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Fyn Inhibition Rescues Established Memory and Synapse Loss in Alzheimer Mice

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Abstract

Objective—Currently no effective disease modifying agents exist for the treatment of AD. The Fyn tyrosine kinase is implicated in Alzheimer's disease (AD) pathology triggered by amyloid- β oligomers (A β) and propagated by Tau. Thus, Fyn inhibition may prevent or delay disease progression. Here, we sought to repurpose the Src family kinase inhibitor oncology compound, AZD0530, for AD.

Methods—The pharmacokinetics and distribution of AZD0530 were evaluated in mice. Inhibition of A β signaling to Fyn, Pyk2 and Glu receptors by AZD0530 was tested by brain slice assays. After AZD0530 or vehicle treatment of wild type and AD transgenic mice, memory was assessed by Morris water maze and novel object recognition. For these cohorts, APP metabolism, synaptic markers (SV2 and PSD-95), and targets of Fyn (Pyk2 and Tau) were studied by immunohistochemistry and by immunoblotting.

Results—AZD0530 potently inhibits Fyn and prevents both A β -induced Fyn signaling and downstream phosphorylation of the AD risk gene product, Pyk2, and of NR2B Glu receptors in brain slices. After 4 weeks of treatment, AZD0530 dosing of APP/PS1 transgenic mice fully rescues spatial memory deficits and synaptic depletion, without altering APP or A β metabolism. AZD0530 treatment also reduces microglial activation in APP/PS1 mice, and rescues Tau phosphorylation and deposition abnormalities in APP/PS1/Tau transgenic mice. There is no evidence of AZD0530 chronic toxicity.

Interpretation—Targeting Fyn can reverse memory deficits found in AD mouse models, and rescue synapse density loss characteristic of the disease. Thus, AZD0530 is a promising candidate to test as a potential therapy for AD.

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INTRODUCTION

Alzheimer's disease (AD) is the most common dementing illness afflicting over 5 million people in the USA¹. Despite growing efforts, there is no effective disease-modifying therapy presently available. The clinical dementia of Alzheimer's disease (AD) is coupled to a distinct pathology, with senile plaques consisting of Amyloid- β (A β) peptide, and with neurofibrillary tangles consisting of hyperphosphorylated Tau protein. Rare autosomal dominant cases of AD provides genetic proof that APP/A β pathways can trigger clinical AD²⁻⁵ while other APP mutations reduce AD risk⁶. Biomarker studies of late onset non-familial AD have revealed that A β dysregulation is the earliest reliably detected change in AD, consistent with A β serving as the trigger for the disease^{4, 7, 8}. Attention has focused on soluble oligomers of A β (A β o) as being specifically neurotoxic⁹⁻¹².

A critical early step in AD is the process by which extracellular A β o interacts with the neuronal surface to trigger downstream pathology, and studies of this pathway have implicated Fyn in AD pathophysiology. TBS-soluble A β derived from human AD stimulates neuronal Fyn via PrP^C¹³⁻¹⁵. Critically, human AD-derived A β species interact with and require PrP^C to suppress synaptic plasticity^{14, 16-18}. Furthermore, dendritic spine destabilization by A β o is not observed in *Prnp*^{-/-} and *Fyn*^{-/-} neurons¹⁴. These studies suggest that Fyn plays a central role in coupling A β o and PrP^C to changes in neuronal function. Consistent with this hypothesis, when Fyn mutants are crossed with APP transgenic mice, Fyn gain-of-function enhances AD-related phenotypes while Fyn loss-of-function ameliorates AD-related phenotypes^{19, 20}.

Similar to studies of A β o, studies of Tau have implicated Fyn mechanistically in AD. Fyn physically associates with Tau, and can phosphorylate tyrosine residues near the amino terminus²¹⁻²⁴. Moreover, the A β o-PrP^C complex-driven activation of Fyn leads to downstream Tau phosphorylation¹⁵. Critically, Fyn and Tau interact genetically to modulate synapse loss, behavioral deficits and electroencephalographic abnormalities in APP transgenic mice^{19, 20, 25}. Without functional Tau, Fyn is uncoupled from NMDA-Rs, and A β toxicity is rescued²⁶. Thus, PrP^C/Fyn signaling appears to couple A β and Tau pathologies.

AZD0530 (saracatinib) is an inhibitor of Src Family kinases (SFK), blocking Src with low nM potency and having activity against Fyn as well^{27, 28}. This inhibition led to its development as a therapy for solid tumors, because Src family kinases regulate tumor cell adhesion, migration and invasion, and cell proliferation²⁷. Clinical tolerability and oral bioavailability have been demonstrated, but Phase II studies have shown only limited benefit as a single agent in specific oncological indications²⁹⁻³³. We sought to repurpose this compound for use as disease-modifying AD therapy. Here we show that AZD0530 potently inhibits Fyn and blocks A β o signaling from Fyn to the AD risk gene product Pyk2. Moreover, orally administered AZD0530 penetrates the CNS in both mice and humans. For transgenic AD mice, AZD0530 treatment effectively rescues memory deficits, and restores synapse density. Not only does the compound block A β o signaling to Fyn in the neuronal synapse, it also reduces microglial activation and, in APP/PS1/Tau triple transgenic mice, AZD0530 suppresses Tau aggregation. Thus, preclinical studies support the testing of AZD0530 efficacy as a disease-modifying therapy for AD.

SUBJECTS/MATERIALS AND METHODS

Enzyme Inhibition Kinetics

Isoforms of Fyn, FynB (PV6346) and FynT (P3042), were obtained from Invitrogen Life Technologies. AZD0530 was generously provided by Astra Zeneca. The activity rate of the isoforms was measured using the Omnia Tyrosine Peptide 5 kit (KNZ3051). Pyk2 (PV4567) was purchased from Invitrogen Life Technologies as well. The binding of Pyk2 to ATP in the presence of AZD0530 was measured using the LanthaScreen Europium Kinase Binding Assay which consisted of Kinase tracer 236 (PV5592), LanthaScreen Eu-Anti-GST antibody (PV5594), and Kinase Buffer (PV3189) all from Invitrogen Life Technologies. All experiments were run on a VICTOR³ plate reader. Statistics to determine the kinetics of the interaction of enzyme and inhibitor were performed on Prism 6.0d.

Mice

All mice were cared for by the Yale Animal Resource Center and all experiments were approved by Yale's institutional animal care and use committee. As described previously^{14, 34, 35}, the C57BL/6J mice, and the APP^{swe}/PSen1^{E9}³⁶ mice on a C57BL/6J background were purchased from Jackson Laboratory. The 3xTg-AD mice³⁷ were a gift of Dr. F. LaFerla, via Dr. P. Lombroso at Yale.

Treatment of Acute Brain Slices

Mouse brains were dissected after decapitation. Acute brain slices (400- μ m thick) were prepared from 3-4 week C57BL/6J wild type male mice using a 1000 Plus Vibratome. Slices were then incubated in artificial cerebral spinal fluid (aCSF: 119 mM NaCl; 2.5mM KCl; 1.3 mM MgSO₄; 26.2 mM NaHCO₃; 11 mM D-glucose; 1.25 mM NaH₂PO₄; 2.4 mM CaCl₂ was added to aCSF after cutting) under constant oxygenation with 5% CO₂ and 95% O₂. Slices were preincubated with AZD0530 for 30-120 min, and subsequently treated with 1 μ M A β (monomer equivalent concentration, estimated oligomer of 10 nM) for 30 min in the same incubation chamber. A β was prepared by a well-characterized procedure including size exclusion chromatography and PrP^C binding^{13, 14, 34, 38}. Brain slices were lysed and homogenized in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA; 0.1% SDS; 0.5% deoxycholic acid; 1X PhosSTOP-Roche; 1X cOmplete-mini-Roche) and centrifuged at 100,000 $\times$ g for 20 min at 4C. Soluble fraction was mixed with Laemmli sample buffer (BIO-RAD) and analyzed by immunoblot.

Brain Tissue Collection

Mice were euthanized and immediately perfused with ice cold PBS for two minutes. The brains were dissected out and the hemispheres were divided. The left hemispheres were weighed and then snap frozen in liquid nitrogen to be used for biochemical analysis. The hemispheres were homogenized in three times the brain weight in a volume of TBS containing PhosSTOP (Roche) and cOmplete-mini (Roche) to extract the cytosolic fraction. The samples were centrifuged for 20 minutes at 100,000 $\times$ g at 4C. The supernatants were collected as TBS-soluble, while the pellets were dissolved in a volume of RIPA buffer containing PhosSTOP (Roche) and cOmplete-mini (Roche) equivalent to the amount used

with TBS. Again this was centrifuged for 20 minutes at $100,000 \times g$ at 4° and the supernatants were collected as the RIPA soluble extract, and the pellet saved as the RIPA-insoluble fraction. The concentration of TBS- and RIPA-soluble fractions was measured by BCA assay (Thermo Scientific).

In order to extract Tau from RIPA-insoluble fractions, the pellet was washed with RIPA-buffer once and resuspended in three volumes (w/v) of 70% Formic acid (Sigma) and then sonicated for 20 sec on ice. It was centrifuged at $100,000 \times g$ for 40 min at $4^{\circ}C$ and the supernatant was lyophilized. The solids material was resuspended in 1 ml of 10 M urea (pH 10) per gram of original tissue, and then neutralized with one-tenth volume of 1 M Trizma-base. After centrifugation at $80,000 \times g$ for 5 min, the supernatant was collected as the RIPA-insoluble fraction and protein content measured by Bradford assay (BIO-RAD).

The right hemispheres were drop-fixed in 4% paraformaldehyde for 24 hours at $4^{\circ}C$. Afterwards, forty μm parasagittal sections were cut from the hemispheres using a Leica WT1000S vibratome for APP/PS1 mice and A β , Iba1, GFAP and pPky2 stains. Alternatively for 3xTg-AD mice, one hemisphere was cryoprotected with sucrose, frozen and cryosectioned at $40 \mu m$.

Immunoblots

Samples were run on precast 4-20% Tris-glycine gel (BIO-RAD) and subsequently transferred to nitrocellulose membrane using iBlot gel transfer stacks (Novex-Life Technologies). After transfer, the membranes were washed in blocking buffer for 1 hour at $23^{\circ}C$ (Licor Odyssey blocking buffer #927-4000). This was followed by incubation overnight at $4^{\circ}C$ with the following primary antibodies: Fyn (Cell Signaling, 1:1000), p-SFK Tyr416 (Cell Signaling, 1:1000), Pyk2 (Cell Signaling, 1:1000), p-Pyk2 (Cell Signaling, 1:1000), phospho-NR2B Tyr1472 (Sigma M2442, 1:100), NR2B (BD Transduction Laboratories #610416, 1:1000), hTau (Abcam Tau-13 Ab24634, 1:5000), Tau (DAKO A0024 (K9JA), 1:5000), pTau-pS199/S202 (Invitrogen 44768G, 1:2000), pTau-pS396 (Invitrogen 44752G, 1:2000), 6E10 (Millipore MAB 1560, 1:1000), β III-tubulin (Abcam 18207, 1:1000), Actin (Cell Signaling, 1:10000). The membranes were then washed three times in TBST, which was then followed by a one hour incubation at room temperature with the appropriate secondary antibody (Odyssey donkey anti-rabbit, donkey anti-mouse, or donkey anti-goat all conjugated to IRDye 680 or IRDye 800). Membranes were washed again with TBST three times. Proteins on membranes were visualized using a Licor Infrared Imaging system. The results were analyzed using ImageJ software and normalized to either actin or β III-tubulin.

AZD0530 Pharmacokinetics

Mice were treated with AZD0530 at 0, 1, 2, 5 and 20 mg/kg/d in divided doses by oral gavage for three days. The daily dose was divided in two equal amounts. The vehicle for the drug was 0.5% w/v HPMC/0.1% w/v Polysorbate 80 and each dose volume was 250 μl . There were 5 mice in each dose group. The CSF was collected at the indicated times after the last dose using the method as described³⁹. The mice were then sacrificed to collect their brains and blood. The blood was collected in heparinized tubes and then centrifuged at $1000 g$ for 10 minutes at $4^{\circ}C$. The plasma (supernatant) was collected. The brain was removed

and one half of the forebrain collected. AZD0530 levels in these samples were determined by LC/MS (MPI Research) as described⁴⁰.

Human CSF was collected as part of Phase Ib multiple ascending dose study of AZD0530 in 24 subjects with mild to moderate AD, MMSE 19-26 (ClinicalTrials.gov identifier NCT01864655). All procedures were conducted with approval of the Yale University Human Investigations Committee, and under an Investigational New Drug submission to the Food and Drug Administration. Five subjects in the study received 125 mg oral AZD0530 daily for 28-35 days, and then underwent lumbar puncture 23-28 hours after their last dose. The CSF AZD0530 level was measured by the same LC/MS method as for the mouse samples.

Behavioral Testing

Animals were randomly assigned to treatment groups and the experimenter was unaware of both genotype and treatment status. All mice utilized in the experiments received twice daily dosing of either vehicle (0.5% w/v HPMC/0.1% w/v Polysorbate 80) or AZD0530 dissolved in vehicle by oral gavage. Mice were pretreated for 1-6 weeks prior to testing and continued to be treated throughout the course of all testing.

Each mouse was handled for 5 minutes for the 5 days leading up to any behavioral testing to reduce anxiety. Morris water maze testing was completed in 3 day blocks. The mice were repeatedly placed in an open water pool about 1 meter in diameter to find a submerged hidden platform. The location of the platform remained fixed in the center of one of the quadrants of the pool throughout the entire testing period. The mice had a total of eight attempts per day to locate the platform, and training was divided into two blocks of four. The first block of four attempts was performed in the morning while the second block of four was done in the afternoon. The order that the mice were tested in remained constant. The mice were gently placed into the pool, facing the wall, at one of four locations located in the opposite hemisphere from where the platform was and the latency to finding the platform was timed. The order of the four locations used to start the mice varied for each block to ensure that the mice would have to rely on spatial cues to find the platform. Once a mouse spent 1 second on the platform the attempt was considered complete and the mouse would be removed from the pool. If a mouse did not find the platform within 60 seconds, it was guided to the platform and allowed to spend 10 seconds on the platform after which it was removed from the pool.

Twenty-four hours after the completion of the last block, the mice were tested in a probe trial. The probe trial consisted of returning the mice to the pool to explore for a single trial of 60 seconds however the platform was removed. The start location was the point in the pool furthest from where the platform originally was placed. The latency to platform testing and the probe trials were recorded on a JVC Everio G-series camcorder and tracked by Panlab's Smart software.

A block of 4 swims to a visible platform was performed after the completion of the probe trial as a test of visual acuity and motivation. Any mouse that had an average latency to the

visible platform that was not within 2 standard deviations from the mean latency was excluded from analysis.

To perform novel object recognition testing, the mice were individually acclimated to a large rat cage for one hour prior to testing. Afterwards the mice were briefly returned to their home cage as the cage, in which the mice were acclimated, was filled with two identical objects equidistant from the center of the cage. The objects were either a 1 mL syringe barrel (BD) or a bottle cap (Pyrex). The starting object was counterbalanced throughout the entire experiment. The mice were then placed into the rat cage. Each mouse had ten minutes to interact with either object for a combined thirty seconds. Interaction was defined as nasal or oral contact with an object. If a mouse did not interact for the necessary thirty seconds, the mouse was excluded from analysis and any further testing. Forty-eight hours later the mice were returned to the rat cage. However this time, one object was the object that the mice had previously been exposed to while the other object in the cage was a novel object. Once again, the mice had ten minutes to accumulate thirty seconds of interaction. The side of the cage that the novel and familiar object was placed was counterbalanced throughout the experiment.

The APP/PS1 mice treated with AZD0530 were tested only in water maze and novel object recognition paradigms. The 3xTg-AD mice treated with AZD0530 were not tested behaviorally. No electrophysiological studies were conducted.

Immunohistology

Forty μ m free-floating sections were washed once in PBS with 0.1% Triton X-100 for five minutes. For pTau (pS199/S202) staining, antigen retrieval step was performed prior to the primary antibody by incubating slices in 300 μ l of citrate buffer (0.01 M citric acid, 0.05% Tween 20 in water, pH 6.0)/ well of 24-well-plate for 10 min at 90C in an oven and cooling down at room temperature for 10 min. Sections were then blocked in 5% normal donkey or horse serum in PBS for one hour and then incubated with primary antibody for 16-48 hours at 4C or 23C. The primary antibodies that were used were as follows: SV2A (Abcam 32942, 1:250), PSD-95 (Invitrogen 51-6900, 1:250), Iba1 (Wako 019-19741, 1:250), GFAP (Abcam 7260 1:500), β -amyloid (Cell Signaling Technology 2454 1:250), pPyk2 (Abcam, 1:1000), pTau (pS199/S202) (Invitrogen, 44786G, 1:2000), PHF-Tau AT180 (Thermo Scientific, MN1040, 1:1000). Sections were then washed 3-5 times in PBS, then incubated for two hours at room temperature in either donkey anti-rabbit, donkey anti-goat, or donkey anti-mouse fluorescent antibody (Invitrogen Alexa Fluor, 1:500) as appropriate. The samples were washed three more times in PBS for five minutes each time. The sections were mounted onto glass slides (Superfrost Plus) and coverslipped with Prolong gold (Molecular Probes) or Vectashield (Vector) antifade aqueous mounting medium, or with Krysalon (Millipore) after dehydration.

For AT180 PHF-tau staining, a peroxidase-coupled second antibody and tyramide amplification (Catalogue number T-20914, Invitrogen) were used to amplify the signal. Before starting a conventional immunohistochemistry, endogenous peroxidase activity was quenched by incubating slices in 3% H_2O_2 in PBS and other steps were performed as the protocol provided by manufacturer.

Imaging and Analysis of Immunohistochemistry

For imaging and analysis of the tissue stained with SV2a and PSD-95 antibodies, an UltraView Vox spinning disc confocal microscope (PerkinElmer) with a 60X 1.3 NA oil-immersion lens was used. The molecular layer of the dentate gyrus was imaged for each mouse and the area occupied by immunoreactive puncta were analyzed with ImageJ. For the imaging and analysis of the tissue stained for Iba1, GFAP, p-Pyk2 or β -amyloid, a Zeiss AxioImager Z1 fluorescent microscope with a 4X or 20X air-objective lens was used. The frontal cortex or the hippocampus was imaged for each mouse and the area occupied by plaques or cell bodies was analyzed by ImageJ. For p-Pyk2, fluorescence intensity of the hippocampus CA3 and CA1 regions was determined by measuring fluorescence (integrated density/area) and subtracting background (integrated density/area). This was performed with ImageJ software and values were normalized to WT, Veh control. For Tau immunostaining, images were captured by Zeiss 710 confocal microscope using 20X lens and three z slices with a 4 μ m interval were projected. ImageJ software was used for quantification.

Chronic Dog Toxicology

The effects of chronic AZD0530 administration over 9 months was assessed under Good Laboratory Practice (GLP) conditions in beagle dogs at MPI Research Inc. (Mattawan, MI). Using a standard, by weight, measured value randomization procedure, 16 male and 16 female animals were assigned to the control and treatment groups. For each dose level, 4 male and 4 female dogs were studied. The vehicle and test article were administered once a day for 273 consecutive days (39 weeks) during the study via oral gavage. The active dose levels were 0.5, 2, and 5 mg/kg/day and administered at a dose volume of 2.5 ml/kg. The control group received the vehicle in the same manner as the treated groups. The following observations and measurements were made: clinical examination, ophthalmoscopic observation, electrocardiogram, food consumption, body weights, hematology, differential leukocytes, coagulation, clinical chemistry and urinalysis. At necropsy after day 273, gross pathology and histopathology were conducted.

RESULTS

AZD0530 Inhibits Fyn, blocks A β o stimulation and prevents downstream Pyk2 signals

As a first step to evaluate AZD0530 for AD, we measured enzyme inhibition kinetics using both hematopoietic-enriched FynT and the brain-enriched FynB isoform (Fig. 1)⁴¹. Cleavage of a fluorometric peptide substrate by purified recombinant Fyn enzyme was monitored. Varying ATP substrate and AZD0530 inhibitor concentrations yields kinetic parameters consistent with a single inhibitor site competitive with ATP, as expected (Fig. 1A). The measured K_i of AZD0530 for Fyn is 8 ± 3 nM with both enzyme isoforms, similar to kinetics previously reported for Src kinase²⁷.

We used an adult brain slice assay to determine whether AZD0530 inhibited Fyn within neuronal networks. Fyn activity was monitored by immunoblot with an antibody specific for the Tyr residue phosphorylated during activation of SFKs (Tyr417 in Fyn), relative to the total amount of Fyn protein (Fig. 1B, C). Our previous studies have demonstrated that the increase in pSFK is Fyn-specific and not due to other Src family kinases in neurons¹⁴.

Addition of AZD0530 reduces basal Fyn activation to 20% of untreated values, and fully blocks the increase otherwise caused by a well-characterized^{13, 14, 34, 38} A β o preparation. These results are similar to our previous studies with dissociated embryonic cortical neurons³⁴, but are more relevant for adult brain degenerative processes.

It has been reported that the AD risk gene product, Pyk2 (also known as PTK2B and FAK2)⁴², is phosphorylated and activated by Fyn, and is localized to neuronal postsynaptic densities where it regulates synaptic plasticity⁴³⁻⁴⁷. Pyk2 and Fyn activations are bidirectional and synergistic^{48, 49}. Therefore, we assessed Pyk2 activation in brain slices exposed to A β o and treated with AZD0530 (Fig. 1B, D). This Tyr402 autophosphorylation site reflects Pyk2 activation state and is distinct from the Fyn phosphorylation site on Pyk2⁴⁴. AZD0530 suppresses basal Pyk2 activation and blocks A β o stimulation. This is not due to a direct action of AZD0530 on Pyk2, because Pyk2 substrate binding is unaffected by AZD0530 at concentrations up to 100 μ M, five orders of magnitude above the Fyn Ki (Fig. 1F).

A known downstream target of Fyn kinase is the NR2B subunit of NMDA-type Glu receptors in neurons, and we previously showed that A β o increases the phosphorylation of Tyr-1472 in NR2B via Fyn¹⁴. In the A β o-treated hippocampal slice assay used for Fyn and Pyk2 assessment, we monitored pY-1472 pNR2B levels relative to total NR2B protein (Fig. 1B, E). AZD0530 treatment suppresses basal phosphorylation and full blocks A β o-induced phosphorylation of NR2B. Thus, AZD0530 potentially inhibits purified Fyn, and prevents Fyn and Pyk2 activation by A β o in brain slices, with the consequence that phosphorylation of the NR2B channel protein is reduced.

CNS Pharmacokinetics of AZD0530

If oral AZD0530 is to be effective for AD, it must penetrate the CNS after peripheral dosing. However, no previous information had been collected for mouse brain AZD0530 levels, and no data had been obtained for CSF levels from any species. We analyzed AZD0530 drug levels after dosing mice with 0, 1, 2, 5 or 20 mg/kg/d AZD0530 divided in b.i.d. oral doses (Fig. 2A). In an initial cohort receiving 6 doses, we collected samples 30-60 minutes after dosing to obtain a peak level. The brain level is at least 50% of plasma levels in mouse. The CSF level is detectable, and about 35% of the brain level. From this analysis, the peak brain level at 5 mg/kg/d averages 27 ng/g, or 50 nM. The peak CSF level at 5 mg/kg/d from one pooled sample is 7 ng/ml, or 13 nM.

Although the half-life of AZD0530 in human plasma is 40 hours, the half-life in mouse may be short, so we measured brain AZD0530 levels at various times after the last dose (Fig. 2B). The apparent brain half-life of AZD0530 in mouse is 16 hours. The trough brain AZD0530 levels collected 12 hours post-dose for a b.i.d. schedule in a cohort of mice at 5 mg/kg/d exhibited a range from 10 ng/g to 25 ng/g (19 to 46 nM). The trough CSF level at 5 mg/kg/d from one pooled mouse sample was 4.6 ng/ml (8.9 nM). The trough CSF range calculated from multiple brain samples is 3.1 ng/ml to 7.6 ng/ml (5.8 to 14 nM) (Fig. 2C). Given that the Ki of AZD0530 for Fyn kinase is 8 nM, these data show that oral delivery at 5 mg/kg/d yields levels expected to inhibit at least 50% of Fyn kinase activity in brain.

We completed a Phase Ib multiple ascending dose study of AZD0530 in 24 subjects with mild to moderate AD (ClinicalTrials.gov identifier NCT01864655). Subjects were treated for a month with doses up to 125 mg/day, and then the CSF was sampled 24 hours after the last dose. Safety, tolerability and imaging information will be reported elsewhere. The trough CSF at 125 mg is 1.4 ng/ml to 7.6 ng/ml, or 2.5 to 14.0 nM (Fig. 2C). Thus, there is close correlation of the AZD0530 levels in CSF between mouse and human AD subjects.

Pyk2 activation in AD transgenic mice is reversed by AZD0530

We sought evidence that AZD0530 alters biochemical events related to AD signaling in the brain. The APP^{swe}/PS1^{?E9} (APP/PS1)³⁶ transgenic mice develop A β plaques and have PrP-interacting A β species by 12 months of age³⁵. As a marker of the Fyn-dependent signaling detectable in brain slices (Fig. 1B, D), we examined activated pPyk2 levels in these animals (Fig. 3). By immunoblot, the hippocampus of aged APP/PS1 shows a five-fold increase in pPyk2 levels relative to WT (Fig. 3A, B). The transgene effect is less pronounced in the cerebral cortex (Fig. 3C) and undetectable in cerebellum (Fig. 3D). Treatment with 5 mg/kg/d AZD0530 reduces activated pPyk2 level to that of WT mice exposed to vehicle. AZD0530 treatment of WT mice does not significantly alter pPyk2 (Fig. 3A-D), suggesting that baseline physiological brain Pyk2 activation is less dependent on Fyn kinase than is Pyk2 activation in acute brain slices, where AZD0530 reduces the level even in the absence of A β (Fig. 1B, D). At the histological level, the transgenic mice show a two-fold increase for p(Y402)Pyk2 within hippocampal CA1 and CA3 cell body layers compared to wild type (WT) mice, and this is reversed by AZD0530 treatment, matching the immunoblot findings (Fig. 3E-G). Thus, chronic oral AZD0530 dosing successfully interrupts A β signaling from Fyn to Pyk2 in vivo.

AZD0530 reverses memory deficits in AD transgenic mice

To test the ability of AZD0530 to ameliorate learning and memory deficits caused by Alzheimer pathophysiology, we treated APP/PS1 transgenic mice and WT controls with 0, 2 or 5 mg/kg/d of AZD0530. The treatment was started when mice were 11-12 months of age, when this mouse strain has developed a pronounced spatial learning and memory deficit³⁵. The oral daily dose was divided on a twice a day schedule and behavioral testing was initiated at 1-2 weeks after the initiation of therapy. The learning trials reveal that WT mice have progressively shortened latencies to a hidden platform, and this is not altered by treatment with 5 mg/kg/d AZD0530. A short treatment course of 1-2 weeks with 5 mg/kg/d of AZD0530 yields no improvement in the impaired rate of acquisition by APP/PS1 mice during the 30 swims to a hidden platform (Fig. 4A), or for memory recall during a 60 sec probe trial 24 hours later (Fig. 4B). Thus, there was no immediate symptomatic benefit of this dose of AZD0530 on performance.

We considered the possibility that Fyn kinase blockade may be beneficial for the AD model mice after more persistent disruption of signaling, and subsequent recovery of brain function through endogenous repair mechanisms. Therefore, we continued treatment for a month, and mice were retested in the Morris water maze (Fig. 4C, D). Treatment with AZD0530 for 3-5 weeks eliminates the pre-existing transgenic deficit, improving latencies to the hidden platform to WT levels during trial blocks 4-6 (Fig. 4C). In a probe trial, the WT mice with or

without 3-5 weeks of AZD0530 treatment prefer the target quadrant, with typical times of 30 seconds compared to an average of 10 sec in each of the non-target quadrants (Fig. 4D). In contrast, the APP/PS1 mice treated with vehicle control, spent much less time in the target quadrant, performing close to the chance level of 15 seconds. APP/PS1 mice treated with 5 mg/kg/d of AZD0530 show the same strong preference for the target quadrant as do WT mice. Thus, after one month the 5 mg/kg/d dose of AZD0530 fully rescues both spatial learning and memory deficits in aged APP/PS1 transgenic mice. The benefit is dose-dependent, as a 2 mg/kg/d dose of AZD0530 fails to improve water maze performance (Fig. 4E).

As a separate test for memory, the same 5 mg/kg/d cohort was tested after 6 weeks of AZD0530 or vehicle treatment for performance in Novel Object Recognition. The animals were familiarized with two objects on the first day of testing, and then observed two days later with one novel object and one familiar object. The time spent exploring each object was scored (Fig. 4F). WT mice spend more time with the novel object, as expected. In contrast, vehicle-treated APP/PS1 mice spent equal time with both objects, consistent with impaired memory of familiarization on the previous day. The AZD0530-treated transgenic mice exhibit performance equal to the WT mice. Thus, treatment of aged APP/PS1 mice with 5 mg/kg/d AZD0530 orally for 3-6 weeks reverses age-dependent memory impairment.

Neuroinflammation, but not A β accumulation, is limited by AZD0530 in APP/PS1 mice

We sought to assess the effect of the memory-rescuing 5 mg/kg/d AZD0530 treatment on brain pathology after 7 weeks. Since we hypothesize that Fyn kinase functions downstream of A β o signaling, no change in APP metabolism or A β accumulation is predicted. Indeed, measures of APP fragments, A β plaque and different A β species show no difference between the AZD0530-treated transgenic mice and the vehicle-treated transgenic mice (Fig. 5).

The glial reaction to A β pathology involves both microglial cells and astrocytes, and these responses are thought to interact with neuronal damage in AD. A blockade of Fyn-mediated synaptotoxic A β o action might therefore be coupled to reduced glial reaction. In addition, Fyn is known to be involved in signaling of immunological responses *per se*. We examined the extent of glial reaction in the brain of mice treated with 5 mg/kg/d AZD0530 for 7 weeks. As expected, microglia detected by Iba1 staining and astrocytes detected by anti-GFAP staining are greatly increased in the brains of aged APP/PS1 transgenic mice (Fig. 6). The extent of microglial induction is significantly reduced in the AZD0530 treated mice (Fig. 6A, B), while there is no significant change in astrocytic reaction (Fig. 6C, D). Thus, while A β burden is not altered by AZD0530, Fyn inhibition reduces the microglial response to A β pathology in APP/PS1. This may reflect both a cell autonomous inhibition of microglial signal transduction, as well as AZD0530 action to inhibit deleterious neuronal Fyn signaling induced by A β pathology coupled with a subsequent decrease in microglial reaction to damaged neuronal synapses.

Synapse density is restored by AZD0530 in AD transgenic mice

Because chronic AZD0530 treatment restores memory function, it may restore synapse density if the damaging effects of A β o are interrupted pharmacologically and endogenous

repair processes are functional. To assess whether Fyn inhibition reverses synaptic damage induced by APP/PS1 transgenes, we measured the density of PSD-95 and SV2a-containing puncta in hippocampal tissue collected after 6 weeks of treatment. The area occupied by presynaptic SV2a and postsynaptic PSD-95 immunoreactivities are reduced by 30-40% in the dentate gyrus of the transgenic mice compared to WT mice at this age in vehicle-treated animals (Fig. 7A-C)^{34, 35}. Treatment with 5 mg/kg/d AZD0530 for 7 weeks does not alter synapse density in WT mice, but fully restores synaptic puncta to WT levels in transgenic mice. Thus, chronic AZD0530 treatment of transgenic AD mice, initiated after their memory is impaired, allows restoration of synaptic density.

AZD0530 limits Tau Aggregation in Triple AD transgenic mice

AD entails both A β and Tau pathology, but APP/PS1 transgenic mice do not manifest Tau abnormalities. However, transgenic mice with mutant Tau transgenes exhibit Tau pathology and co-expression of APP/PS1 mutant transgenes may accelerate Tau changes⁵⁰. We treated triple APP/PS1/Tau transgenic (3xTg-AD)³⁷ mice at 11 month of age with 5 mg/kg/d AZD0530 for 5 weeks and then assessed Tau biochemically and histologically in the brain (Fig. 8). At this age, 3xTg-AD mice begin to accumulate A β and Tau pathology but do not exhibit synaptic loss or Morris water maze deficits (data not shown). The levels of total Tau, phosphorylated pS199/S202-Tau and pS396-Tau protein are not altered in TBS-soluble fractions by AZD0530 treatment (Fig. 8A, B). Similarly, the levels of human Tau transgene mRNA assessed by qRT-PCR are not changed by AZD0530 (data not shown). In the 3xTg-AD transgenic mice, there is no effect of AZD0530 on APP/A β metabolism (data not shown). Thus, the substrates for interaction between A β and Tau pathologies are not altered by AZD0530 treatment of triple transgenic mice.

Once pathological Tau hyperphosphorylation and misfolding are initiated, protein solubility decreases. The RIPA extraction of TBS-insoluble material from brain shows significantly reduced levels of total Tau, pS199/S202-Tau and pS396-Tau in transgenic mice treated with AZD0530 compared to 3xTg-AD mice treated with vehicle (Fig. 8A, C). The 50% reduction in these measures observed in the 3xTg-AD mice with AZD0530 treatment yields levels indistinguishable from non-transgenic mice. Tau protein also accumulates in RIPA-resistant fractions of the 3xTg-AD mouse brain (Fig. 8D). The amount of total Tau and human transgenic Tau in the RIPA-resistant fraction is suppressed to one third of vehicle-treated levels in the AZD0530-treated transgenic mice. The pS199/S202-Tau and AT180 anti-Tau antibodies recognize pathological phosphorylation states immunohistologically. Pathological staining of CA1 cell bodies with these antibodies is detectable in vehicle-treated 3xTg-AD mice but is essentially eliminated in the AZD0530 treated mice (Fig. 8E, F). Thus, in the 3xTg-AD model, AZD0530 treatment reduces Tau pathology.

Chronic AZD0530 treatment is well tolerated

If AZD0530 is to be utilized for disease modification in AD therapy, then effective doses must be tolerated over extended periods. Previous toxicological studies in rat and dog have been limited in duration. We conducted a nine month toxicological study in dogs to support chronic dosing in clinical situations. The dose levels were 0, 0.5, 2 and 5 mg/kg/d. Based on previous pharmacokinetic studies these doses are most similar to 0, 12.5, 50 and 125 mg

daily oral doses in human. Periodically throughout the treatment period the following observations were collected: clinical examination, ophthalmoscopic observation, ECG, food consumption, body weights, hematology, differential leukocytes, coagulation, clinical chemistry and urinalysis. After 273 days of treatment, necropsy included gross pathology and histopathology. Pharmacokinetic studies for AZD0530 showed that the AUC_{0-24hr} and C_{max} values for Week 39 at 5 mg/kg/day, sexes combined, were 5,490 hr*ng/mL and 389 ng/mL, respectively.

No significantly abnormal measurement or evidence of pathology was detected in any group, including the 5 mg/kg/d cohort. Selected observations for body weight and hematological parameters are proved in Fig. 9. In conclusion, 39 consecutive weeks of oral administration of AZD0530 by gavage to male and female dogs at 0.5, 2, and 5 mg/kg/day were tolerated at all dose levels tested. As a result, the no-observed-adverse-effect-level (NOAEL) was considered to be greater than 5 mg/kg/day.

DISCUSSION

The major finding of this study is that a Fyn kinase inhibitor, AZD0530, enters the brain and reverses memory deficits associated with familial AD-derived mutant transgenes in mice. Critically, the rescue of learning and memory impairment is coupled to full restoration of synapse density by chronic Fyn inhibition. The kinase inhibitor can block downstream signaling from Fyn to the AD risk gene product, Pyk2. These primary actions are consistent with an interruption of A β signaling via PrP^C and mGluR5 as described^{14, 34}. In addition, oral treatment with AZD0530 reduces microglial activation without changing plaque density. For a mouse strain with both A β and Tau pathology, Fyn kinase inhibition reduces the phosphorylation and aggregation of Tau protein to control levels. The microglial and Tau effects may be secondary to blockade of A β signaling in neurons, or reflect additional pathological pathways blocked by AZD0530. Together, the findings show symptomatic improvement in mouse AD models with a clinical stage compound.

The specificity and pharmacokinetics of AZD0530 appear appropriate to achieve Fyn kinase inhibition as a target for AD. We show that the molecule has high potency against FynB and FynT. Previous work has shown that it also inhibits other Src Family kinases but has little or no potency against a panel of other kinases. The only other detectable activity is against Abl kinase, but the Ki is approximately 15-fold higher^{27, 28}. At the concentrations that we observed *in vivo* with our dosing regimen, the predicted inhibition of Abl is at most 10%, so Abl inhibition is highly unlikely to play a role in our studies. The half-life of the compound in humans is documented to be between one and two days, supporting once daily clinical dosing²⁷. Here, we show that the molecule enters the CNS and is detectable in CSF of mouse and human at concentrations that inhibit Fyn. Thus, the compound can be used to target Fyn kinase with the goal of developing a new pharmacology for AD.

Our previous work has implicated a PrP^C-mGluR5-Fyn complex in mediating the ill effects of A β on synapse maintenance and function^{14, 34}. Indeed, AZD0530 does not alter the levels of various forms of APP or A β , including A β plaque and A β themselves. Instead, the ability of A β to increase Fyn kinase activity in brain slices is abrogated by AZD0530.

Previously, we showed that NR2B is a downstream target of Fyn in the post-synaptic density¹⁴, and here we find that AZD0530 prevents A β -induced NR2B phosphorylation. Recent work has implicated another target of Fyn, Pyk2 (PTK2b, FAK2), in AD risk genetically through a massive GWAS of the disease⁴². Fyn and Pyk2 activation is cooperative and synergistic^{48, 49}. Critically, the Pyk2 kinase is enriched in the postsynaptic density and titrates synaptic plasticity^{43, 45, 46}. It can alter F-actin status, similar to the closely related FAK enzyme and crucial to dendritic spine dynamics^{44, 51}. Importantly, we show that AZD0530 blocks Fyn-dependent phosphorylation and activation of Pyk2 in neuronal slices. Furthermore, the ability of A β to activate Pyk2 is eliminated by AZD0530. The APP/PS1 transgenic mice exhibit increased Pyk2 activation in the hippocampus similar to acute A β treatment of brain slices, and this activation is eliminated by oral AZD0530 treatment. These findings provide a mechanistic basis for the linkage between Pyk2 genetic variation and AD risk. Moreover, oral treatment with AZD0530 appears to interrupt a pathological A β signaling cascade in brain synapses.

To test whether AZD0530 protects against synapse loss related to AD transgenes *in vivo*, we examined the hippocampus of mice treated with AZD0530. By the age of 12 months, this strain has lost one-third of normal synapse density in the dentate gyrus. Critically, treatment with the Fyn kinase inhibitor allows a full restoration of synapse density after 7 weeks. The full synapse rescue alleviates a central functional pathology of AD, and is consistent with the *in vitro* data showing that AZD0530 blocks A β signaling. Importantly, it occurs with no alteration of A β plaque burden in the brain, fully distinguishing this therapy from interventions focused on A β itself, such as anti-A β antibodies or secretase inhibitors. The return of synapse density to WT levels implies that endogenous repair processes are active once the A β toxicity is blocked. Consistent with the notion that healthy adult brain can form new synapses to recover from A β -induced synaptic loss, previous *in vivo* imaging studies of WT mouse brain have demonstrated that new transient dendritic spines are formed at a rate of 5-10% per week even in the aged brain⁵²⁻⁵⁴. If the synaptotoxic effects of A β are blocked by AZD0530, then these transient spines may be stabilized to form new synapses and restore brain anatomy. Thus, AZD0530 appears to permit brain repair after mutant APP/PS1 transgene-triggered damage in this transgenic mouse strain.

While A β species may initiate AD, it is clear that misfolded and hyperphosphorylated Tau plays a role downstream, with Tau aggregation in neurofibrillary tangles and with spreading of the disease along axonal pathways. Fyn is a direct binding partner for Tau, and can contribute Tyr phosphorylation of Tau^{23, 24, 26}. Their interaction is bidirectional, in that Tau is required for appropriate Fyn localization. Importantly, it has been shown that Tau and Fyn interact genetically in mediating the expression of mutant APP related phenotypes in transgenic mouse models of AD^{19, 20, 25}. We studied a triple transgenic mouse with both A β and Tau pathology to test whether AZD0530 would alter Tau pathology. We observed significant decreases in Tau phosphorylation and aggregation within insoluble complexes after drug treatment. This effect is likely to involve more than one mechanism. By interrupting A β signaling, AZD0530 is expected to eliminate the acceleration of Tau pathology by the APP/PS1 mutant proteins in this model. By altering Fyn–Tau direct interactions, the compound has the potential to limit phenotypes directly dependent on the

mutant Tau allele. Future studies in transgenic mice with Tau pathology but no A β pathology, will shed further light on the relative role of these mechanisms. Consistent with a central role for Fyn in AD pathology, the net effect of both pathways is a reduction in Tau pathology.

Brain inflammation involving microglia is recognized as an important factor in AD, as highlighted by recent genetic studies of TREM2 in AD^{55, 56}. Both Fyn and Pyk2 have been implicated in aspects of inflammation and Fyn is known to be expressed in microglial cells^{57, 58}. A β deposition generates microglial activation near plaques either directly or indirectly through neuronal damage and microglial recruitment. In the AZD0530 treated mice, A β deposition is not altered but microglial activation is attenuated. This reduction of inflammation may occur by both cell autonomous and non-autonomous mechanisms. Inhibition of Fyn within microglia has the potential to prevent their activation by A β deposition directly. Interruption of synaptotoxic neuronal signaling by AZD0530 may eliminate a damage signal that would otherwise recruit microglial cells for engulfment of damaged synapses. By both mechanisms, AZD0530 treatment may reduce brain inflammation triggered by A β pathology. The relative balance between AZD0530 action in neurons versus microglia is not yet defined, though the net effect is clearly beneficial.

Symptoms of AD derive from impaired cognitive function. We monitored both spatial memory and object recognition memory in mice expressing human mutant APP and PS1. After one to two weeks of AZD0530, drug levels have reached steady state since more than 15 half-lives have elapsed, but memory is not improved by AZD0530. However, after a month or more of treatment with the Fyn kinase inhibitor, the age-dependent memory deficit in these mice is fully rescued in both memory tests. The time-dependence of the memory improvement implies that recovery is not due to a symptomatic action of Fyn kinase inhibition on synapses impaired by A β o signaling. Instead, the delayed action suggests that blockade of A β o synaptotoxicity allows a recovery of functional synapse density by formation of new synapses. The restoration of synapse density observed at 7 weeks support this view. The dose-dependence suggests that achieving 50-75% inhibition of Fyn kinase is required for the beneficial effects on learning and memory. The doses here are substantially lower than the doses utilized in oncology indications. It remains possible that much higher doses of AZD0530 and near complete inhibition of Fyn would be deleterious for memory function. In constitutive Fyn null mice, the developmental versus adult role of Fyn has not been separated, but long term potentiation and behavior are impaired⁵⁹.

Taken together, the current study reveals that pharmacological reduction of Fyn kinase activity with AZD0530 beneficially modifies the course of transgenic mouse models of AD. Central to the benefit of the drug is interruption of damaging A β o signals at the synapse. We show that inhibition of Fyn kinase reduces downstream activation of the AD risk gene product, Pyk2, amongst other synaptic targets of Fyn. Treatment leads to restoration of synapse density and a gradual full recovery of behavioral deficits. Blockade of A β o signaling in synapses is not the only benefit of AZD0530 treatment; microglial inflammation and Tau pathology are also reduced. These later effects may be independent benefits or secondary to synapse preservation by Fyn blockade. Based on these data, AZD0530 is a candidate disease-modifying therapy for AD. Indeed, the compound reaches the CSF of AD

patients in levels beneficial in transgenic mouse models. A Phase 2a study of AZD0530 in AD is being initiated.

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Figure 1. AZD0530 potently inhibits Fyn activity and indirectly blocks Pyk2

(A) The enzyme kinetics of the interaction between FynB and AZD0530 shown as ATP varies indicate a competitive model of inhibition. The best fit of the data indicates that the K_i is $8.3 \text{ nM} \pm 3.4 \text{ nM}$. (B) Immunoblot analysis of RIPA-soluble extracts from brain slices incubated with or without Aβ and with or without AZD0530 for the indicated antigens for 30 min. (C) Densitometric analysis of the immunoblot from B showing that Aβ treatment induces significantly increased Src family kinase phosphorylation, which can be significantly reduced by incubating with AZD0530. Data are mean ± SEM of 8-9 brain slices per condition. (** p<0.01, *** p<0.001, one-way ANOVA with post-hoc LSD comparisons as noted). (D) Densitometric analysis of the immunoblot from B showing that Aβ treatment also induces significantly increased Pyk2 phosphorylation, which can also be significantly reduced by AZD0530. Data are mean ± SEM of 10-11 brain slices per condition. (*** p<0.001, **** p<0.0001, one-way ANOVA with Tukey's multiple comparisons as noted). (E) Densitometric analysis of the immunoblot from B showing that Aβ treatment induces significantly increased NR2B phosphorylation, which is reduced by AZD0530. Data are mean ± SEM of 3 brain slices per condition. (* p<0.05, *** p<0.001, one-way ANOVA with Tukey's multiple comparisons as noted) (F) The LanthaScreen

binding assay shows that AZD0530 minimally inhibits Pyk2. The IC_{50} of this interaction is greater than 250 μ M.

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Figure 2. AZD0530 effectively crosses the blood brain barrier and has a long half life within the brain

(A) AZD0530 can be found in plasma, brain, and CSF of mice that received 6 oral doses given over a 3 day time period. Higher doses correlated with higher levels in all compartments. The data points for plasma and brain are the mean $\pm$ SEM of 5 mice. The data points for CSF are single values for samples pooled from 5 mice. (B) AZD0530 levels within the brain fall over time with an estimated half-life of 16 hours. Each data point is the mean $\pm$ SEM of 5 mice that received 5 mg/kg/d for 3 days prior to sacrifice. (C) Mice treated with 5 mg/kg/d for 3 days achieve comparable trough levels in CSF to that of humans treated with 125 mg/d for one month. Data are mean $\pm$ SEM of the calculated CSF value of 5 mice derived from their brain levels, and the actual CSF value of 5 humans.

Figure 3. Pyk2 levels are returned to WT levels by treatment with AZD0530

(A) Immunoblot analysis of RIPA-soluble lysates from brain hemispheres from mice treated with AZD0530 or vehicle for two weeks stained with an anti Pyk2 antibody and an anti p-Pyk2 antibody. Genotypes and treatment status are indicated above each lane and each lane is from a separate mouse. (B) Densitometric analysis of immunoblot experiment normalized to actin levels from A. Data are mean $\pm$ SEM $n=3-6$ mice per group. There was a significant elevation of the ratio of p-Pyk2 to Pyk2 in the vehicle treated APP/PS1 mice in the hippocampus compared to all other treatment groups and the ratio in the AZD0530 treated APP/PS1 mice normalized (ANOVA with Tukey post-hoc testing performed $***p<0.001$). (C-D) Densitometric analysis of immunoblot experiments normalized to actin levels from the same mice used for A however looking in the cortex and the cerebellum. The pattern seen in the cortex matches what was seen in the hippocampus in that there is an elevated ratio of p-Pyk2 to Pyk2 in the vehicle treated APP/PS1 mice which is returns to the WT baseline in the AZD0530 treated APP/PS1 mice (ANOVA with Tukey post-hoc testing performed $*p<0.05$, $**p<0.01$). In the cerebellum, no group of mice showed any elevated ratio (ANOVA $p>0.05$). (E) WT and APP/PS1 mice at 11 months of age were treated with Vehicle or 5 mg/kg/d AZD0530 for 7 weeks and then the brain was collected for analysis.

Immunofluorescence staining of p-Pyk2 in the CA1 regions of the hippocampus is illustrated. Scale bar, 25 μm . **(F-G)** The intensity of pPyk2 in the cell body layer of the CA3 and CA1 hippocampus was measured. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, one-way ANOVA with post-hoc Tukey comparisons as noted. Data are mean $\pm$ SEM from $n=6$ mice per group.

Figure 4. AZD0530 reverses learning and memory deficits in APP/PS1 mouse model after 3-5 weeks treatment

(A) Spatial learning is plotted as the latency for a cohort of 12 month old mice take to find a hidden platform after receiving treatment for 1-2 weeks prior to testing. Mean $\pm$ SEM $n=17-26$ mice per groups. Performance differed over the last 12 trials by genotype but not treatment status (two way RM-ANOVA for APP/PS1 $***p<0.001$; for short pretreatment with AZD0530 $p>0.05$). Tukey post hoc analysis indicated that both APP/PS1 groups differed from the C57BL/6 group ($***p<0.001$) while short pre-treatment with AZD0530 of APP/PS1 did not cause the mice to perform differently than the vehicle treated APP/PS1 group ($p>0.05$). (B) Performance during a 60 second probe trial, completed 24 hours after the training in the Morris water maze is completed, is measured by percent time spent in the quadrant where the platform was located previously. The dashed line indicates random chance at 25%. Mean $\pm$ SEM $n=9-14$ mice per group. Target quadrant differed by genotype but not by treatment status (two way ANOVA for APP/PS1 $***p<0.001$; for short pretreatment with AZD0530 $p>0.05$). By Fisher's LSD post-hoc pairwise comparisons vehicle treated APP/PS1 and AZD0530 treated APP/PS1 did not differ from each other significantly ($p>0.05$) while both were significantly different from vehicle treated C57BL/6 ($*p<0.05$ and $**p<0.01$ respectively). (C) Spatial learning is plotted as the latency a cohort

of 13 month old mice take to find a hidden platform after receiving treatment for 3-5 weeks prior to testing. Mean $\pm$ SEM n=17-27 mice per group. Performance differed over the last 12 trials by genotype as well as treatment status (two way RM-ANOVA for APP/PS1 ***p<0.001; for long pretreatment with AZD0530 *p<0.05). There was an interaction between genotype and treatment. (two-way ANOVA , APP/PS1 $\times$ AZD0530 p<0.05). The vehicle treated APP/PS1 group significantly differed (**p<0.01, ***p<0.001) from the other groups in Fisher's LSD post hoc pairwise comparisons while all other comparisons were not different (p>0.05). For the indicated trial blocks, the vehicle treated APP/PS1 group differed from the other groups (*p<0.05, **p<0.01, ***p<0.001) while none of the other groups differed from each other. **(D)** A 60 second probe trial was performed 24 hours after training in mice that received 3-5 weeks of pretreatment prior to testing. Random chance is 25%. Mean $\pm$ SEM n=9-14 mice for each group. A one-way ANOVA indicated that differences existed between the groups (***p<0.001). By Fisher's LSD post hoc, pairwise comparisons the untreated APP/PS1 group differed from the others (*p<0.05, ***p<0.001), whereas none of the other groups differed from each other (p>0.05). **(E)** Spatial learning was testing in a cohort of mice that was treated for 3-5 weeks with a lower dose of AZD0530 and plotted as the latency to finding a hidden platform. Mean $\pm$ SEM n=8-13 mice per groups. Performance among the groups differed over the last 12 trials of the training (one way RM-ANOVA, ***p<0.01). Tukey post-hoc pairwise comparisons indicate that APP/PS1 treated with the lower dose of AZD0530 performed significantly worse than both higher dose treated APP/PS1 group and the vehicle treated WT group (**p<0.01) while the two latter groups performed similarly (p>0.05). **(F)** Object recognition learning is plotted as the time a 13 month cohort of mice pre-treated for 6 weeks spent interacting with a novel object compared to time spent interacting with a familiar object. Mean $\pm$ SEM n=6-10 mice per group. Vehicle treated APP/PS1 mice showed no preference for a novel object over a familiar object (two-tailed Student's t-test p>0.05) while the other groups all showed a distinct preference for the novel object (two-tailed Student's t-test **p<0.01 ***p<0.001).

Figure 5. APP and Aβ levels are not altered by treatment with AZD0530

(A) Immunoblot analysis of TBS-soluble lysates from brain hemispheres stained with anti-Aβ antibody 6E10. Genotypes and treatment status are indicated above each lane and each lane is from a separate mouse. The molecular weight of the standard is shown to the left of the blot. (B) Densitometric analysis of immunoblot experiment normalized to βIII-tubulin levels from A. Data are mean ± SEM n=4 mice per group. There was no difference after treatment between the two APP/PS1 groups (two-tailed Student's t-test, $p>0.05$). (C) Treatment with AZD0530 did not alter total Aβ monomer levels as detected by an Aβ ELISA (Tukey post hoc comparison testing, $p>0.05$). (D) Immunofluorescent detection of Aβ in 13 month APP/PS1 mice treated with vehicle or AZD0530 in the hippocampus using a confocal microscope and a 4X objective lens. Scale bar, 100 μm. (E) Fractional analysis of the immunoreactive area stained for Aβ with antibody 2454. There was no difference between the two groups (two-tailed Student's t-test $p>0.05$).

Figure 6. Microgliosis is reduced in APP/PS1 mice after 7 weeks of treatment with AZD0530
(A) The cortex of the indicated groups was stained with Iba1 and imaged with a confocal microscope with a 20X objective. Scale bar, 50 μ m. **(B)** Fractional area of immunoreactivity for Iba1 in the cortex of the groups indicated in A. A one-way ANOVA with Tukey post hoc comparisons show that the vehicle treated APP/PS1 group differs from all groups including the AZD0530 treated APP/PS1 group (* p <0.05, *** p <0.001). The AZD0530 treated APP/PS1 also differed from the two C57Bl6 groups (* p <0.05). Mean $\pm$ SEM 5 mg/kg/d AZD0531 APP/PS1 n =6 mice, Vehicle APP/PS1 n =9 mice, 5 mg/kg/d AZD0530 WT n =5 mice, Vehicle WT n =5 mice. **(C)** The cortex of the indicated groups was stained with GFAP and imaged with a confocal microscope with a 20X objective. Scale bar, 50 μ m. **(D)** Fractional area of immunoreactivity for GFAP in the cortex of the groups indicated in C. A one-way ANOVA with Tukey post hoc comparisons show that the vehicle treated APP/PS1 differs from both C57Bl6 groups (** p <0.01, *** p <0.001) while not differing significantly from the AZD050 treated APP/PS1 group (p >0.05). Mean $\pm$ SEM 5 mg/kg/d AZD0531 APP/PS1 n =6 mice, Vehicle APP/PS1 n =9 mice, 5 mg/kg/d AZD0530 WT n =4 mice, Vehicle WT n =4 mice.

Figure 7. Synaptic markers recover to WT levels after 7 weeks of treatment with AZD0530
(A) The dentate gyrus of the hippocampus of the indicated groups was stained with PSD-95 and imaged with a confocal microscope with a 60X objective. **(B)** Fractional area of immunoreactive puncta for PSD-95 in the dentate gyrus of the groups indicated in A. A one-way ANOVA with Fisher's LSD post hoc pairwise comparisons show that the vehicle treated APP/PS1 group differs from the other groups including the AZD0530 treated APP/PS1 group (* $p < 0.05$) while no other group exhibited differences from each other ($p > 0.05$). Mean $\pm$ SEM $n = 5-11$ mice per group. **(C)** Fractional area of immunoreactive puncta for SV2a in the dentate gyrus of the hippocampus imaged with a 60x objective. A one-way ANOVA with Fisher's LSD post hoc pairwise comparisons show that the vehicle treated APP/PS1 differs from the remaining groups (* $p < 0.05$). Mean $\pm$ SEM $n = 5-11$ mice per group.

CA1 of hippocampus showed that the pTau-positive area was decreased by AZD0530 treatment (t-test, $**p < 0.01$, $***p < 0.001$; Error bars show SEM).

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Figure 9. No Toxic Effect of Chronic AZD0530 in Dogs

Dogs were treated for 273 days with 0, 0.5, 2 or 5 mg/kg/d of AZD0530. (A) The body weight of the male dogs as a function of age is reported. (B-E) Hematological and coagulation parameters are reported for 8 dogs at each dose level prior to and after 9 months of AZD0530. All data are mean $\pm$ sem. There were small changes in various parameters as the dogs aged, but there was no statistically significant difference between AZD0530 groups and vehicle by one-way ANOVA with Tukey post hoc correction.