homogenates of individual mice. (G,H) Quantification of GFAP and TNFα protein levels in midbrains of non-treated and ATH434-treated mice. The data is shown in arbitrary units (a.u.). N = 9–10. Error bars indicate SD. [Color figure can be viewed at wileyonlinelibrary.com]
ATH434 had a neuroprotective effect in SNC (Fig. 1C–F). Stereological counting showed a similar number of dopaminergic (TH+) neurons in the SNC of treated mice compared to the baseline group and a significant neuronal loss in non-treated mice (Fig. 1C,D). These observations were confirmed by CV staining (Fig. 1E). There was a significant correlation between the neuroprotective effect of ATH434 and the motor improvement (TH+ neurons vs. slips per step: $R^2 = 0.21$, $P = 0.0463$; CV neurons vs. slips per step: $R^2 = 0.39$, $P = 0.0101$) (Fig. 1F).

ATH434 also prevented the loss of medium spiny neurons (MSNs) in the STR (Fig. 2). Stereological counting of DARPP32+ MSNs showed similar numbers in ATH434-treated and baseline PLP-α-syn mice, while non-treated animals suffered a significant reduction in the number of MSNs over the six-month experimental period (Fig. 2A–C). The neuronal protection in STR was confirmed by CV staining (Fig. 2D).

ATH434 Reduces α-Syn Aggregation in PLP-α-Syn Mice

Previous findings demonstrated the ability of ATH434 to modulate α-syn aggregation in vitro and in vivo\(^{11}\); therefore, we assessed its potential to reduce α-syn accumulation in MSA mice. Immunohistological analysis showed a significant reduction of GCI-like inclusions in the SNC of ATH434-treated mice compared to non-treated animals, with levels similar to the baseline group (Fig. 3A,B). Moreover, the decrease of α-syn aggregates significantly correlated with the neuroprotective effect observed in the SNC (TH+ neurons vs. GCI density: $R^2 = 0.47$, $P = 0.0012$; CV neurons vs. GCI density: $R^2 = 0.46$, $P = 0.004$) (Fig. 3C).

In addition to the effect on the number of inclusions, western blot analysis showed a significant reduction in the ratio of total and phosphorylated α-syn oligomers versus monomer in ATH434-treated mice compared to the non-treated group (Fig. 3D–H). Thus, the results confirmed the modulatory effect of ATH434 on α-syn oligomerization, with a shift from oligomeric to monomeric α-syn in treated animals. Interestingly, lower levels of total α-syn oligomeric species significantly correlated with the rescue of striatal neurons (DARPP32+ neurons in STR vs. α-syn oligomers/monomers ratio: $R^2 = 0.75$, $P = 0.0001$; CV neurons in STR vs. α-syn oligomers/monomers ratio: $R^2 = 0.30$, $P = 0.0426$) (Fig. 3F).

ATH434-Treated Mice Show Increase of Microglial Phagocytic Activation That Is Not Associated With Neuroinflammation

To assess the effect of ATH434 treatment on microglial activation, we performed immunofluorescence microscopy for CD68 in SN brain sections, a lysosomal marker indicative of phagocytic activity of microglia.\(^{26}\) ATH434-treated PLP-α-syn mice showed a significant increase in CD68 levels in the SN compared to non-treated animals (Fig. 4A,B). In addition, confocal microscopy demonstrated the presence of α-syn within CD68+ lysosomes in microglia (Fig. 4C). The increase in CD68 levels was not associated with an increase of the microglial marker CD11b (Fig. 4D,E), astrogliaisis (Fig. 4F,G) or the pro-inflammatory cytokine TNFα (Fig. 4F,H). Moreover, there was a significant correlation between the increase of microglial lysosomal marker CD68 and the reduction in the intracellular GCI-like aggregates ($R^2 = 0.27$, $P = 0.0066$), the lower levels of oligomeric species ($R^2 = 0.25$, $P = 0.0363$), the preservation of MSNs in the STR (DARPP32+ neurons: $R^2 = 0.31$, $P = 0.0239$; CV neurons: $R^2 = 0.28$, $P = 0.0345$) and the motor improvement ($R^2 = 0.28$, $P = 0.0137$). Finally, there was a positive, but not significant, correlation between CD68 levels and the neuroprotective effect of ATH434 in the SNC (TH+ neurons: $R^2 = 0.21$, $P = 0.0832$; CV neurons in SNC: $R^2 = 0.24$, $P = 0.1023$) (Figure S1A–G).

ATH434 Reduces the Level of Ferric Iron (Fe\(^{3+}\)) in the SNC of PLP-α-Syn Mice

To assess the Fe\(^{3+}\) in the SNC of PLP-α-syn mice, we applied a classical Perls Prussian stain and estimated the distribution of the resulting blue precipitates in non-treated versus ATH434-treated MSA mice. In SNC of PLP-α-syn mice at 12 months of age, intracellular Fe\(^{3+}\) (Fig. 5A) was significantly reduced after the treatment.
with ATH434, a known moderate affinity iron chelator.\textsuperscript{11}

**Discussion**

Although the origin and exact role of fibrillary α-syn inclusions and soluble oligomeric species in MSA are still under discussion, several \textit{in vivo} and \textit{in vitro} studies have shown the ability of pathological α-syn to spread through the CNS, to induce cellular dysfunction, neuroinflammation, motor symptoms and neurodegeneration.\textsuperscript{27-39} Based on this evidence and the results from preclinical studies, numerous ongoing clinical trials are focused on targeting or modulating α-syn oligomerization and aggregation.\textsuperscript{9} In particular, the use of small molecules that modulate α-syn accumulation presents many advantages such as their high oral bioavailability, blood–brain barrier penetration, ability to act inside cells,\textsuperscript{40} and promising therapeutic potential in preclinical models of α-synucleinopathies by reducing α-syn inclusions, neurodegeneration and improving motor function.\textsuperscript{7-13}

We show that the small molecule ATH434, a novel quinazolinone inhibitor of iron-mediated α-syn protein accumulation,\textsuperscript{11} reduces GCI-like inclusions and α-syn oligomeric species in MSA mice. We also show that ATH434 has a neuroprotective effect in the STR and the SNc of PLP-α-syn mice, preventing the loss of medium spiny and dopaminergic neurons respectively in these brain regions. In this regard, there was a significant correlation between the reduction of GCI-like inclusions in the SNc, the dopaminergic neuroprotective effect in the SNc and the motor improvement. Therefore, our findings suggest that the neuroprotective effect of ATH434 in this brain region and on the motor function is associated with its modulatory effect on α-syn aggregation. We also identified a shift from oligomeric towards monomeric α-syn species in ATH434-treated mice with a significant lowering of the pathological phosphorylated high-molecular weight forms. These findings, together with the observed lower density of GCIs in the treated animals, suggest that the ATH434 effect is linked to preventing the formation of new α-syn aggregates during the progression of the disease rather than disaggregation of already existing aggregates at the beginning of the therapy. Interestingly, there was a significant correlation between the reduction in α-syn oligomers and the neuroprotective effect of ATH434 in the STR, suggesting that soluble α-syn pathogenic species may contribute to the MSN degeneration in MSA mice. Our data corroborate observations from a previous study, where ATH434 reduced α-syn aggregation \textit{in vitro} and \textit{in vivo}.\textsuperscript{11} In this earlier work, administration of ATH434 in toxin-based and transgenic mouse models of PD prevented the loss of dopaminergic neurons in SNc and ameliorated motor symptoms. These effects were associated with a decrease in oxidative stress, SNc iron and a reduction of α-syn pathological species.\textsuperscript{11} Altogether, these findings indicate that ATH434 is able to modulate α-syn accumulation in different pathological conditions, leading to neuroprotection and motor improvement.

PLP-α-syn mice recapitulate the natural history of the human MSA-P, developing SND and neuroinflammation with aging, among other disturbances.\textsuperscript{22} At early stages—2 months old—these animals show GCI-like inclusions in the brain without signs of neuroinflammation, neurodegeneration or motor symptoms. However, with aging, MSA mice develop progressive neurodegeneration in motor areas that becomes evident at 4–6 months of age in SNc and at 12 months of age in STR, accompanied by α-syn oligomer formation, progressive neuroinflammation and motor dysfunction.\textsuperscript{20,22} Considering that MSA patients are usually diagnosed once the motor symptoms have appeared and the neurodegenerative process has started\textsuperscript{41} we decided to administer ATH434 to MSA mice at 6 months of age, when initial motor symptoms are already present,\textsuperscript{22} and to evaluate its therapeutic potential on the disease progression up to 12 months of age. The fact that ATH434 was able to halt SND and preserve motor function at this stage of the disease in MSA mice constitutes a promising rationale for future clinical trials in MSA.

Here we also show that ATH434-treated mice presented an increase of the microglial lysosomal marker CD68. This prompted us to characterize further the neuroinflammatory status of the animals receiving ATH434. Molecular and histological evaluation demonstrated that the increase of CD68 immunoreactivity observed in ATH434-treated mice was not associated with further increase of astrogliosis, microgliosis or proinflammatory markers. In contrast, we observed a positive correlation between CD68 levels in SN and the neuroprotective effect of ATH434, and a negative correlation between CD68 and α-syn inclusions and oligomers (Figure S1). Furthermore, we demonstrated the presence of pathological α-syn within CD68+ lysosomes of microglia. Thus the results support an increase of microglial phagocytic activity linked at least partly to the clearance of α-syn through the lysosomal pathway,\textsuperscript{4,42-44} without further increase of the toxic proinflammatory signaling in the brains of MSA mice receiving ATH434 therapy.

Iron accumulation constitutes another pathological feature of MSA. Several studies have demonstrated increased levels of iron in brain areas affected by the disease.\textsuperscript{45} Increased iron in the SN is also a feature of preclinical PD models.\textsuperscript{11,46} In addition, preclinical studies have shown that, either by direct interaction or indirectly (ie, through the generation of oxidative stress), iron is able to induce α-syn aggregation.\textsuperscript{47-52} Here we
show that ferric iron in the SNC of PLP-α-syn mice is lowered by ATH434 treatment, similar to previous observations in a murine PD model in which ATH434 reduced MPTP-induced elevations of iron.\textsuperscript{11} Taken together, these new results provide the first evidence for the possible involvement of iron in the neurodegeneration process in the PLP-α-syn mouse model. Further studies however will be needed to characterize the stages of iron involvement during the progression of the disease. Our findings corroborate the neuroprotective effects of ATH434 in α-synucleinopathy, which may be attributed to its iron chelator activity as well as its effect on limiting α-syn aggregation.

In summary, our study confirms the neuroprotective potential of ATH434 previously observed in PD models\textsuperscript{11} and indicates that oral administration of ATH434, which recently completed a phase I trial in healthy volunteers,\textsuperscript{13} could constitute a promising therapeutic strategy to slow down disease progression in MSA, supporting further development in human clinical trials with patients suffering from MSA or related α-synucleinopathies.\textsuperscript{14}

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\textbf{Data Availability Statement}

The data that supports the findings of this study are available in the main and supplementary material of this article.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.