Supplementary Material

Reducing tau aggregates with anle138b delays disease progression in a mouse model of tauopathies

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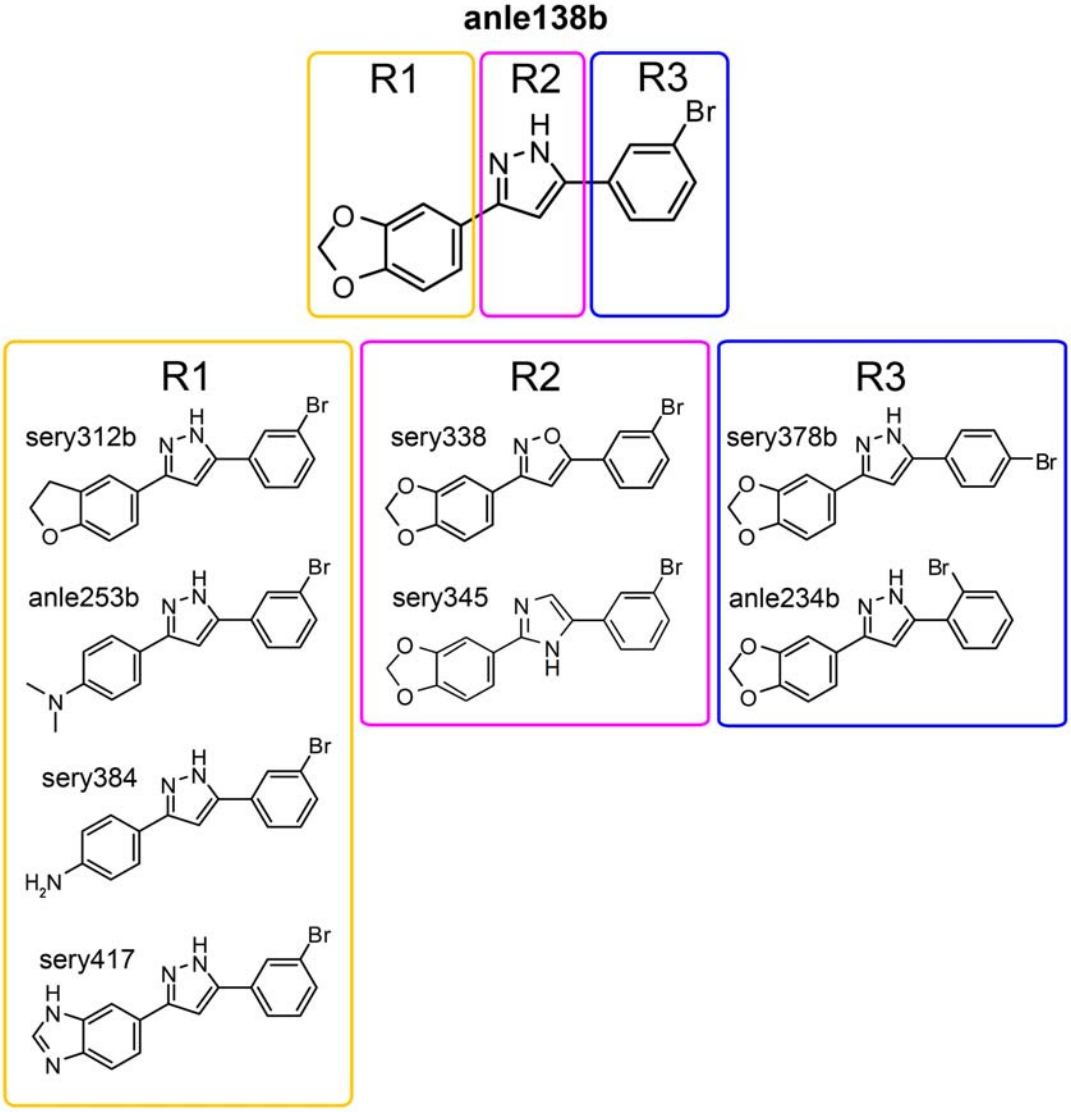
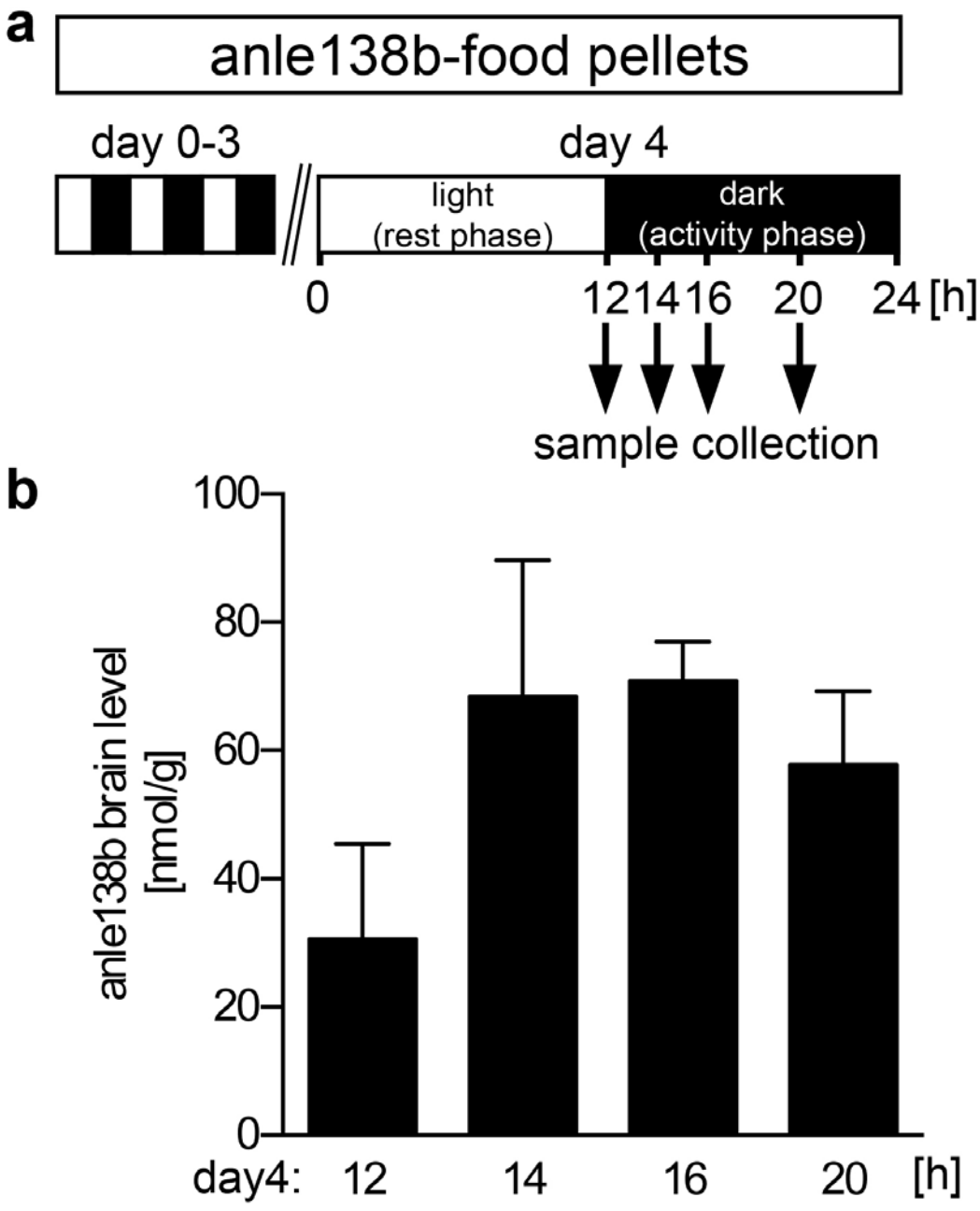
Supplement Figure 1:



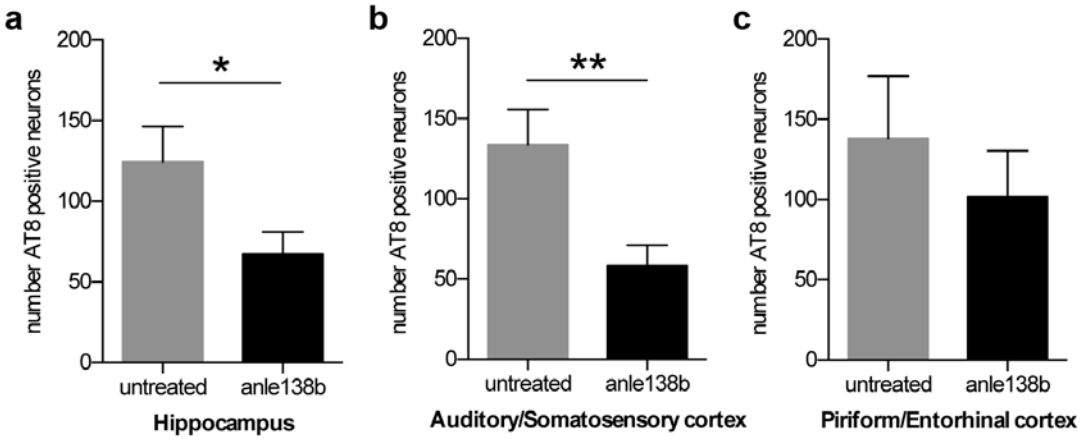
Chemical structure of anle138b and related compounds. Shown are the structures of anle138b related compounds with systematic variations of the substituents on the 3-phenyl (R1) and 5-phenyl ring (R3) as well as the pyrazole ring (R2).

Supplement Figure 2:



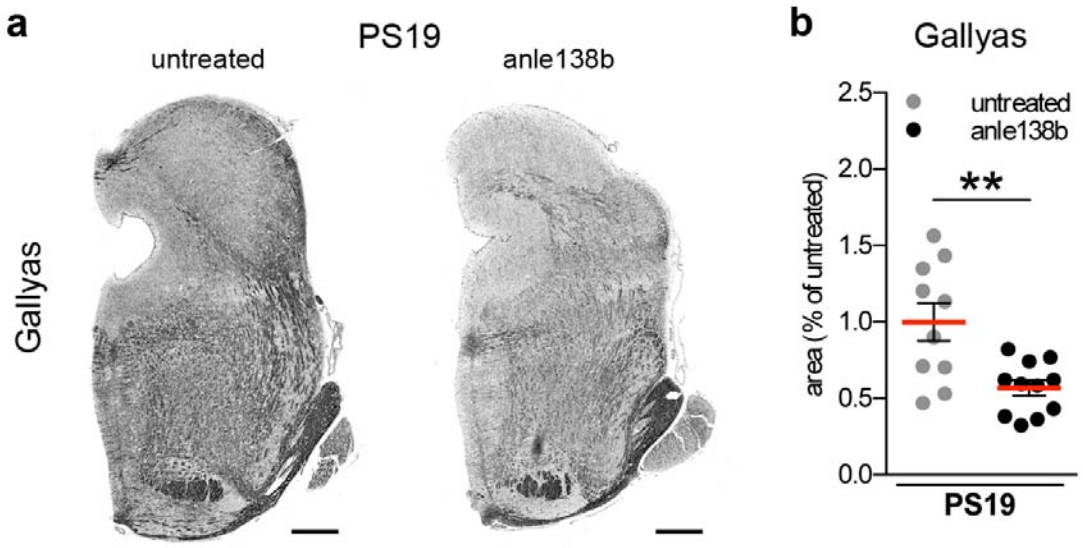
Anle138b effectively crosses the blood-brain-barrier. **a** Experimental scheme for pharmacokinetic analysis of compound brain level after anle138b treatment. **b** Brain compound level in ad libitum fed mice on day 4 at 12h, 14h, 16h and 20h (n = 3 mice for each time point).

Supplement Figure 3



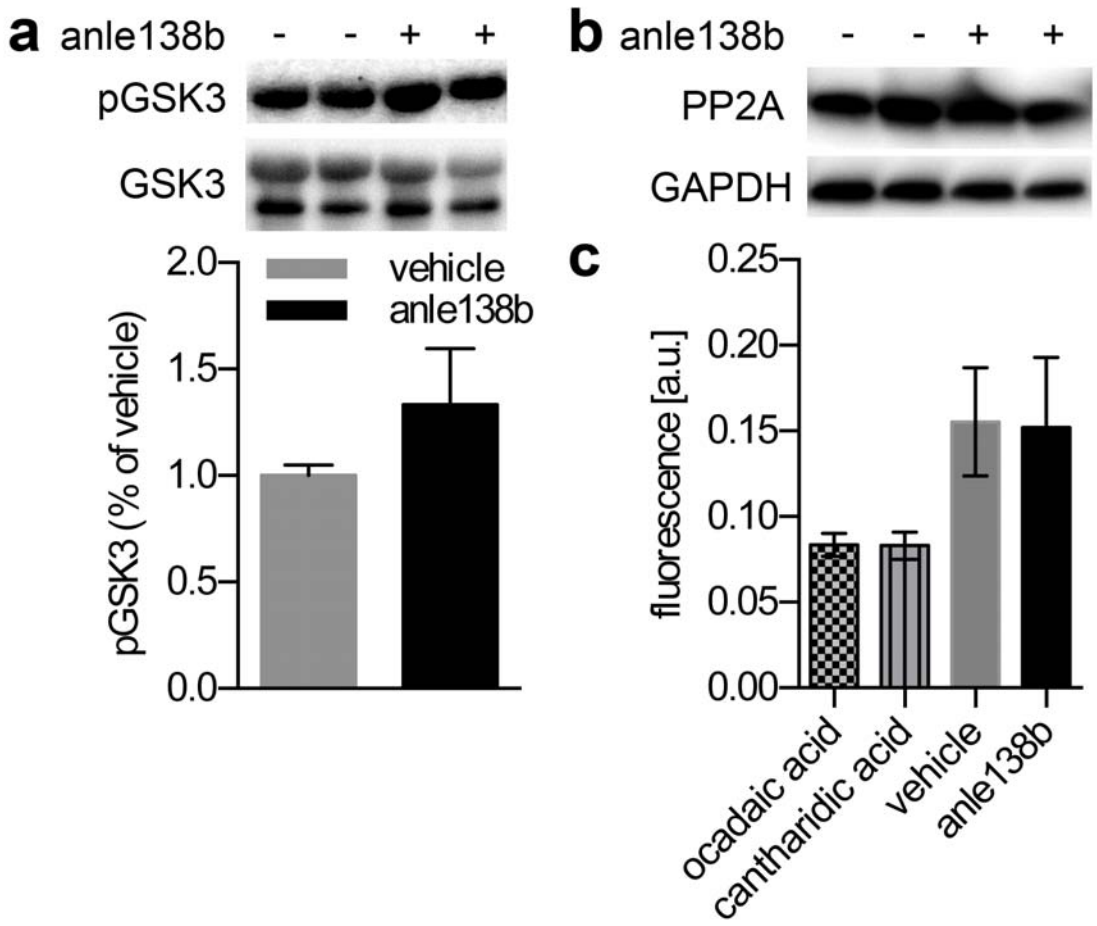
Anle138b treatment decreased the number of AT8-positive neurons in PS19 mice. **a-c** The number of AT8-positive neurons in the hippocampus (**a**), auditory/somatosensory cortex (**b**), and piriform/entorhinal cortex (**c**) in PS19 mice. The number of AT8-positive neurons was significantly reduced in the hippocampus and the auditory/somatosensory cortex (n = 10-11 mice/group; 10-12 months). Asterisks indicate significant differences relative to untreated PS19 mice (*, $p < 0,05$; **, $p < 0.01$; t-test).

Supplement Figure 4:



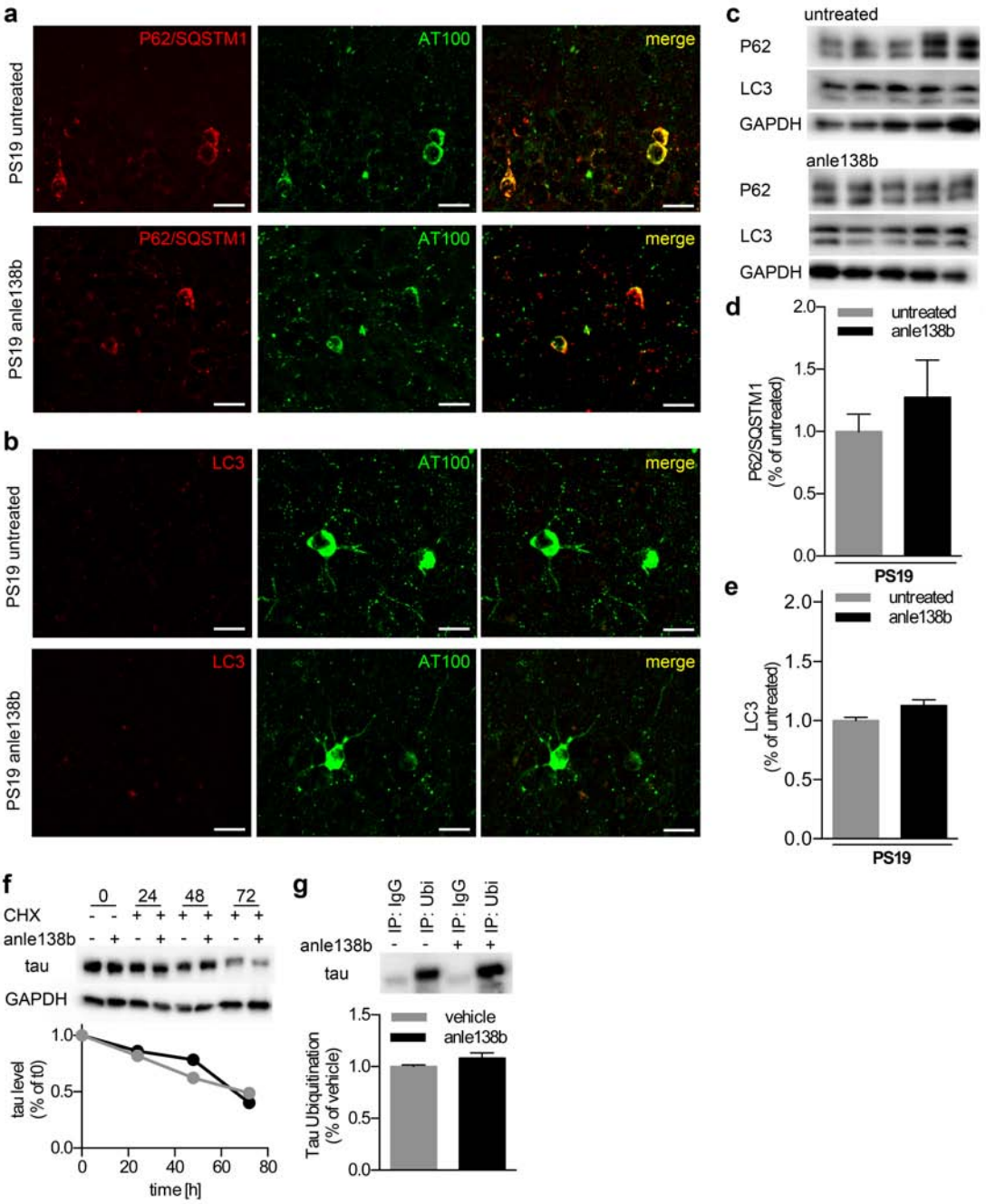
Anle138b treatment reduces tau pathology in the brain stem of PS19 mice. **a** Gallyas silver staining showed tangle tau pathology in the brain stem of PS19 mice. **b** Quantification of tangle tau pathology in treated and untreated PS19 mice (n = 10-11 mice/group; 10-12 months). Asterisks indicate significant differences relative to untreated PS19 mice (**, $p < 0.01$; t-test). Scale bars: 500 µm.

Supplement Figure 5:



Kinase and Phosphatase activity is unaffected after anle138b treatment. **a** Phosphorylation of GSK3 is slightly but not significantly elevated in anle138b-treated primary neurons (n = 5; p>0.05, t-test). **b** PP2A expression is unchanged after anle138b-treatment in primary neurons. **c** Analysis of PP2A activity in primary neurons revealed that the activity is unaltered in anle138b-treated neurons compared with the vehicle-treated group (n = 3; p > 0.05, t-test).

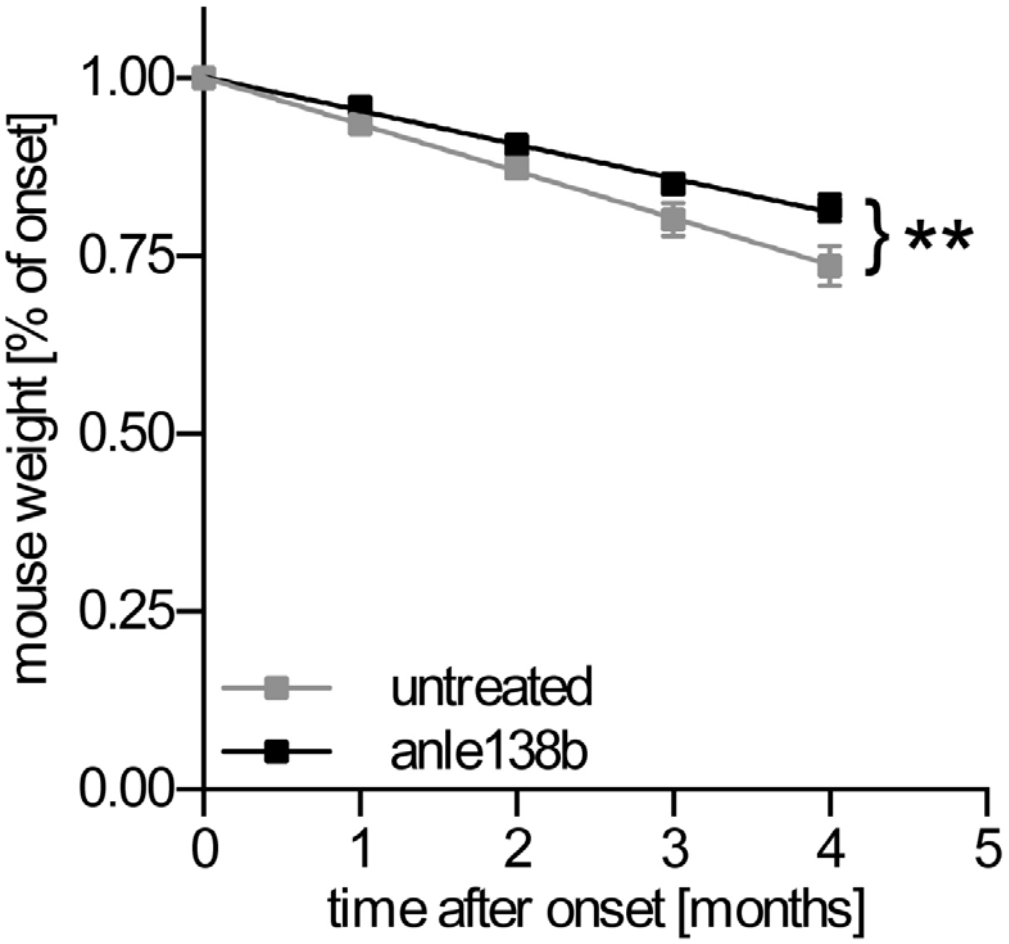
Supplement Figure 6:



Autophagy and tau degradation is unaffected after anle138b treatment. **a** P62/SQSTM1 and AT100-positive tau inclusions are co-localized in untreated as well as anle138b-treated PS19 mice. **b** LC3 expression is not increased after anle138b-treatment in PS19 mice. **c** Immunoblots of P62/SQSTM1 and LC3 in brains of PS19 mice. **d+e** Densitometric analysis of P62/SQSTM1 and LC3 levels in brains of PS19 mice revealed that expression of these autophagic marker were unchanged in

anle138b-treated PS19 mice compared with untreated animals (n = 5 mice/ group; $p>0.05$, t-test). **f** The effect of anle138b on tau stability was analyzed in time-course experiments with the translation inhibitor cycloheximide (CHX). Tau degradation in primary neurons was unchanged after anle138b treatment compared with vehicle-treated neurons. **g** The effect of anle138b on tau ubiquitination was examined by immunoblots with the antibody tau5 (total tau) after immunopurification (IP) of ubiquitinated proteins. Anle138b treatment did not affect the level of ubiquitinated tau in primary neurons (n=2; $p>0.05$, t-test).

Supplement Figure 7:



Effect of anle138b treatment on mouse weight of PS19 mice. Treatment with anle138b significantly ameliorated weight loss of PS19 mice. The mouse weight was normalized to the peak of body weight. The peak of body weight was defined as the onset of disease. (**; n=43-45 mice/group; $p < 0.01$; F test).

Supplementary Methods

Pharmacokinetic analysis of brain concentrations of anle138b

Mice (B6SJLF1, Jackson Laboratory) were kept in groups of 4 animals in each cage in an inversed day/night-cycle for 2 weeks before starting and during the experiment (light 18:00 to 6:00, dark 6:00 to 18:00) with free access to food and water. Age ranged from 78 d to 96 d. The normal food was exchanged to anle138b-containing food (2 g anle138b/kg food; Ssniff, Soest, Germany) three days before killing animals to get a steady state. Animals were killed by cervical dislocation during their active phase on the fourth day at time points 12 h, 14 h, 16 h and 20 h. Brains were taken out and immediately frozen in liquid nitrogen. Samples were stored at -80 °C.

The tissues were thawed at 4 °C prior to use. It was homogenized twice in 5 mL of acetonitrile at maximum speed for 3 minutes using a homogenizer (IKA ULTRA-TURRAX Tube drive workstation, Germany). The homogenate was ultrasonicated at 30 °C for 5 minutes and centrifuged at 5000 x g for 10 minutes. An aliquot (100 µL of supernatant was injected into HPLC system. Briefly, analytical high performance liquid chromatography (HPLC) was performed using a Waters HPLC system with a Waters 996 Photodiode Array Detector. All separations involved a mobile phase of 0.1 % trifluoroacetic acid (TFA) (v/v) in water (solvent A) and 0.1 % TFA in acetonitrile (solvent B). HPLC was performed using reversed-phase (RP) column Eurospher RP 18, 100 Å, 5 µm, 250 × 4.6 mm at flow rates of 1 mL/min with a gradient of solvent B from 0 % to 100 % in 50 minutes. The effluent was monitored for UV absorption at 260 nm. Samples were quantified using peak area ratio of compounds to external standard.

Primary cultures

Primary cortical neurons were isolated from NMRI mouse embryos at day E14.5. Cortices were collected in DMEM (Invitrogen) and cells were dissociated by incubation with Trypsin/EDTA for 6.5 min at 37 °C. Cells were then diluted in neurobasal medium containing B27 supplement (Gibco / Life Technologies). Cells were plated at a density of 8.0 x 10^5 onto 0.2 mg/ml poly-D-lysin and 2 µg/ml Laminin (Sigma) coated 6 well plates and incubated at 37 °C with 5 % CO_2. 30 min after seeding a change of medium was performed.

Western blot

Cell pellets were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 mM EDTA, complete protease inhibitors (Roche)) and sonicated. Samples were mixed 1:1 with 2 x concentrate of sample buffer consisting of 2 % SDS, 20 % glycerol, 20 mM Tris-Cl, pH 6.8, 2 mM ethylene diamine tetraacetic acid (EDTA), 160 mM dithiothreitol (DTT), and 0.1 mg/ml bromphenol blue dye and boiled for 5 min at 95 °C. Proteins were resolved on 10 % SDS gels and blotted onto PVDF-membranes (Roche). Antibodies were purchased from the following companies: Tau-5 (Life technologies), anti-human PHF pSer202/Thr205 (Fisher Scientific), Tau 1 (Millipore), pGSK3 (Cell Signalling), GSK3 (Cell Signalling), GAPDH (Cell Signalling).

PP2A activity assay

PP2A-holoenzymes, which were purified by immunoprecipitation using a PP2A-A-subunit antibody (Millipore), were incubated with or without 100 µM anle138b or 40 nM okadaic acid for 1 hour. Afterwards PP2A phosphatase activity was determined

using a malachite green assay kit (PP2A Immunoprecipitation Phosphatase Assay Kit, Millipore) following the manufacturer's instructions.

Immunohistochemistry

Mice were transcardially perfused with cold PBS after being deeply anesthetized. The brains were removed and weighed. From all animals one brain hemisphere was fixed in 4 % paraformaldehyde for 24 h for histological examination. After fixation the brain tissue was sectioned into 50 µm tissue slices using a Leica VT1000S vibratome (Leica, Wetzlar, Germany). Immunohistochemistry was performed as described by Gogolla et al. [1] with minor modifications. Briefly, the sections were incubated with permeabilization solution (1 % Triton X-100 in PBS) for 12 h under constant agitation. Subsequently, the slices were blocked with 20 % BSA in PBS buffer for 6 h. Then the tissue sections were stained with the following primary antibodies in 3 % BSA/PBS overnight: tau protein (AT8, 0.3 µg/mL; AT100, 1µg/mL, ThermoScientific), and autophagic marker (anti-LC3, 1:1000, Novus Biologicals; P62/SQSTM1, 1:1000, Abcam). After three washes with 3 % BSA/PBS the slices were incubated with a correspondent secondary antibody in 3 % BSA/PBS for 3 h (LifeTechnologies). Finally, the slices were mounted using fluorescence mounting medium (Dako, Hamburg, Germany) and images were acquired on an inverted Zeiss LSM700 confocal microscope and Zeiss AxioScan.Z1 (Zeiss, Germany). For quantitative analysis of tau pathology with the tau stain AT8, we counted the number positively stained cell bodies in matched brain sections. For each mouse, three sections per brain were counted. Quantification was done with the ZEN software package (Zeiss, Germany).

CHX timecourse

To investigate the effect of anle138b on protein stability, time course experiments with the translation inhibitor cycloheximide (CHX) were performed. Primary cortical neurons were treated with or without 10 µM anle138b in the presence or absence of CHX, f.c. 40 µM. Cells were lysed after 24h, 48h, or 72h of CHX-treatment and the degradation of Tau protein over time was analyzed on a western blot.

Immunoprecipitation

To investigate the effect of anle138b on tau ubiquitination, we immunopurified ubiquitinated proteins and detected ubiquitinated tau protein on a western blot. Therefore, primary cortical neurons were treated with or without 10 µM anle138b for 24h, harvested and lysed in a buffer containing 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 % NP40, and complete protease inhibitors (Roche) using the precellys cell homoginator. Immunoprecipitation was carried out using anti-ubiquitin antibodies (Santa Cruz) in combination with protein G agarose beads (Roche) according to the manufacturer's instructions. Ubiquitinated Tau protein was detected on western blots using Tau- antibodies.

Animals and treatment

PS19 human tau transgenic mice [2] were obtained from The Jackson Laboratories (Stock number 8169, The Jackson Laboratory) and group housed separated by gender in individually ventilated cages (IVCs) under specified pathogen-free (SPF) conditions. Age and sex matched non-transgenic littermates were used for all experiments and equal numbers of male and female mice were randomly assigned to the experimental groups. Mice had unlimited access to food and water, the light and

dark cycle was 12h/12h and the temperature was kept constant at 22°C. The compound anle138b was administered with drug-supplemented food pellets (2 g compound/kg food; Ssniff, Soest, Germany). The compound was added during the manufacturing process of the food pellets as dry powder without a vehicle. Therefore, control mice were treated with non-supplemented food pellets. The treatment was initiated after weaning and lasted until the time of sacrifice. Trained animal caretakers blinded to the study design assessed body weight monthly. The mouse weight was normalized to the peak of body weight development. The peak of body weight was defined as the onset of disease. The experimental procedures were in accordance with guidelines established by the DZNE and were approved by the government of North-Rhine-Westphalia.

References

1 Gogolla N, Galimberti I, DePaola V, Caroni P (2006) Staining protocol for organotypic hippocampal slice cultures. Nat Protoc 1: 2452-2456 Doi 10.1038/nprot.2006.180

2 Yoshiyama Y, Higuchi M, Zhang Bet al. (2007) Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron 53: 337-351 Doi 10.1016/j.neuron.2007.01.010