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Preclinical development of a high affinity α-synuclein antibody, MEDI1341, that can enter the brain, sequester extracellular α-synuclein and attenuate α-synuclein spreading *in vivo*

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ABSTRACT

There are no approved drug therapies that can prevent or slow the progression of Parkinson's disease (PD). Accumulation and aggregation of α-synuclein protein is observed throughout the nervous system in PD. α-Synuclein is a core component of Lewy bodies and neurites that neuropathologically define PD, suggesting that α-synuclein may be a key causative agent in PD. Recent experimental data suggest that PD progression may arise due to spreading of pathological forms of extracellular α-synuclein throughout the brain *via* a cellular release, uptake and seeding mechanism. We have developed a high affinity α-synuclein antibody, MEDI1341, that can enter the brain, sequester extracellular α-synuclein and attenuate α-synuclein spreading *in vivo*. MEDI1341 binds both monomeric and aggregated forms of α-synuclein. *In vitro*, MEDI1341 blocks cell-to-cell transmission of pathologically relevant α-synuclein preformed fibrils (pffs). After intravenous injection into rats and cynomolgus monkeys, MEDI1341 rapidly enters the central nervous system and lowers free extracellular α-synuclein levels in the interstitial fluid (ISF) and cerebrospinal fluid (CSF) compartments. Using a novel lentiviral-based *in vivo* mouse model of α-synuclein spreading in the brain, we show that treatment with MEDI1341 significantly reduces α-synuclein accumulation and propagation along axons. In this same model, we demonstrate that an effector-null version of the antibody was equally as effective as one with effector function. MEDI1341 is now in Phase 1 human clinical trial testing as a novel treatment for α-synucleinopathies including PD with the aim to slow or halt disease progression.

1. Introduction

Parkinson's disease (PD) is an age-related movement disorder with an incidence of approximately 1% in people over 60 years of age. It is the second most common neurodegenerative condition after Alzheimer's disease, and a recent Global Burden of Disease Study estimated PD prevalence at > 6 million people (Disease, Injury, and Prevalence, 2016).

The cardinal clinical symptoms of PD are motor-related disturbances including resting tremor, rigidity, bradykinesia and postural

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instability. However, PD is a complex disorder and there are also a wide range of non-motor symptoms that affect patients including cognitive deficits, dementia, hyposmia, sleep disturbances including rapid eye movement sleep behaviour disorder (RBD), mood disorders including depression and anxiety, autonomic dysfunction including cardiovascular and gastrointestinal problems such as constipation, and fatigue and somnolence. Some of these non-motor symptoms appear to characterise the premotor or prodromal phase of the disease (Kalia and Lang, 2015).

Current drug therapies for PD primarily treat the motor-related symptoms of the disease that result from the progressive degeneration of dopaminergic neurons in the substantia nigra region of the brain; and include L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (receptor) agonists, catechol-*O*-methyltransferase (COMT) inhibitors, and monoamine oxidase type B (MAO-B) inhibitors (Tarrants, Denarie, Castelli-Haley, Millard, and Zhang, 2010). There are currently no marketed or available therapies that can halt or slow the progression of PD.

The defining hallmark pathologies of PD are Lewy bodies and Lewy neurites, which are insoluble proteinaceous inclusions found inside neurons of the PD brain revealed upon post-mortem histopathological examination. α-Synuclein is the major protein component of Lewy bodies and Lewy neurites (Spillantini et al., 1997). This suggests that α-synuclein is involved in the disease process (Goedert, Spillantini, Del Tredici, and Braak, 2013). However, the precise mechanisms by which α-synuclein contributes to neurodegeneration, including understanding of the molecular basis for the conversion of normal physiological forms of α-synuclein into various pathological structures, and how this may trigger the disease or promote disease progression remains unknown.

It has recently been suggested that prion-like transmission or spreading of α-synuclein Lewy pathology may be a critical mediator of PD progression and neurodegeneration. Initially this was based on early clinical studies reporting that Lewy body pathology was observed within embryonic mesencephalic neuronal grafts therapeutically transplanted into the striatum of PD patients over a decade previously (Li et al., 2008; Kordower, Chu, Hauser, Freeman, and Olanow, 2008). The principle of prion-like cell-to-cell transmission or spreading of α-synuclein pathology as a key mechanism that drives the neurodegenerative process and progression of PD may explain the stereotypical and non-random progressive pattern of Lewy pathology observed in ascending order between the peripheral nervous system (PNS) and the central nervous system (CNS) involving neuroanatomically connected brain nuclei and neuronal pathways that was originally proposed by Braak and colleagues (Braak et al., 2003; Visanji, Brooks, Hazrati, and Lang, 2013). An alternative α-synuclein-driven 'threshold hypothesis' for PD progression has recently been proposed (Engelender and Isacson, 2017). In support of the Braak hypothesis, numerous experimental models have recently demonstrated cell-to-cell transmission of α-synuclein in cultured cells, or *in vivo* spreading and propagation of α-synuclein pathologies between neuroanatomically connected brain regions in mice and monkeys, which appear to involve transmembrane release, uptake and cell seeding processes (recently reviewed by (Recasens and Dehay, 2014)). In these preclinical models, it is proposed that the prion-like propagation or transmission of α-synuclein and its aggregated forms to recipient cells or host animals occurs over a period of time; has an extracellular phase; involves uptake into cells through mechanisms that remain to be fully determined but that may involve transmembrane endocytosis; and appears to occur between anatomically connected brain regions suggesting that transmission of α-synuclein pathology occurs along axons and across synapses (McCann, Cartwright, and Halliday, 2015). Precisely how the α-synuclein spreading process contributes to the degeneration of affected neuronal pathways (axons, synapses, cell bodies) is an area of intense investigation and debate, and direct clinical evidence (*e.g.* by neuroimaging) that spreading plays a causal role in PD is still lacking (recently discussed in (Manfredsson, Tansey, and Golde, 2018)).

Passive immunization studies in α-synuclein transgenic mice developed as experimental models of PD have shown that monoclonal antibodies directed against α-synuclein can reduce α-synuclein pathology and related deficits (Bae et al., 2012; Games et al., 2014; Masliah et al., 2011). More recently, *in vivo* mouse models of α-synuclein spreading have been developed and shown that systemic (passive) treatment of mice with preclinical anti-α-synuclein antibodies can reduce α-synuclein propagation in the brain (Tran et al., 2014). These preclinical mouse models of α-synuclein spreading have helped to validate immunotherapy as a potential approach to treating PD.

Here we report in-depth results from the preclinical development phase of a novel high affinity, α-synuclein-specific therapeutic antibody, MEDI1341. We propose that MEDI1341 will have utility as a treatment to halt or slow the progression of α-synucleinopathies including PD, based around the spreading hypothesis. We show that MEDI1341 can enter the brain of rats and monkeys and rapidly sequester extracellular α-synuclein. We demonstrate that MEDI1341 can block the cell-to-cell transmission of α-synuclein pffs *in vitro*, and abrogate the *in vivo* accumulation and axonal propagation of α-synuclein in a recently established lentiviral-based mouse model of α-synuclein spreading (Spencer et al., 2017).

2. Materials and methods

A concise overall study plan is detailed below. Full Materials and Methods can be found in the online Supplementary Materials.

This study was designed to isolate α-synuclein-specific monoclonal antibodies (mAbs) for therapeutic use by screening and characterizing the properties of these mAbs for binding to α-synuclein including disease-relevant forms and assessing α-synuclein specificity and species cross-reactivity. Based on these screens a single lead mAb was identified, then affinity optimized (which was guided by *in silico* PK/PD modelling) and tested for *in vitro* and *in vivo* efficacy.

Human α-synuclein specific mAbs were isolated by selection of phage displayed antibody libraries with recombinant monomeric human α-synuclein protein. Library outputs were tested as scFv for specificity for α-synuclein over closely related synuclein family members (β-synuclein and γ-synuclein), as well as binding to rodent and cynomolgus monkey α-synuclein using homogenous time resolved fluorescence-based epitope competition assays. Human α-synuclein specific VH and VL gene sequence unique clones cross-reactive to rodent and cynomolgus monkey α-synuclein were epitope binned using truncated variants of α-synuclein and characterized for their ability to bind to both monomeric and aggregated forms of recombinant α-synuclein by ELISA. Binding to disease relevant forms of α-synuclein was determined by immunohistochemistry and biochemistry on PD brain tissue. The lead mAb, asyn0087, was *in vitro* affinity matured by generating NNS block randomization libraries of the antibody complementarity determining regions (CDRs) to select for a high affinity (74pM) α-synuclein C-terminal specific antibody, named MEDI1341. MEDI1341 was then tested in *in vitro* and *in vivo* models representing the cellular transmission and spreading of α-synuclein and its aggregated forms, respectively, for the ability to block these pathologically relevant processes that are now strongly believed to contribute to the neurodegenerative process in Parkinson's disease. In a series of target engagement studies, MEDI1341 was also tested for the ability to enter the brain and sequester extracellular α-synuclein in the CNS.

2.1. Ethical approvals

Human brain tissue used to generate data in Fig. 2A was sourced *via* Tissue Solutions, Glasgow, UK and all samples were procured from a specialist brain bank and collected under institutional review board (IRB) ethical approval and with informed donor consent. Human brain tissue used to generate data in Fig. 2B was covered by Cambridge LREC ethical approval (ref n° 09/40). Experimental handling and use of all human brain tissue was according to the UK Human Tissue Act 2006.

2.2. In vivo animal studies

PK/PD animal studies were conducted at Brains On-Line, Netherlands and Charles River Laboratories, France. *In vivo* α-synuclein spreading studies in mice were carried out at University of California San Diego (UCSD), USA. All studies were conducted according to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the testing facilities concerned and in compliance with the AstraZeneca Animal Welfare and Bioethics policies. Detailed methods and materials for each *in vivo* study can be found in Supplementary Materials.

2.3. Statistical analyses

Mean experimental data ± standard deviation (SD) or ± standard error of the mean (SEM) were graphed using GraphPad Prism software. Distribution of the data was assessed by the Kolmogorov-Smirnov test. *Trans*-well cell-cell α-synuclein transmission model, free (unbound) α-synuclein ISF/CSF bioassay and *in vivo* α-synuclein immunohistochemical data were analysed by parametric one-way analysis of variance (ANOVA) and if significance was achieved then post-hoc analysis was performed using Dunnett's multiple comparisons test comparing treatments to control group. When only two groups of data were experimentally compared, where statistically appropriate, an unpaired two-tailed *t*-test was employed. All statistical analyses were performed using GraphPad Prism statistical analysis software. Differences between antibody treatment groups and vehicle control groups were defined as statistically significant when $P < .05$. *In vivo* spreading mouse model experiments and analyses were performed blinded to the rater and the antibody treatment groups were also blinded to the rater during the in-life portion of the study.

3. Results

3.1. Antibody target affinity modelling for optimal α-synuclein sequestration and clearance in vivo

We hypothesized that potent *in vivo* sequestration and clearance of all forms of extracellular α-synuclein including monomers, which could be critical basic building blocks for aggregate formation, may have the greatest efficacy for preventing pathological α-synuclein spreading and slowing disease progression in PD and other α-synucleinopathies. We used *in silico* PK/PD modelling, which incorporated knowledge of human antibody PK, α-synuclein turnover and antibody:α-synuclein binding kinetics in both periphery and CNS, in a semi-mechanistic compartment model to predict the target antibody affinity required to potently suppress extracellular monomeric α-synuclein levels in the brain. Model outputs indicated that an antibody target affinity of < 100pM would be required to potently suppress free monomeric α-synuclein levels in the brain/CSF by between 30% and 90% across a viable clinical dose range and dose interval when administered by the intravenous (iv) route (see Fig. S1 for model characteristics and parameters). In summary, achieving an antibody affinity of < 100pM was the target for antibody optimization.

3.2. Initial antibody selections and specificity screening

α-Synuclein specific antibodies were isolated from naive human phage display libraries by selection on recombinant human α-synuclein. From these selections, asyn0087 was identified. This clone was α-synuclein specific, recognized an epitope at the C-terminus of α-synuclein present on both monomeric and aggregated forms of the protein; it was cross-reactive to cynomolgus monkey and rodent α-synuclein and it recognized disease relevant forms of α-synuclein in PD brain by IHC. This clone had an affinity for monomeric α-synuclein too low to be measured accurately by Octet or BIAcore (estimated at > 1 μM) and therefore required further affinity optimization.

3.3. Antibody affinity optimization and characterisation

To improve the affinity of asyn0087, random mutagenesis libraries of individual complementarity determining regions (CDRs) of both the antibody heavy and light chains were generated. The CDRs are the hypervariable loops of the antibody structure that are responsible for antigen binding. Four of the six CDRs were chosen for mutagenesis, namely heavy chain CDR2 and CDR3 and light chain CDR1 and CDR3 (see Fig. S2A), based on the need to achieve significant affinity improvements (~10,000-fold) for the target antigen. Affinity driven selections were performed on soluble biotinylated human α-synuclein.

From these iterative rounds of library selection, screening, and recombination, a potent α-synuclein specific, cynomolgus and rat α-synuclein cross-reactive clone was identified, aslo0452. This clone was derived from two rounds of library recombination and screening. For the first round of recombination, new libraries were prepared in which two CDRs were simultaneously randomized by combining previously selected CDR mutant populations (*i.e.* HCDR2 with HCDR3, and LCDR1 with LCDR3). This process can produce new variants with potentially additive or synergistic improvements through the random pairing of selected CDR variants followed by further selection. This was followed by a second round of recombination, where a new library was prepared in which all four CDRs were simultaneously randomized by combining previously selected CDR mutant populations HCDR2 with HCDR3 and LCDR1 with LCDR3, again followed by further selection and screening.

Aslo0452 retained all of the attributes of the parental clone, asyn0087, and demonstrated a 1000-fold improvement in affinity (K_D, equilibrium dissociation constant) at 1.1 nM (95% CI = 0.3–2.3 nM) by Octet.

Single point mutagenesis was performed on aslo0452 in each of the CDRs where improvements in IC_{50} potency had been observed. Each amino acid position chosen was mutated individually through all 20 possible amino acid residues, and again screened for improvement in IC_{50} compared to aslo0452 IgG. Multiple residues across four CDRs (HCDR2, HCDR3, LCDR1 and LCDR3) were identified and combined on the aslo0452 backbone, and again assessed for improved IC_{50} compared to aslo0452. From this final round of mutagenesis, MEDI1341 was identified. The affinity of MEDI1341 (K_D, equilibrium dissociation constant) for monomeric human α-synuclein, when measured as an IgG using the KinExA platform, was 74 pM (95% CI = 15–177 pM) (see Fig. S2B for KinExA binding curve data). The CDR sequence differences between the lead isolate, asyn0087, and the final affinity optimized lead are summarized in Fig. S2C. The affinity optimization and screening cascades are summarized in Fig. S2D.

MEDI1341 binds to the C-terminal region of α-synuclein. Fig. 1A shows that MEDI1341 binds to full length α-synuclein (1–140) and all truncated forms encompassing the C-terminal region (aa 61–140, aa 96–140, ΔNAC (Non-Aβ component of Alzheimer's disease amyloid, missing aa 61–95) with the exception of a truncated form that lacks amino acids 103–129 (Δ103–129), suggesting that MEDI1341 has an epitope located in this region of the protein.

MEDI1341 is specific for α-synuclein over the other synuclein family members, β-synuclein and γ-synuclein. This was determined using an HTRF epitope competition assay that measured MEDI1341 binding to biotinylated human α-synuclein in solution. α-Synuclein, β-synuclein and γ-synuclein were titrated in the assay, and the selectivity of MEDI1341 was measured as the degree of inhibition of biotinylated human α-synuclein binding to MEDI1341. IC_{50} values obtained with α-synuclein, β-synuclein and γ-synuclein proteins are shown in Fig. 1B. No binding of MEDI1341 to β-synuclein and γ-synuclein was observed at concentrations up to 5 μM.

Immunohistochemical staining of the human SH-SY5Y neuroblastoma cell line with MEDI1341 or with two commercially available anti α-synuclein antibodies 4D6 and 4B12 revealed strong

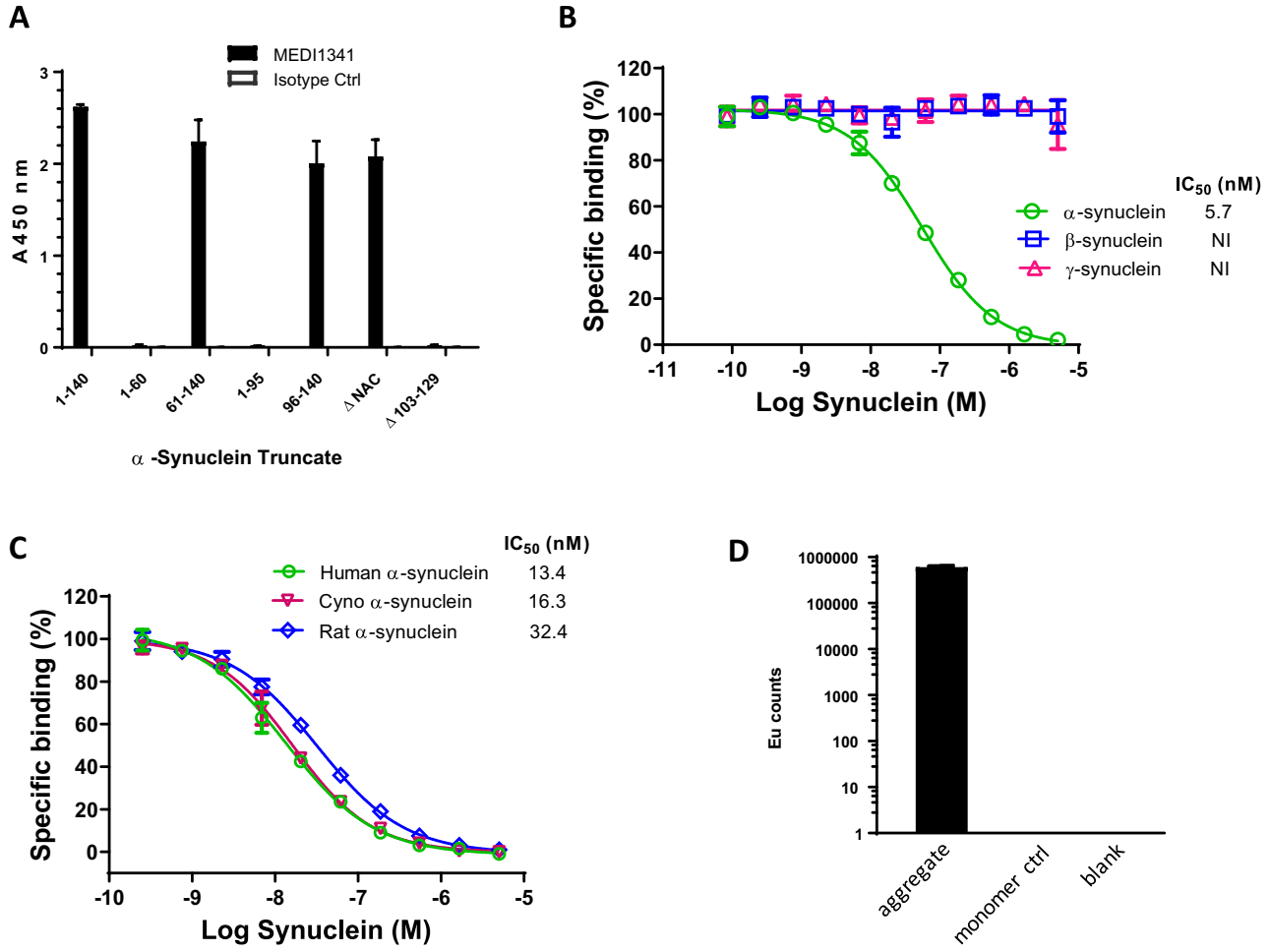

Fig. 1. Epitope binning, specificity and selectivity of MEDI1341. (A) Binding of MEDI1341 to recombinant α-synuclein and a range of truncated forms representing defined regions of the protein was determined by ELISA. 1–140: full length α-synuclein; 1–60: N-terminal region only; 61–140: Non-Aβ component of Alzheimer's disease amyloid (NAC) plus C-terminal region; 1–95: N-terminal and NAC regions; 96–140: C-terminal region only; ΔNAC: NAC region deleted (aa61–95); Δ103–129: α-synuclein missing amino acids 103–129. (B) MEDI1341 is specific for α-synuclein as determined by an epitope competition HTRF assay by titration with unlabeled α-synuclein, β-synuclein and γ-synuclein. IC_{50} values for each synuclein family member are given (NI = no inhibition). (C) The species cross-reactivity profile of MEDI1341 was determined in a similar HTRF assay by titration of unlabeled human, cynomolgus monkey, and rat α-synuclein. IC_{50} values for each species of α-synuclein are given. (D) MEDI1341 detects recombinant human aggregated α-synuclein by pair-wise ELISA using a mouse and human version of MEDI1341 as the capture and detection antibody, respectively. When monomeric α-synuclein (monomer ctrl) was used as the target antigen no assay signal was observed, indicating the specificity of the assay for detecting aggregated α-synuclein.

immunoreactive signals within the cell soma. In contrast, immunohistochemical staining of human BT20 and SKBR3 breast cancer cell lines, which are devoid of α-synuclein or express it at extremely low levels, respectively (Lawrence et al., 2015) with MEDI1341, 4D6 or 4B12 showed no observable immunoreactive signal for α-synuclein in the cell soma above background signals seen with an isotype control antibody (see Fig. S3). These data further indicate that MEDI1341 is specific for α-synuclein.

MEDI1341 binding to human, cynomolgus monkey and rat α-synuclein was assessed using a similar HTRF based epitope competition assay. IC_{50} values are shown in Fig. 1C. MEDI1341 binds to human and cynomolgus α-synuclein with IC_{50} values of 13.4 nM and 16.3 nM, respectively, and binds to rat α-synuclein with an IC_{50} value of 32.4 nM, which is approximately 2–3 fold lower than binding to human α-synuclein.

MEDI1341 binds to aggregated forms of human α-synuclein as determined using a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA®) antibody capture assay (Fig. 1D). The DELFIA assay specifically measures the capture of aggregated human α-synuclein in a pair-wise enzyme-linked immunosorbent assay (ELISA) using a mouse and human version of MEDI1341 as the capture and detection antibody, respectively. In the assay, only aggregated human α-synuclein is captured by MEDI1341 and detected, due to the presence of multiple copies of the same epitope on an aggregate. The specificity of the DELFIA assay for detecting aggregated α-synuclein was confirmed by replacing it with monomeric α-synuclein as the target antigen in the assay. Only one binding epitope is present in the monomer and so the detecting MEDI1341 antibody cannot bind in the presence of capture antibody, thus no monomer signal was observed in the assay.

MEDI1341 recognises disease relevant forms of human α-synuclein as shown by immunohistochemical staining of fresh frozen PD brain tissues (Fig. 2A). More specifically, MEDI1341 detected Lewy bodies, Lewy neurites and Lewy dots of the substantia nigra and amygdala in PD brain tissue. An isotype-matched control antibody demonstrated no staining in PD brain tissues (Fig. 2A - panel ix). MEDI1341 also detected α-synuclein in normal brain sections (Fig. 2A – panel viii and inset), which is to be expected given that α-synuclein is brain-enriched, especially at presynaptic nerve terminals. At the biochemical level, we demonstrated by immunoblotting that MEDI1341 recognises soluble α-synuclein isolated from control human brain samples and also binds sarkosyl-insoluble higher molecular weight α-synuclein aggregates extracted from the brains of patients with PD or dementia with Lewy

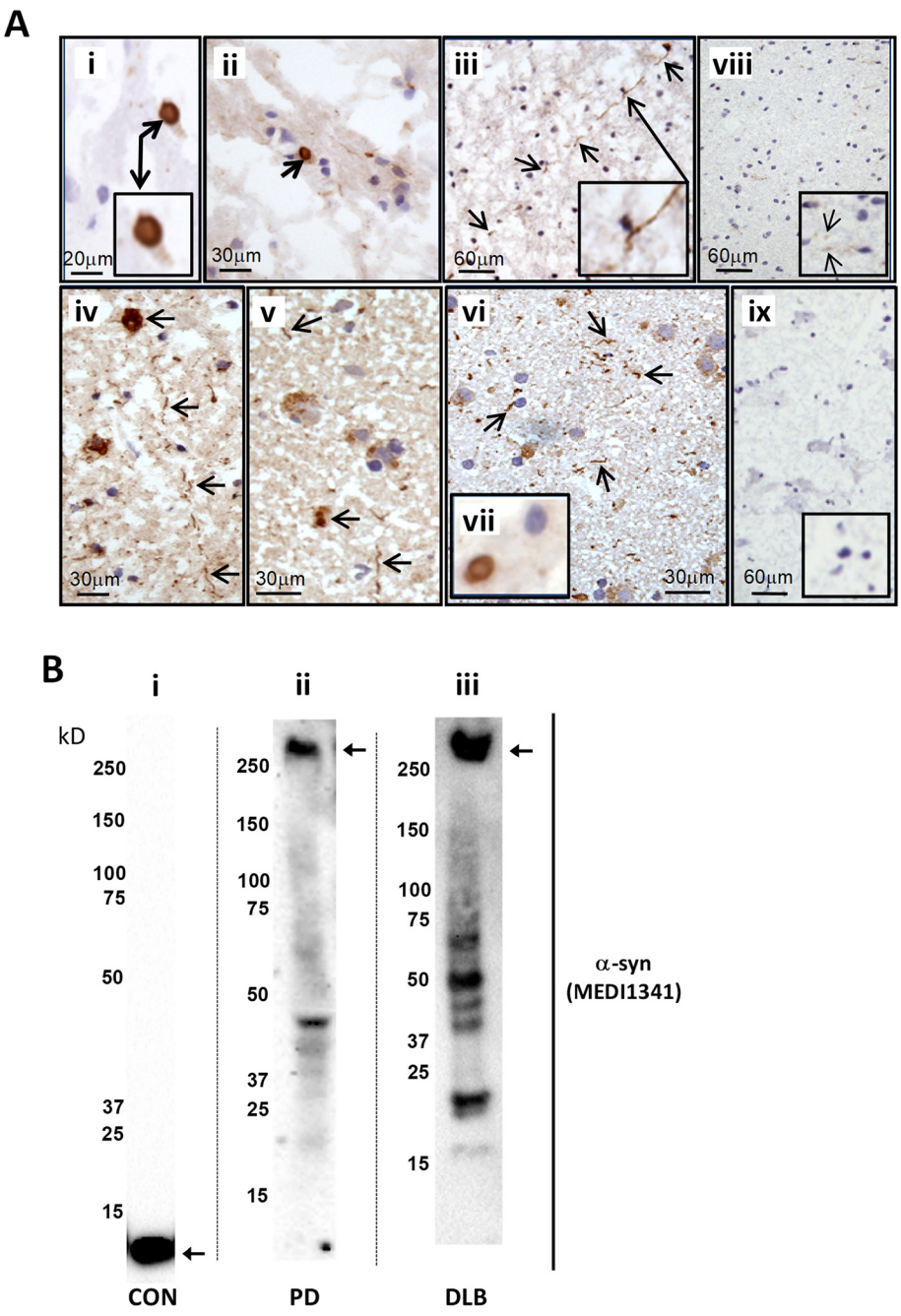

Fig. 2. α-Synuclein immunohistochemistry and biochemistry with MEDI1341 in Parkinson's disease and normal brain tissues. (A) Panels (i-iii) show MEDI1341 staining Lewy bodies (panels i and ii) and Lewy neurites (panel iii) of substantia nigra in Parkinson's disease brain sections. Inset in panel (i) and black arrows in panels (i) and (ii) indicate intense Lewy body staining. Arrows and inset in panel (iii) show Lewy neurite pathology stained by MEDI1341. Panels (iv, v, vi, vii) show MEDI1341 staining Lewy bodies, Lewy neurites and Lewy dots of the amygdala in Parkinson's disease brain sections, as indicated by black arrows. Panel (viii and inset) shows low-level neuropil staining of α-synuclein in cells of the temporal cortex in a normal control brain section with MEDI1341. Panel (ix and inset) shows immunohistochemistry with an isotype matched control antibody demonstrating no staining of the amygdala in Parkinson's disease brain sections. (B) PBS/Triton X-soluble control human brain fraction (CON) (i), and sarkosyl- insoluble brain fractions isolated from a Parkinson's disease (PD) patient (ii) or from a dementia with Lewy bodies (DLB) patient (iii) were probed on immunoblots for α-synuclein (α-Syn) using MEDI1341. Arrows in (i-iii) indicate the major immunoreactive α-synuclein signal on each blot.

bodies (DLB) (Fig. 2B). MEDI1341 immunoblots shown in Fig. 2B were stripped and reprobed with a commercially available anti-α-synuclein antibody LB509 that also has its epitope located within the C-terminal region of α-synuclein. Similar to MEDI1341, LB 509 bound to soluble monomeric α-synuclein isolated from control human brain and also recognized an insoluble higher molecular weight (> 250 kD) α-synuclein band extracted from PD brain (see Fig. S4).

3.4. *MEDI1341 inhibits cell-to-cell transmission of α-synuclein in an in vitro human model*

We assessed whether MEDI1341 can inhibit cellular transmission of α-synuclein aggregates using human recombinant *preformed α-synuclein fibrils* (pffs) in a trans-well SH-SY5Y cell culture assay system where SH-SY5Y "donor" cells are treated with 0.2 μM pre-sonicated pffs for 24 h before assessing pff transmission and uptake into "recipient" SH-SY5Y cells (see Fig. 3A).

Before testing the ability of MEDI1341 to block cell-to-cell transmission of pffs, we first determined which species of α-synuclein MEDI1341 recognises in the pffs preparation. We found that MEDI1341 could immunoprecipitate monomers and higher molecular weight oligomeric α-synuclein species in the range of 28 to 200 kD from the trans-well culture medium of pff-treated donor SH-SY5Y cells, as detected by immunoblotting (Fig. 3B – lane 6). No α-synuclein signal was observed on immunoblots when MEDI1341 immunoprecipitations were performed using media from non pff-treated donor SH-SY5Y cells (Fig. 3B – lane 2) or when immunoprecipitations were performed using an isotype control antibody (Fig. 3B – lanes 3 & 5). In the pff transmission assay, addition of MEDI1341 to the cell culture medium led to a robust concentration-dependent decrease in the number of α-synuclein-positive puncta in the recipient cells when compared to isotype control mAb-treated cells (Fig. 3C, D). The transmission of pffs from donor cells to

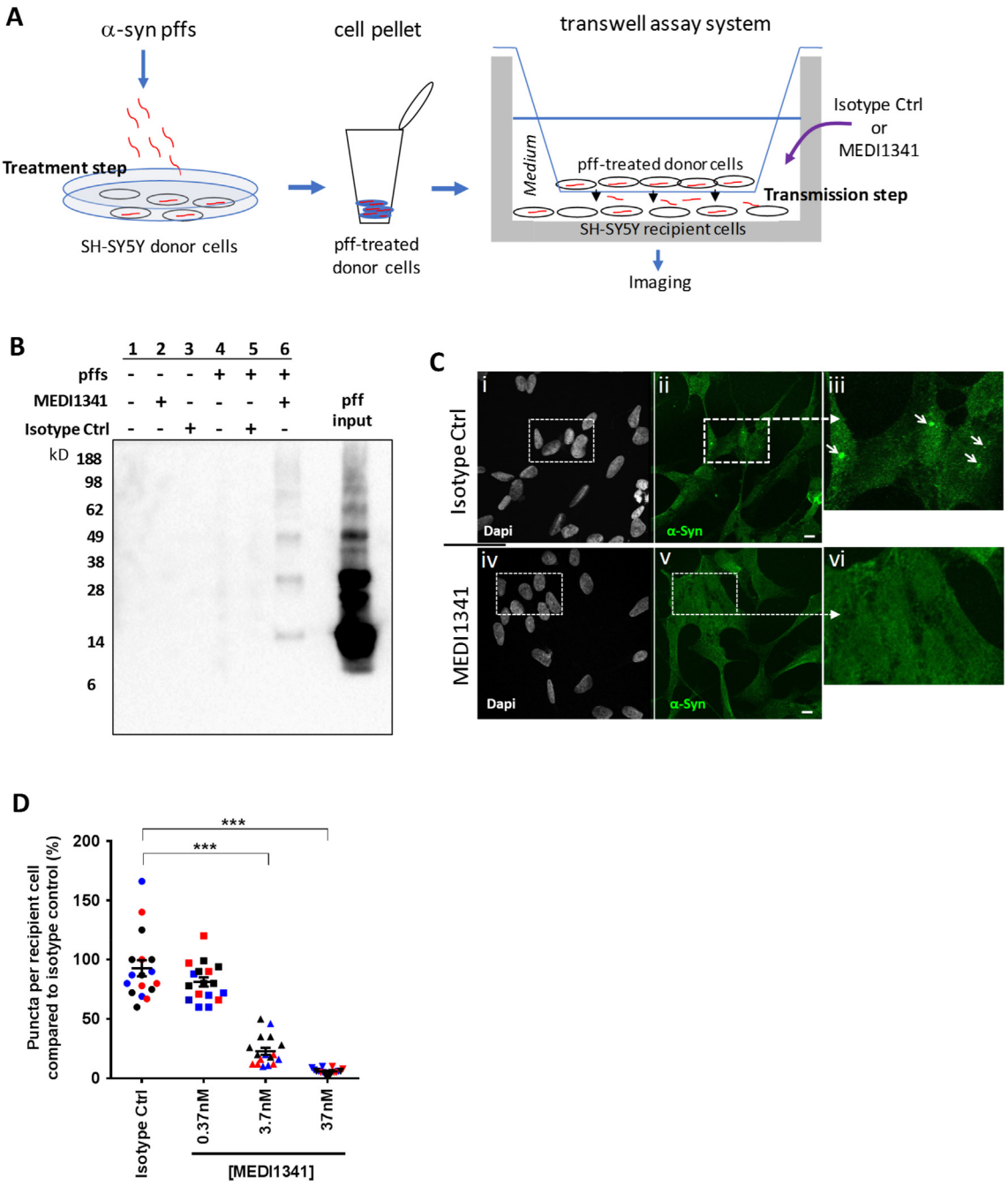

Fig. 3. MEDI1341 binds to α-synuclein preformed fibrils (pffs) and inhibits pff transmission in human cells. (A) Schematic of the SH-SY5Y trans-well α-synuclein cell-to-cell transmission assay. (B) Culture medium from non-pff-treated (lanes 1–3) or pff-treated (lanes 4–6) SH-SY5Y cells was left alone (lanes 1 & 4) or incubated with isotype control antibody (lanes 3 & 5) or with MEDI1341 antibody (lanes 2 & 6). Protein G immunoprecipitations were performed followed by immunoblotting for α-synuclein using an anti-α-synuclein antibody. (C) SH-SY5Y recipient cells from the pff trans-well transmission assay were immunofluorescently stained for α-synuclein (α-Syn) and counterstained with Dapi nuclear stain, followed by confocal imaging microscopy. Pff transmission assay culture medium was pretreated with 37 nM isotype control antibody (panels i, ii, iii) or 37 nM MEDI1341 antibody (panels iv, v, vi) for 24 h prior to imaging. Representative confocal images show numerous α-synuclein puncta in recipient cells with isotype control antibody treatment (ii, iii – white arrows) and an absence of α-synuclein puncta in recipient cells with MEDI1341 treatment (v, vi). Scale bar in panels (ii, v) = 10 μm. (D) Quantification of recipient cell α-synuclein puncta obtained from analysis of immunofluorescence images represented in (C). Isotype control antibody and MEDI1341 antibody data are represented as scatter dot plots and the mean ± SEM values for each treatment are also indicated and represent 3 independent experiments. Each color represents data from different experiments. ***$P < .001$ *vs* Isotype control antibody; 1-way ANOVA with Dunnett's multiple comparisons post-test.

recipient cells was inhibited at 3.7 nM and 37 nM MEDI1341 by 70% and 87%, respectively (Fig. 3D). Thus, in a human *in vitro* cell model of α-synuclein cell-to-cell propagation, when MEDI1341 is added to the culture medium it binds to extracellular monomeric and oligomeric/filamentous α-synuclein (pffs) and blocks transmission, strongly reducing the levels of α-synuclein aggregates found in the recipient SH-SY5Y cells.

We tested whether the MEDI1341-mediated reduction in α-synuclein pff puncta in recipient SH-SY5Y cells could be explained by MEDI1341 being taken up into recipient cells and sterically hindering the binding of the sheep anti-α-synuclein antibody used for the confocal immunofluorescence analysis. To address this, we performed the pff

transmission assay under control conditions (without any antibody treatments during the course of the experiment) and then immunostained recipient cells with sheep anti-α-synuclein antibody in the absence or presence of MEDI1341, and counted the number of pff puncta. We found that the presence of MEDI1341 had no statistically significant effect on the number of puncta observed in recipient cells (Fig. S5). This confirmed that the MEDI1341-mediated reduction in α-synuclein puncta in recipient cells in the experimental transmission study was most likely due to the inhibition of pff transmission between donor and recipient cells *via* MEDI1341 sequestration of pffs in the trans-well media and inhibition of pff uptake into recipient cells.

3.5. In vivo pharmacokinetic and target engagement properties of MEDI1341

To aid selection of an appropriate antibody dosing regimen (dose (mg/kg) and frequency) for downstream proof-of-mechanism (PoM) and efficacy studies assessing the ability of MEDI1341 to prevent α-synuclein spreading *in vivo*, we first assessed the pharmacokinetic (PK) and pharmacodynamic properties of MEDI1341 in the periphery and CNS (CSF and ISF compartments) following iv administration to rats. The parameters assessed included antibody half-life ($t_{1/2}$) in plasma, antibody concentration/exposure in the brain, and target engagement in the CNS determined by an assay measuring free α-synuclein levels. The mean plasma pharmacokinetic parameters after iv administration of MEDI1341 are fully summarized in Table S1A. When administered as a single iv dose of 3 mg/kg, MEDI1341 exhibited a mean clearance (CL) of 6.53 mL/day/kg and a mean terminal volume of distribution (V_z) of 44.2 mL/kg, which resulted in a plasma $t_{1/2}$ of 4.79 days. Comparable values were determined after iv administration of MEDI1341 at 10, 30 and 100 mg/kg dose levels. Our calculated plasma $t_{1/2}$ of MEDI1341 in rats falls within a recently published $t_{1/2}$ range of 3–10 days for a variety of different human antibodies in rats following iv administration (Clynes, Towers, Presta, and Ravetch, 2000). The mean maximum observed concentration (C_{max}) and mean area under the concentration-time curve from time zero up to the last measurable timepoint (AUC_{last}) of MEDI1341 increased in a dose proportional manner from 3 mg/kg to 100 mg/kg with the exception of mean AUC_{last} for the 100 mg/kg dose which increased in a slightly less than dose proportional manner (Fig. 4A).

The mean CSF pharmacokinetic parameters of MEDI1341 after iv administration to rats are summarized in Table S1B. Briefly, following iv administration of MEDI1341, the mean CSF C_{max} increased in essentially a dose proportional manner from 3 mg/kg to 100 mg/kg whereas the mean AUC_{last} in CSF increased in a slightly less than dose proportional manner between doses (Fig. S6). Comparing mean AUC_{last} CSF/plasma data for the 3 mg/kg to 100 mg/kg dose range indicated that the CSF concentration of MEDI1341 was approximately 0.3% of that observed in plasma.

To assess whether systemically administered MEDI1341 can enter the CNS and sequester extracellular α-synuclein we developed a biomarker/target engagement assay that measures the levels of free (unbound) extracellular α-synuclein in the cerebrospinal fluid (CSF) and brain interstitial fluid (ISF) after removal of extracellular α-synuclein bound to MEDI1341 (Fig. S7A and supplementary methods). Following iv administration of MEDI1341 to rats, a dose- and time-dependent reduction in free α-synuclein concentrations was observed in CSF over the 4-day time course of the study, with peak suppression of free α-synuclein levels across the MEDI1341 dose range occurring between 2 h and 24 h post-dose (Fig. 4B). The maximum suppression of free α-synuclein levels in CSF after MEDI1341 treatment was 81.5 ± 4.6% at a dose of 100 mg/kg. MEDI1341 suppressed free α-synuclein levels for the duration of the study at all doses tested, but there was a gradual time-dependent return towards baseline (vehicle levels) across the entire dose range. The suppression of free α-synuclein levels in CSF 4 days after iv dosing of 100 mg/kg MEDI1341 was still 45 ± 4.7% (Fig. 4B).

CSF is a translationally and clinically relevant compartment for biomarker measurements. However, to assess whether MEDI1341 can bind to and sequester extracellular α-synuclein directly in the brain parenchyma, we used a push-pull *in vivo* microdialysis method to monitor α-synuclein levels in the interstitial fluid (ISF) that bathes the brain (Fig. S7B). Serial ISF samples were taken from the prefrontal cortex of rats that had been iv dosed with vehicle or MEDI1341 and free α-synuclein levels were determined using an α-synuclein-specific ELISA. A single dose of 30 mg/kg MEDI1341 elicited a rapid, robust and statistically significant reduction in free α-synuclein levels in ISF (Fig. 4C). A maximum suppression of approximately 75% was observed by 4 h post-MEDI1341 dose when compared to vehicle-treated rats, which was maintained at the timepoints measured (up to 10 h) (Fig. 4C). We determined the concentration of MEDI1341 (30 mg/kg) in ISF following iv dosing and compared this to the measured plasma concentration of MEDI1341 in order to obtain an indication of the percentage of iv-administered MEDI1341 that enters the brain (ISF). Maximal antibody concentration in the ISF was observed between 3 h and 5 h post-MEDI1341 dose (Fig. 4D), which correlates well with the observed timing of maximal suppression of free α-synuclein levels in the ISF by MEDI1341 (4 h – Fig. 4C). Comparison of the mean plasma AUC *versus* the mean ISF AUC indicated that ~0.4% of MEDI1341 in the plasma crossed the blood-brain-barrier (BBB) into the ISF. The ISF MEDI1341 concentration correlates well with the estimated CSF concentration of MEDI1341 (0.3%) generated from analysis of the plasma and CSF data described in Fig. 4A and S6, respectively.

3.6. Pharmacokinetic and target engagement studies with MEDI1341 in monkeys

As part of a pre-clinical study in cynomolgus monkeys conducted to support first time in human clinical trials with MEDI1341, we assessed the exposure of MEDI1341 in serum and CSF samples. MEDI1341 was administered once weekly for 13 weeks to monkeys at iv doses of 25, 75 and 150 mg/kg/week. Serum and CSF samples were taken at various timepoints and analysed for MEDI1341 concentration. Data from week 1 and week 13 of the study showed that levels of MEDI1341 in serum and CSF increased in a dose proportional manner across the 25 to 150 mg/kg range in terms of C_{max} and area under the concentration-time curve from pre-dose to 168 h post-dose ($AUC_{0\text{-}t}$). Across all 3 dose groups, the average CSF concentrations of MEDI1341 were approximately 0.2% of those observed in serum, which is consistent with the CNS exposure of MEDI1341 observed in our preclinical rat PK/PD studies.

We also assessed CNS target engagement of MEDI1341 in monkeys with respect to sequestration and lowering of free α-synuclein levels in the CSF. CSF was collected from the cisterna magna 3 days after the final dose was administered, followed by analysis of free α-synuclein levels in CSF. Our data showed that MEDI1341 elicited a dose-dependent suppression of free α-synuclein levels in the CSF with a maximum suppression of 88 ± 1.8% at the top dose of 150 mg/kg (Fig. 4E).

3.7. MEDI1341 blocks α-synuclein accumulation and spreading in vivo

We recently developed a novel mouse model of α-synuclein spreading. The model involves intrahippocampal injection of a lentiviral human α-synuclein construct which leads to high level expression of the protein, followed by the accumulation and spread of human α-synuclein to the contralateral hippocampus *via* neuroanatomically connected axons (Spencer et al., 2017). We tested our clinical candidate anti-α-synuclein antibody MEDI1341 in this model for its ability to attenuate α-synuclein spreading (see Fig. S8 for study design).

In order to mitigate potential formation of neutralizing anti-drug-antibodies following chronic weekly dosing of mice with a human antibody, the human antibody variable domains (heavy and light chain) of MEDI1341 were engineered onto a mouse immunoglobulin G1

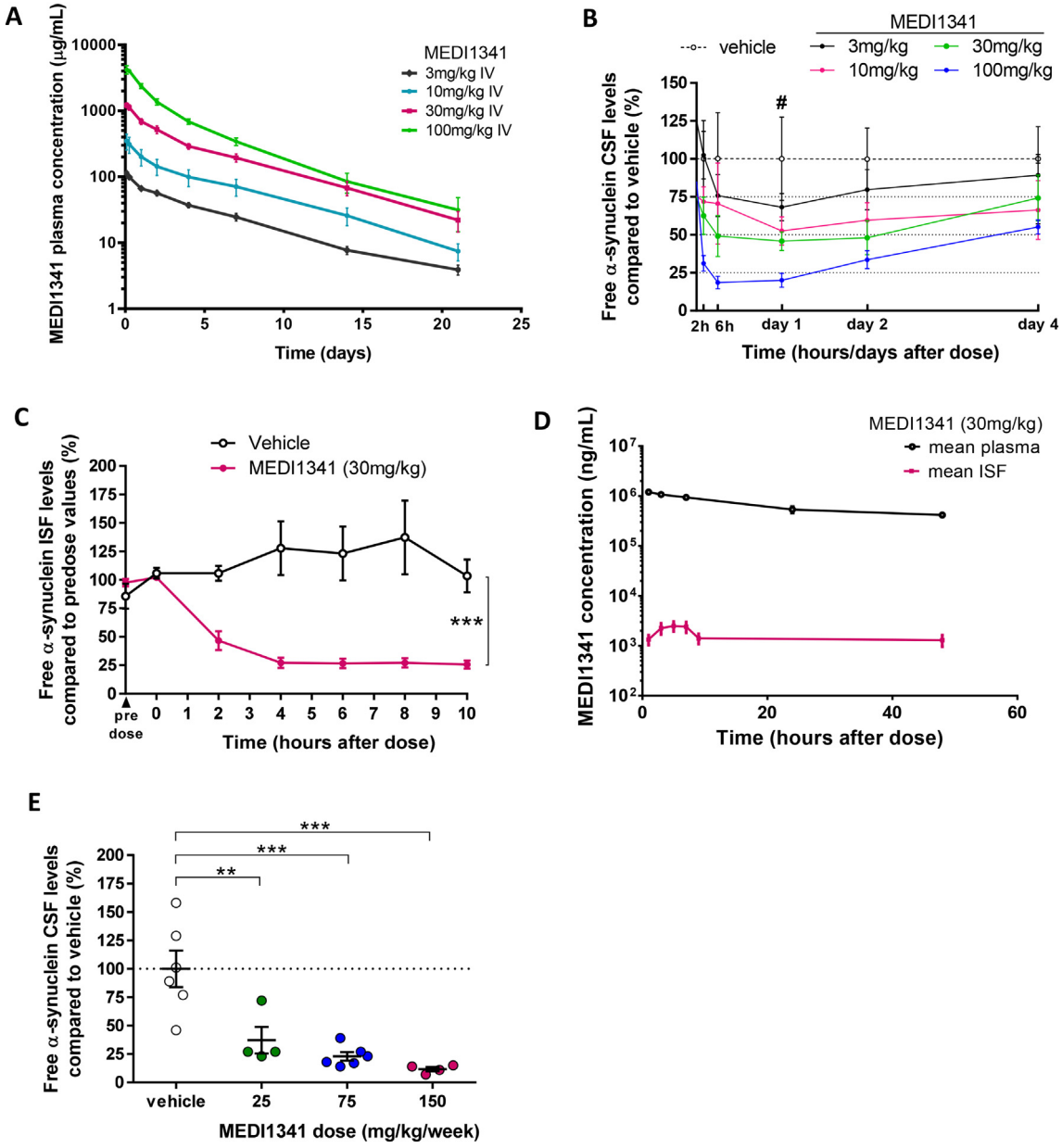

Fig. 4. MEDI1341 pharmacokinetics and target engagement in the CNS. (A) Log-linear plots showing MEDI1341 mean plasma concentration over time profiles after single dose iv administration to rats. Mean ± SEM values are shown. (B) Plots showing free α-synuclein cerebrospinal fluid (CSF) levels over time after single dose iv administration of vehicle control or MEDI1341 to rats. Free α-synuclein levels represent the pool of unbound α-synuclein remaining in experimental samples after removal of α-synuclein bound to MEDI1341 by immunoprecipitation with protein A beads. Vehicle control samples were similarly treated with protein A beads. For each treatment, data shown are the mean ± SEM value relative to vehicle at each timepoint (n = 4–9 rats per treatment group). # $P < .05$ (10 mg/kg, 30 mg/kg) and $P < .001$ (100 mg/kg) MEDI1341 *vs* vehicle control at day 1; 1-way ANOVA with Dunnett's multiple comparisons post-test. (C) Plots showing free α-synuclein interstitial fluid (ISF) levels over time after single dose iv administration of vehicle control or MEDI1341 to rats. For each treatment, data shown are the mean ± SEM value relative to pre-dose levels (n = 8–10 rats per treatment group). ***$P < .001$ MEDI1341 *vs* vehicle control at 10 h timepoint; unpaired two-tailed t-test. (D) Log-linear plots showing MEDI1341 mean plasma and interstitial fluid (ISF) concentration over time profiles after single dose iv administration of 30 mg/kg to rats. Mean ± SEM values are shown. (E) Plots showing free α-synuclein CSF levels after multiple dose iv administration of vehicle control or MEDI1341 to cynomolgus monkeys. α-Synuclein levels were measured 3 days after the last of 13 once-weekly doses. For each treatment, results shown are individual data points (circles), plus the mean ± SEM value relative to vehicle control (n = 4–6 animals per treatment group). **$P < .01$ (25 mg/kg) and ***$P < .001$ (75 mg/kg,150 mg/kg) MEDI1341 *vs* vehicle control; 1-way ANOVA with Dunnett's multiple comparisons post-test.

(IgG1) backbone. Further, to test the role of Fc-mediated antibody effector function(s) such as phagocytosis in any observed MEDI1341 efficacy *in vivo*, we evaluated two versions of MEDI1341; one which retained antibody Fc effector function, and MEDI1341-D265A which has an aspartic acid to alanine point mutation at position 265 in the mouse IgG fragment crystallizable (Fc) domain that results in loss of interaction between this isotype and low-affinity IgG Fc receptors (FcγRIIB and FcγRIII) that are present on cells including microglia. This mutation abolishes antibody Fc-mediated effector mechanisms (Clynes, Towers, Presta, and Ravetch, 2000). Mice were injected with lentiviral vector expressing human α-synuclein (LV-α-syn) into the right hippocampus followed one week later by intraperitoneal (ip) administration with MEDI1341 or the effector null version, MEDI1341-D265A, or with an isotype control mouse IgG1, weekly for 13 weeks. All IgGs were dosed at 20 mg/kg. Immunocytochemical analyses of isotype control antibody-treated mice revealed that α-synuclein immunoreactivity on the

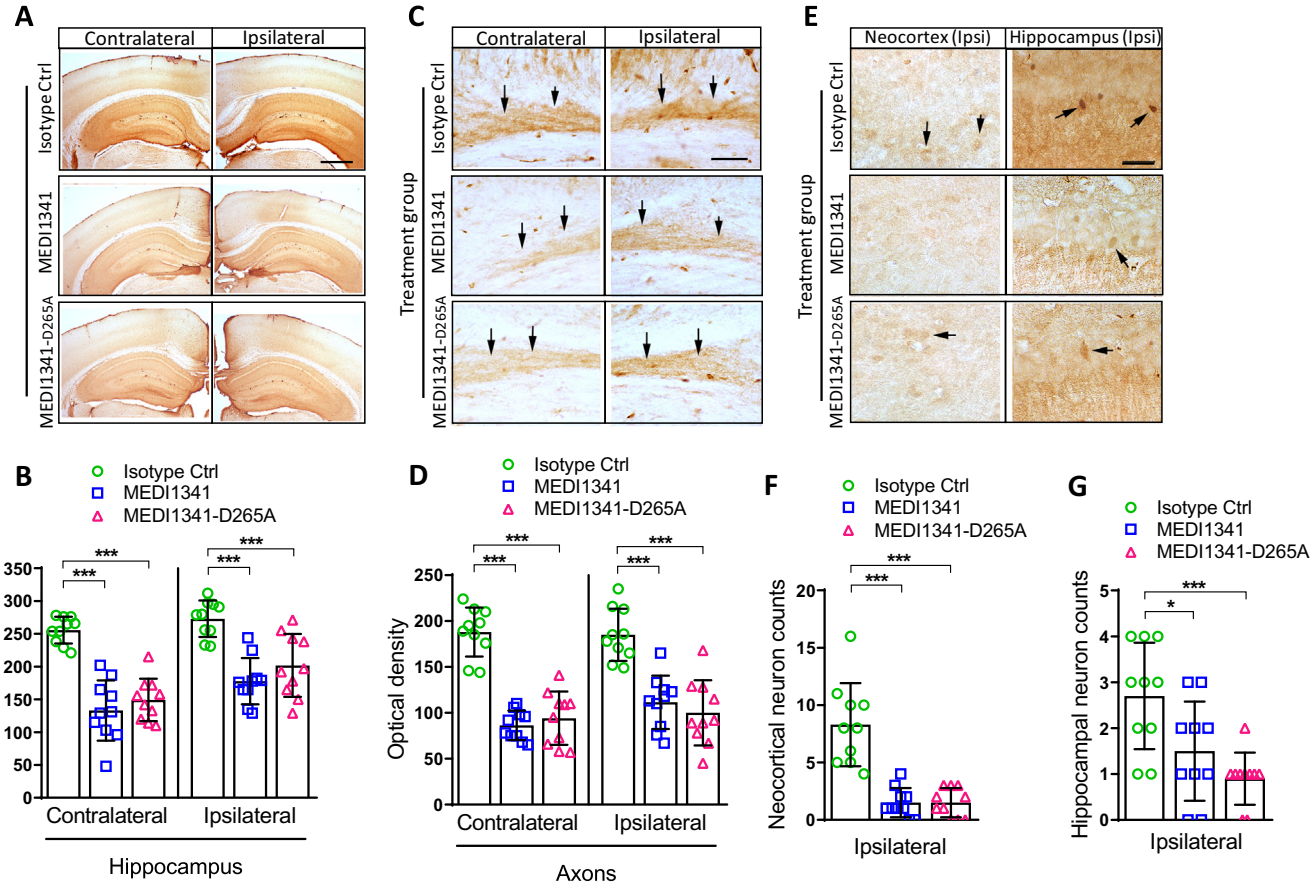

Fig. 5. MEDI1341 inhibits α-synuclein spreading and accumulation *in vivo*. (A) Immunohistochemical staining of coronal brain sections from mice injected with LV-α-syn into the right hippocampus (ipsilateral) and passively immunized with isotype control (Ctrl) antibody, MEDI1341 or MEDI1341-D265A effector null antibody. Representative images are shown. (B) Automated image analysis and quantification of α-synuclein immunoreactivity in the ipsilateral and contralateral hippocampal coronal sections represented in (A). (C) Immunohistochemical staining of α-synuclein along ipsilateral and contralateral trans-hippocampal axons (black arrows) in mice injected with LV-α-syn followed by passive immunization with isotype Ctrl antibody, MEDI1341 or MEDI1341-D265A. Representative images are shown. (D) Automated image analysis and quantification of α-synuclein immunoreactivity in the ipsi and contralateral trans-hippocampal axons represented in (C). (E) Immunohistochemical staining of α-synuclein deposits in ipsilateral (Ipsi) CA1 hippocampal neurons (right panels) and layer 5 neocortical neurons (left panels) following passive immunization of mice with isotype Ctrl antibody, MEDI1341 or MEDI1341-D265A. Examples of cellular α-synuclein deposits are indicated by black arrows. Representative images are shown. (F) Automated image analysis and quantification of α-synuclein deposits in ipsilateral layer 5 neocortical neurons represented in (E). (G) Automated image analysis and quantification of α-synuclein deposits in ipsilateral CA1 hippocampal neurons represented in (E). Data shown in (B), (D), (F), (G) are scatter dot plots with the mean ± SD value of 10 independent antibody treatments (n = 10 mice per antibody treatment group). *P < .05 and ***P < .001 *vs* Isotype Ctrl; 1-way ANOVA with Dunnett's post-test. Data shown in (F) and (G) represent the number of α-synuclein positive cells (neurons) per 0.1 sq. mm. Scale bar in (A) = 250 μm and scale bars in (C) and (E) = 25 μm.

LV-α-*syn*-injected ipsilateral side of the hippocampus was intense in the neuropil (Fig. 5A – top right panel). Similarly, the contralateral non-injected side of the hippocampus also displayed high levels of α-synuclein immunoreactivity in control antibody-treated mice (Fig. 5A – top left panel), indicating that the lentivirally-expressed α-synuclein had been efficiently transferred from the injected right hippocampus to the left hippocampus during the 4-month course of the experiment. We have shown previously in this model that only expressed α-synuclein protein spreads to the contralateral side, with no evidence of transfer of the lentivirus itself (Spencer et al., 2017).

We found that ipsilateral and contralateral hippocampal levels of α-synuclein in mice passively immunized with either MEDI1341 or the effector null mutant version MEDI1341-D265A were significantly lower than the levels in mice treated with isotype control mouse IgG1 (Fig. 5A, B and see Fig. S9 for additional representative hippocampal images). At higher magnification, α-synuclein immunoreactive deposits were observable along ipsilateral axons of the injected side and contralateral axons of the non-injected side of mice (Fig. 5C; black arrows show trans-hippocampal axons with associated α-synuclein deposits), suggesting that α-synuclein spreading to the contralateral hippocampus may principally occur along trans-hippocampal axonal connections. Immunocytochemical analysis of these trans-hippocampal tracts revealed that axonal α-synuclein deposits in mice passively immunized with MEDI1341 or MEDI1341-D265A were significantly lower than the levels in mice treated with isotype control mouse IgG1 (Fig. 5C). MEDI1341 or MEDI1341-D265A treatment led to a reduction in contralateral axonal α-synuclein levels of approximately 50% (Fig. 5D). In LV-α-*syn*-injected mice, at higher magnification strong α-synuclein immunoreactivity was detected in the neuropil of the ipsilateral hippocampus (Fig. 5E – top right panel) and to a lesser extent in the neuropil of the ipsilateral neocortex. (Fig. 5E – top left panel). In addition, intense deposits of α-synuclein were detected in a number of identifiable neuronal cell bodies (soma) in the CA1 region of the ipsilateral hippocampus and weaker α-synuclein immunoreactivity was detected in layer 5 neurons of the ipsilateral neocortex (Fig. 5E - top two panels, black arrows). Immunocytochemical analysis of these ipsilateral regions revealed that mice treated with MEDI1341 or MEDI1341 D265A had significantly less hippocampal CA1 neurons and neocortical layer 5 neurons containing α-synuclein deposits compared to isotype control mouse IgG-treated mice and the intensity of the α-

synuclein immunoreactivity in the surrounding neuropil and neurons was also significantly reduced in MEDI1341 and MEDI1341-D265A-treated mice (Fig. 5E, F, G). Finally, we addressed whether our observed reduction in ipsilateral and contralateral hippocampal α-synuclein levels after systemic MEDI1341 treatment in the human LV α-synuclein *in vivo* spreading model, could be explained by intraneuronal accumulation of MEDI1341 over the course of the study leading to masking of the anti-α-synuclein Syn-1 mAb epitope used in our immunocytochemistry protocol for quantifying α-synuclein levels, since MEDI1341 also binds with high affinity to α-synuclein. In a set of control experiments, we performed α-synuclein immunofluorescence in SH-SY5Y cells (a neuronal cell line that expresses endogenous human α-synuclein) with the Syn-1 mAb in the absence or presence of MEDI1341. After fixation and permeabilization procedures to access the intracellular compartments, SH-SY5Y cells were stained with Syn-1 alone or after preincubation of the cells with MEDI1341, followed by image analysis to quantify intracellular α-synuclein levels. We found that the intracellular levels of α-synuclein detected with Syn-1 alone were not diminished when cells were preincubated with MEDI1341 before Syn-1 immunostaining. (Fig. S10). We conclude from this control experiment that the reduction in α-synuclein levels and spreading observed *in vivo* after MEDI1341 treatment was not due to MEDI1341-mediated masking of the Syn-1 mAb-mediated α-synuclein signal in our immunocytochemical analyses.

Taken together, our spreading model data indicate that passive immunization of mice with MEDI1341 robustly attenuated the trans-axonal transfer of α-synuclein from one side of the hippocampus to the other, when compared with an isotype control antibody. Importantly, the effector null D265A mutant version of MEDI1341 was equally effective as MEDI1341 at reducing α-synuclein accumulation and spreading in the model.

4. Discussion

Current treatments for Parkinson's disease (PD) largely address dopamine-related motor disturbances and do not appear to have any meaningful impact on the underlying disease process (Kalia and Lang, 2015). The mechanisms underlying PD pathophysiology are not known, however, it is believed that α-synuclein plays a significant and central role (Goedert, Spillantini, Del Tredici, and Braak, 2013). Preclinical scientific evidence from animal models of PD supports a role for oligomeric or aggregated species of α-synuclein as key neurotoxic mediators leading to neurodegeneration in affected neuronal networks, and this may occur through a pathological spreading mechanism involving propagation of extracellular α-synuclein species (Bengoa-Vergniory, Roberts, Wade-Martins, and Alegre-Abarrategui, 2017). Passive immunization with experimental α-synuclein antibodies can abrogate α-synuclein spreading in animal models (Bae et al., 2012; Games et al., 2014; Masliah et al., 2011). If these preclinical animal findings translate to humans, it is anticipated that antibody-mediated sequestration and clearance of extracellular pathological α-synuclein will block its cellular uptake and attenuate spreading and the neurodegenerative process in PD and other α-synucleinopathies such as MSA. However, the forms of extracellular α-synuclein and precise mechanisms that are actually responsible for pathological spreading have not been fully resolved (Grozdanov and Danzer, 2018). With this uncertainty in mind, we hypothesized that the most efficient prevention of α-synuclein spreading throughout the CNS and hence the greatest clinical efficacy may occur with a high affinity α-synuclein antibody that can robustly sequester, neutralize and clear all forms of extracellular α-synuclein including monomeric species that may be key building blocks for oligomerization and aggregation, along with targeting pre-existing oligomers and aggregates. Our immunotherapeutic strategy appears to mechanistically differ from other α-synuclein antibodies currently in clinical testing (Weihofen et al., 2018; Schenk et al., 2017; Brundin, Dave, and Kordower, 2017).

We used *in silico* PK/PD modelling, which incorporated knowledge of human antibody PK, α-synuclein turnover and antibody-α-synuclein binding kinetics in both periphery and CNS, to predict the target antibody affinity required to potently suppress extracellular monomeric α-synuclein levels in the brain across a range of viable clinical doses. Our modelling approach predicted that an antibody with an affinity of < 100 pM would be required to achieve this goal. During our antibody lead optimization process, the most promising antibody clones in terms of maintaining selectivity on α-synuclein and those that showed the greatest affinity gains, were targeted to the C-terminal region of α-synuclein. As part of our antibody lead optimization process, we selected antibodies for further development based on minimal-to-no binding to other synuclein family members (β- and γ-synuclein), which could potentially act as a sink and reduce the potency of the antibody with respect to sequestering α-synuclein. Antibodies also needed to bind across multiple pharmacologically relevant species (rat, monkey and human) to enable critical translational pharmacology and toxicology testing required to support future clinical development. One clone was finally selected as our lead clinical candidate antibody, MEDI1341. We have shown that MEDI1341 has the desired high affinity for α-synuclein (74pM), is highly selective, and robustly suppresses free monomeric α-synuclein levels in the CNS of rats and cynomolgus monkeys. Our unbiased approach to the species of α-synuclein that MEDI1341 should target for greatest efficacy is further reflected in our data showing that it binds to a wide range of α-synuclein forms including soluble monomers, pffs secreted from SH-SY5Y cells, α-synuclein aggregates present in PD and DLB brain tissue, and from the fact that it appears to efficiently target *in vivo*-generated α-synuclein forms responsible for spreading in our lentiviral α-synuclein mouse model.

Neuronal activity can regulate the release of α-synuclein into the extracellular space during normal physiological conditions (Yamada and Iwatsubo, 2018; Emmanouilidou et al., 2011; Emmanouilidou et al., 2010). However, of pathological relevance and in line with our antibody targeting strategy, secretion of both monomeric and oligomeric/aggregated forms of α-synuclein is elevated under conditions of cellular stress such as proteasomal and mitochondrial dysfunction (Emmanouilidou et al., 2010; Lee et al., 2013; Jang et al., 2010; Danzer et al., 2012; Lee, Patel, and Lee, 2005). With respect to understanding the molecular mechanisms responsible for the spreading of pathological α-synuclein, cell-to-cell α-synuclein transmission models have been developed recently. For example, it has been shown that preformed recombinant α-synuclein fibrils (pffs) and α-synuclein oligomers can be internalised by cultured cells and neurons, and direct transfer of α-synuclein from donor to recipient cells with the formation of α-synuclein inclusions similar to Lewy pathology has also been demonstrated (Danzer et al., 2007; Volpicelli-Daley et al., 2011; Luk et al., 2009). A lentiviral-based α-synuclein *in vitro* transmission model with donor cell release of lentivirally-expressed α-synuclein and uptake into recipient cells has also been recently been published (Games et al., 2014). In our studies, we developed a pff-based cell-to-cell α-synuclein transmission model in a human-derived SH-SY5Y neuroblastoma cell line. The SH-SY5Y cell line has neuronal characteristics and expresses some dopaminergic markers and thus serves as a useful *in vitro* model for the study of neuronal function in relation to PD, recently reviewed in (Xicoy, Wieringa, and Martens, 2017). We showed that MEDI1341 can strongly attenuate the transmission of α-synuclein pffs from donor SH-SY5Y cells to recipient SH-SY5Y cells. The most obvious explanation for this blocking effect is that MEDI1341, when added to the cell culture medium of the pff transmission assay, binds to pffs released from the donor cells and prevents or slows their uptake into the recipient SH-SY5Y cells. The cellular release and uptake of pathologically relevant α-synuclein may play an important role in extracellular α-synuclein spreading *in vivo* (Grozdanov and Danzer, 2018).

CNS extracellular α-synuclein, be it monomeric, aggregated or a combination of the two, may be critically involved in the α-synuclein spreading process. Thus, it was important to demonstrate that MEDI1341 can enter the brain and sequester this extracellular pool of

α-synuclein before assessing its ability to interfere with α-synuclein spreading *in vivo*. We developed an antibody target engagement bioassay that quantifies how MEDI1341 affects free extracellular monomeric α-synuclein levels in ISF and CSF. Development and validation of bioassays sensitive enough to quantify oligomeric/aggregated forms of extracellular α-synuclein present in CSF or ISF and any sequestration effects by antibodies remains a significant challenge to the field. We found that upon single dose or repeat dose IV administration of MEDI1341 to rodents and monkeys, a small proportion of systemically circulating antibody (0.2–0.4% on average) entered the brain. These findings are in agreement with recently published data that similarly assessed CNS exposure of peripherally administered antibodies in rats and monkeys (Wang et al., 2018), and also align with the observed CNS penetration of an α-synuclein antibody in a recently published human clinical study (Jankovic et al., 2018). Our *in vivo* microdialysis study in rats with serial sampling of ISF over time from prefrontal cortex followed by quantification of MEDI1341 levels is to our knowledge one of the first reports assessing how much antibody directly and dynamically enters the brain parenchyma following systemic iv administration; previous reports have typically measured antibody levels in the CSF compartment as a surrogate measure of brain/CNS penetration, as exemplified in (Wang et al., 2018). Our calculated ISF and CSF exposures of MEDI1341 were very similar *i.e.* 0.4% and 0.3% of serum levels, respectively, which may not be surprising given the known communication between ISF and CSF (Lei, Han, Yuan, Javeed, and Zhao, 2017). Despite the relatively small amount of MEDI1341 entering the CNS from the periphery, it led to a rapid, dose-dependent sequestration of extracellular α-synuclein, robustly lowering the free levels of the protein at the highest doses tested. This MEDI1341-mediated suppression of free extracellular α-synuclein concentrations in the CNS was maintained over a period of several days following single iv doses to rats and over a period of weeks after repeat iv doses to monkeys.

Testing the ability of MEDI1341 to interfere with α-synuclein spreading *in vivo*, we demonstrated that peripherally administered MEDI1341 robustly attenuates the trans-axonal propagation of LV α-synuclein from one side of the hippocampus to the other in a novel mouse model of α-synuclein propagation. In this model, LV α-synuclein is expressed on one side of the hippocampus (ipsilateral) and transfer or propagation to the contralateral hippocampus is observed over time. However, it must be considered that ipsilateral to contralateral propagation of both mouse α-synuclein and LV-expressed human α-synuclein, through a cross-seeding mechanism, may be occurring in the model; we did not distinguish between the two species with our use of anti-α-synuclein mAb Syn-1 for immunocytochemical analysis of α-synuclein spreading, and MEDI1341 binds to human and rodent α-synuclein.

Our spreading model data supports previous reports showing that α-synuclein can propagate between connected brain regions or circuits. (Luk et al., 2012a; Luk et al., 2012b; Recasens et al., 2014; Watts et al., 2013; Masuda-Suzukake et al., 2013). With respect to *in vivo* mechanisms of α-synuclein spreading or propagation, we believe that the α-synuclein detected in the contralateral hippocampus of our LV α-synuclein model involves trans-synaptic spreading of pathological α-synuclein and is not simply due to physiological transfer of α-synuclein along axons. We have previously demonstrated the presence of dystrophic axons and abnormal patterns of α-synuclein accumulations in and around axons in our model (Spencer et al., 2017). Furthermore, in addition to the presence of α-synuclein in axons along commissural fibre tracts and the corpus callosum connecting both hippocampi, we previously reported the presence of intraneuronal α-synuclein aggregates (3–6 μm in size) within the molecular layer of dentate gyrus and subiculum on the contralateral (non-injected side) hippocampus. This is suggestive of trans-synaptic propagation of α-synuclein that would arguably have had to be transmitted across synapses in order to be localized there (Spencer et al., 2017). We observed that MEDI1341 treatment also significantly lowered ipsilateral (injected side) α-synuclein levels over the 3-month course of the study. This may be explained by the following scenario: LV α-synuclein-infected neurons/neuropil in and around the hippocampal injection site express and release α-synuclein into the extracellular space, which is followed by a local paracrine effect whereby locally connected neuronal networks take up the released LV α-synuclein and cause local spreading throughout the ipsilateral neuropil. MEDI1341 treatment sequesters and clears this local LV-α-synuclein propagation on the ipsilateral side, and this process may in part also contribute to the block of trans-axonal contralateral spreading observed with MEDI1341 immunotherapy.

Although we have demonstrated that MEDI1341 can enter the brain, sequester extracellular α-synuclein and attenuate α-synuclein spreading in the brain, a clear relationship between the extent and duration of antibody-mediated targeting and clearance of extracellular α-synuclein in the CNS that is required to prevent α-synuclein spreading *in vivo* is still lacking. Further animal studies assessing antibody PK/PD and effects on α-synuclein spreading are warranted to answer this important question.

Roles for extracellular free α-synuclein, exosomal α-synuclein and α-synuclein transmission *via* tunneling nanotubules have all been suggested to be involved in the spreading process (McCann, Cartwright, and Halliday, 2015). Our *in vitro* and *in vivo* spreading model data strongly support there being a major role for extracellular free α-synuclein in the transmission process. If exosomal or tunneling nanotube mechanisms were primary routes for spreading, our MEDI1341 antibody may have been less effective than it was. This is because large proteins such as antibodies do not readily cross membranes, thus they would not gain access to α-synuclein contained within exosomes or nanotubule structures. However, it is reasonable to assume that multiple mechanisms may be involved in the spreading process.

The data from our LV α-synuclein spreading model showed that systemic treatment of mice with two different versions of MEDI1341; one with retained effector function or one without effector function, similarly and robustly reduced the levels of α-synuclein detected along axons and blocked ipsi-to-contralateral transfer of α-synuclein between the hippocampal hemispheres. It seems most likely that antibody-mediated clearance mechanisms underlie these observed effects. However, precisely how the MEDI1341-α-synuclein complexes were cleared from the brain extracellular space remains to be fully determined. Our observation that an effector-null version of MEDI1341 blocked α-synuclein spreading equally as well as our MEDI1341 version with retained effector function suggests that microglial-directed clearance mechanisms, such as phagocytosis, are not required or essential for prevention of α-synuclein spreading, at least in our *in vivo* model. It is possible that a proportion of the extracellular α-synuclein assemblies bound to MEDI1341 were taken up by neurons and then degraded by the proteasome and the AAA-ATPase p97/VCP after antibody-mediated engagement of the cytosolic Fc receptor tripartite motif protein 21 (TRIM21), as has recently been shown for antibody-bound tau aggregate assemblies (McEwan et al., 2017). Another route for clearance of extracellular α-synuclein from the brain may involve bulk flow of MEDI1341 antibody-antigen complexes from ISF to the CSF and then into the periphery; a recent report has shown that rapid bulk flow of antibody-antigen complexes from the brain parenchyma to the periphery may be a significant clearance route (Noguchi et al., n.d.).

Our preclinical assessment of whether or not antibody effector function is required to block α-synuclein spreading was also performed to provide an important guide as to which antibody format (effector-retained or effector-null) to employ in human clinical testing. The fact that an effector-null version of MEDI1341 blocked α-synuclein spreading in our mouse model has the benefit that when used in humans it will significantly reduce or eliminate completely the risk of Fc-mediated effector cytotoxicity functions being engaged in the PD brain that may already be in a neuroinflammatory state.

In the CNS, cellular α-synuclein is enriched at presynaptic nerve terminals and appears to have neurophysiological roles in synaptic

vesicle biology, neurotransmission, synaptic plasticity and some cognitive functions (Burre, Sharma, and Sudhof, 2018). Antibodies do not readily enter cells, or do so with low efficiency, so we anticipate that MEDI1341 immunotherapy should have minimal to no impact on the abundant intracellular α-synuclein pools present in neurons and therefore not alter cellular α-synuclein functions in the brain. The biological roles of extracellular α-synuclein, if any, are not known. Whether chronic or prolonged sequestration of extracellular α-synuclein by MEDI1341 would pose a safety risk for humans will require further investigation. Extracellular α-synuclein has been implicated in trans-synaptic propagation of α-synuclein 'Lewy' pathology and neurodegeneration in PD and other synucleinopathies (Lee, Bae, and Lee, 2014). In a pathological setting like PD, we propose that sequestration and removal of extracellular α-synuclein with immunotherapy may be clinically beneficial by ameliorating α-synuclein spreading. Interestingly, α-synuclein immunotherapy might also mitigate α-synuclein-mediated synaptotoxicity, since a recent preclinical study has indicated that extracellular oligomeric α-synuclein impairs synaptic plasticity *via* a mechanism involving cellular prion protein (PrPC) (Ferreira et al., n.d.).

A biomarker that can detect and monitor α-synuclein pathology in the PD or MSA brain is currently lacking. Development of an α-synuclein positron emission tomography (PET) ligand could have critical utility in: patient diagnosis and selection for clinical trials, monitoring of disease progression, testing of the Braak spreading hypothesis in humans, and assessing drug response in clinical trials. Global research and development efforts in this important α-synuclein biomarker area are ongoing.

In conclusion, we have presented preclinical data for a novel α-synuclein specific antibody, MEDI1341, that we are developing as a treatment for α-synucleinopathies including PD. MEDI1341 is a high affinity (74 pM) and highly selective α-synuclein antibody that interacts with multiple forms of α-synuclein (monomeric, aggregated). We have shown that MEDI1341 can rapidly enter the brain, and, once there, robustly sequester extracellular α-synuclein. MEDI1341 blocks cell-to-cell transmission of α-synuclein pffs *in vitro*, and inhibits α-synuclein propagation *in vivo*, with an effector null version equally as effective as one with retained effector function. We believe that the combined properties and characteristics of MEDI1341 described above are unique and differentiate it from other α-synuclein antibodies currently in human clinical testing (Weihofen et al., 2018; Schenk et al., 2017; Brundin, Dave, and Kordower, 2017). MEDI1341 is now in early clinical development. A phase 1 clinical study testing the safety, tolerability and PK of MEDI1341, as well as assessing its ability to sequester α-synuclein in the CNS, has recently begun.

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Author contributions

DJS, MP, LI, LC, MS, IG, MM, BD, EM, KT, AB, TV, CD, IC designed the studies. DJS, LC, LI, GR, AN, JR, JPA, FC, EC, GF, CL, AB, ER performed the experiments. MP, DJS wrote the manuscript. JR, LB, EM analysed the immunohistopathology.

Declaration of Competing Interest

All authors except LC, ER, BS, EM, ES were employed by AstraZeneca or MedImmune when the work was executed and may currently hold AstraZeneca stock or stock options. MP, DJS and LI are named as inventors on patent W02017207739 filed by MedImmune Ltd., which discloses, in part, in this manuscript.

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Appendix A. Supplementary data

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