

Rifampicin is a candidate preventive medicine against amyloid- β and tau oligomers

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Amyloid- β , tau, and α -synuclein, or more specifically their soluble oligomers, are the aetiologic molecules in Alzheimer's disease, tauopathies, and α -synucleinopathies, respectively. These proteins have been shown to interact to accelerate each other's pathology. Clinical studies of amyloid- β -targeting therapies in Alzheimer's disease have revealed that the treatments after disease onset have little benefit on patient cognition. These findings prompted us to explore a preventive medicine which is orally available, has few adverse effects, and is effective at reducing neurotoxic oligomers with a broad spectrum. We initially tested five candidate compounds: rifampicin, curcumin, epigallocatechin-3-gallate, myricetin, and scyllo-inositol, in cells expressing amyloid precursor protein (APP) with the Osaka (E693 Δ) mutation, which promotes amyloid- β oligomerization. Among these compounds, rifampicin, a well-known antibiotic, showed the strongest activities against the accumulation and toxicity (i.e. cytochrome *c* release from mitochondria) of intracellular amyloid- β oligomers. Under cell-free conditions, rifampicin inhibited oligomer formation of amyloid- β , tau, and α -synuclein, indicating its broad spectrum. The inhibitory effects of rifampicin against amyloid- β and tau oligomers were evaluated in APP_{OSK} mice (amyloid- β oligomer model), Tg2576 mice (Alzheimer's disease model), and tau609 mice (tauopathy model). When orally administered to 17-month-old APP_{OSK} mice at 0.5 and 1 mg/day for 1 month, rifampicin reduced the accumulation of amyloid- β oligomers as well as tau hyperphosphorylation, synapse loss, and microglial activation in a dose-dependent manner. In the Morris water maze, rifampicin at 1 mg/day improved memory of the mice to a level similar to that in non-transgenic littermates. Rifampicin also inhibited cytochrome *c* release from the mitochondria and caspase 3 activation in the hippocampus. In 13-month-old Tg2576 mice, oral rifampicin at 0.5 mg/day for 1 month decreased amyloid- β oligomer accumulation, tau hyperphosphorylation, synapse loss, and microglial activation, but not amyloid deposition. Rifampicin treatment to 14–15-month-old tau609 mice at 0.5 and 1 mg/day for 1 month also reduced tau oligomer accumulation, tau hyperphosphorylation, synapse loss, and microglial activation in a dose-dependent fashion, and improved the memory almost completely at 1 mg/day. In addition, rifampicin decreased the level of p62/sequestosome-1 in the brain without affecting the increased levels of LC3 (microtubule-associated protein light chain 3) conversion, suggesting the restoration of autophagy-lysosomal function. Considering its prescribed dose and safety in humans, these results indicate that rifampicin could be a promising, ready-to-use medicine for the prevention of Alzheimer's disease and other neurodegenerative diseases.

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Abbreviations: CMC = carboxyl methyl cellulose; DMSO = dimethyl sulphoxide; EGCG = epigallocatechin-3-gallate; ELISA = enzyme-linked immunosorbent assay; GST = glutathione-S-transferase; GuHCl = guanidine hydrochloride; LC3 = microtubule-associated protein light chain 3; NMR = nuclear magnetic resonance; PICUP = photo-induced cross-linking of unmodified proteins; SDS = sodium dodecyl sulphate

Introduction

Neurodegenerative diseases are characterized by the accumulation of insoluble aggregates of misfolded proteins in the CNS. For example, Alzheimer's disease shows extracellular deposits of amyloid- β aggregates and intracellular inclusions of hyperphosphorylated tau aggregates. Intracellular tau inclusions are also a hallmark of tauopathies (Lee *et al.*, 2001), which include Pick's disease, corticobasal degeneration, progressive supranuclear palsy, argyrophilic grain disease, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). A different type of neurodegenerative disorder, α -synucleinopathies (Galvin *et al.*, 2001), which include diffuse Lewy body disease, Parkinson's disease, and multiple system atrophy, exhibit intracellular inclusions of α -synuclein aggregates. Recent evidence suggests that these proteins aggregate, more specifically their soluble oligomers, impair synaptic and cognitive function (Lambert *et al.*, 1998; Walsh *et al.*, 2002; Cleary *et al.*, 2005; Diógenes *et al.*, 2012; Lasagna-Reeves *et al.*, 2012; Martin *et al.*, 2012) and mediate the cell-to-cell propagation of neuropathology (Danzer *et al.*, 2009; Langer *et al.*, 2011; Lasagna-Reeves *et al.*, 2012). Thus, reducing neurotoxic oligomers would appear to be a rational strategy to treat these diseases.

Clinical studies of amyloid- β -targeting therapies in Alzheimer's disease have revealed that the treatments after disease onset have little effect on the cognition of patients (Imbimbo and Giardina, 2011; Panza *et al.*, 2014). In addition, amyloid- β , tau, and α -synuclein have been shown to synergistically interact to accelerate each other's pathology (Clinton *et al.*, 2010; Larson *et al.*, 2012; Umeda *et al.*, 2014). These findings suggest that the treatment of neurodegenerative diseases should be started prior to the onset of clinical symptoms (Sperling *et al.*, 2011) and that not only the aetiologic molecule but also other associated proteins should be targeted together, desirably with one drug. This notion prompted us to develop a preventive medicine that is orally available, has few adverse effects, and is effective at reducing extracellular and intracellular neurotoxic oligomers with a broad spectrum.

To explore such drugs, we consulted the literature for natural and semi-natural small compounds that have inhibitory activity against amyloid- β aggregation. Natural small compounds are presumed to have less adverse effects and are cheaper for long-term preventive treatment. We selected five compounds as candidates: rifampicin (Tomiyama *et al.*, 1994, 1996, 1997), curcumin (Lim *et al.*, 2001; Ono *et al.*, 2004; Yang *et al.*, 2005), epigallocatechin-3-gallate (EGCG) (Bastianetto *et al.*, 2006; Rezai-Zadeh *et al.*, 2008), myricetin (Ono *et al.*, 2003; Hamaguchi *et al.*, 2009), and scyllo-inositol (McLaurin *et al.*, 2000, 2006). These compounds have been shown to interact with amyloid- β to affect its aggregation *in vitro* and, except rifampicin, ameliorate amyloid pathology in mouse models when orally administered. Rifampicin is a well-known antibiotic used in humans and is known to penetrate into the brain following oral administration (No authors, 2008). Among these candidates, we found that rifampicin had the strongest activities against the accumulation and toxicity of intracellular amyloid- β oligomers in cultured cells. Under cell-free conditions, rifampicin inhibited the oligomer formation of amyloid- β , tau, and α -synuclein, indicating its broad spectrum. When orally administered to three different mouse models of Alzheimer's disease and tauopathy, rifampicin reduced the accumulation of amyloid- β oligomers and tau oligomers, respectively, and improved the memory of the mice. Our results indicate that rifampicin could be a promising, ready-to-use medicine for the prevention of Alzheimer's disease and other neurodegenerative diseases.

Materials and methods

Immunocytochemical analysis of amyloid- β oligomers in cultured cells

Rifampicin, myricetin, and EGCG were purchased from Sigma-Aldrich, and curcumin and scyllo-inositol were purchased from Wako Pure Chemical Industries and Tokyo Chemical Industry, respectively. The stock solutions of

rifampicin, myricetin, and EGCG were prepared at 100 mM in dimethyl sulphoxide (DMSO); curcumin was prepared at 10 mM in ethanol and scyllo-inositol at 1 mM in Opti-MEMTM I medium (Life Technologies). COS-7 cells cultured in Opti-MEMTM I medium were transfected with APP_{OSK} construct using the Lipofectamine[®] PlusTM reagent (Invitrogen), as described previously (Umeda *et al.*, 2011). On the day after the transfection, each compound was added to the culture to a final concentration of 100 μ M. The cells were further cultured for 2 days, and then fixed with 4% paraformaldehyde and treated with 0.05% Tween-20, as described previously (Umeda *et al.*, 2011). The cells were stained with amyloid- β oligomer-selective NU-1 antibody (Lambert *et al.*, 2007) followed by fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) and counterstained with DAPI (Molecular Probes). FITC fluorescence intensity in a constant field (400 μ m \times 400 μ m) of the culture was quantified using NIH ImageJ software, and the number of DAPI-positive cells in the same field was counted. Amyloid- β oligomer accumulation was assessed by dividing the FITC intensity with the cell number.

Biochemical analysis of amyloid- β oligomers in cultured cells

COS-7 cells transfected with APP_{OSK} construct were treated with rifampicin at 100 μ M for 2 days, as described above. Conditioned media were collected for amyloid- β enzyme-linked immunosorbent assay (ELISA). Cells in a 10 cm dish were harvested and lysed by sonication in 100 μ l of 1% TritonTM X-100 in 50 mM Tris-HCl, pH 7.6, and 150 mM NaCl (Tris-buffered saline, TBS) containing 1/100 volumes of protease inhibitor cocktail (P8340; Sigma-Aldrich). The cell lysates were subjected to western blot with mouse monoclonal 82E1 antibody (IBL) for amyloid- β , rabbit polyclonal C40 antibody (Tomiya *et al.*, 2010) for APP, and rabbit polyclonal anti-actin antibody (Sigma-Aldrich). Signals were visualized using an LAS-3000 luminescent image analyzer (Fujifilm). The levels of amyloid- β ₄₀, amyloid- β ₄₂, and amyloid- β oligomers in conditioned media were measured using Human β Amyloid(1-40) ELISA kit Wako II; Human β Amyloid(1-42) ELISA kit Wako, High-sensitive (both from Wako); and Human Amyloid β Oligomers (82E1-specific) Assay kit-IBL (IBL), respectively.

Photo-induced cross-linking of unmodified proteins assay

Synthetic amyloid- β ₄₀ and amyloid- β ₄₂ peptides (Peptide Institute) were dissolved to 50 μ M in 10% (v/v) 60 mM NaOH and 90% (v/v) 10 mM phosphate buffer, pH 7.4. Recombinant tau-441 protein (rPeptide) was dissolved to 50 μ M in 20 mM Tris buffer, pH 7.4, while recombinant α -synuclein protein (rPeptide) was dissolved to 50 μ M in 10 mM HEPES buffer, pH 7.4. After sonication for 1 min, these protein solutions were centrifuged for 10 min at 16 000g. A stock solution of glutathione-S-transferase (GST; Sigma-Aldrich) was prepared by dissolving the lyophilizate to a concentration of 250 μ M in 60 mM NaOH. Prior to use, the GST solution was diluted to 50 μ M with 10 mM phosphate buffer, pH 7.4.

Rifampicin (Sigma-Aldrich) was dissolved to 2.5 mM in ethanol and then diluted to 50 and 200 μ M with 10 mM phosphate buffer, pH 7.4. The protein solutions were mixed with the rifampicin solutions at 1:1 volume and immediately cross-linked by photo-induced cross-linking of unmodified proteins (PICUP), as described previously (Ono *et al.*, 2009, 2012). The samples were subjected to SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) with 10–20% Tricine gels under reducing conditions, and the oligomer formation of each protein was visualized by silver staining using the SilverQuest Silver Staining Kit (Invitrogen).

To confirm the PICUP results, amyloid- β oligomers were measured by ELISA. Amyloid- β ₄₂ peptide with the Osaka mutation (Peptide Institute) was dissolved to 500 μ M in 60 mM NaOH and diluted to 25 μ M in phosphate-buffered saline (PBS) containing rifampicin at 25 μ M or 100 μ M. The mixtures were subjected to sandwich amyloid- β ₄₂ ELISA (Wako) and direct oligomer ELISA (see below).

Rifampicin treatment of model mice

Rifampicin was dissolved by sonication to 1 and 2 mg/ml in 0.5% low-viscosity carboxyl methyl cellulose (CMC; Sigma-Aldrich) just before use every day. Five hundred microlitres of either rifampicin solution was orally administered using feeding needles to male APP_{OSK} mice, Tg2576 mice (Taconic), and tau609 mice ($n = 6$ –12 per group) every day for 1 month. Age-matched transgenic and non-transgenic littermates ($n = 6$ –17 per group) administered CMC solution without rifampicin were used as controls. All animal experiments were approved by the ethics committee of Osaka City University (Osaka, Japan) and performed in accordance with the Guide for Animal Experimentation, Osaka City University.

Behavioural test of model mice

Spatial reference memory in mice was examined using the Morris water maze, as described previously (Umeda *et al.*, 2013, 2015). The tests were performed under unblinded conditions: the experimenter knew which mice were treated with rifampicin. Daily oral administration of rifampicin was continued during the behavioural test.

Immunohistochemical analysis of amyloid and tau pathologies in model mice

After the behavioural tests, brain sections were prepared and immunohistochemical staining was performed as described previously (Umeda *et al.*, 2014, 2015). For APP_{OSK} mice, amyloid- β oligomers and phosphorylated tau were stained with mouse monoclonal 11A1 (IBL) and PHF-1 antibodies (a kind gift from Dr Peter Davies, Albert Einstein College of Medicine), respectively. For Tg2576 mice, amyloid deposits were stained with those antibodies and rabbit polyclonal β 001 antibody (Tomiya *et al.*, 2010). For tau609 mice, tau oligomers and phosphorylated tau were stained with rabbit polyclonal T22 antibody (EMD Millipore) and mouse monoclonal PHF-1, AT8 (Thermo Scientific), and Ta1505 antibodies (Umeda *et al.*, 2015), respectively. Synaptophysin

and microglia were stained with mouse monoclonal antibody to synaptophysin (SVP-38; Sigma-Aldrich) and rabbit polyclonal antibody to Iba-1 (Wako Pure Chemical Industries), respectively. Amyloid deposition, amyloid- β oligomer accumulation, tau oligomer accumulation, tau hyperphosphorylation, synapse loss, and microglial activation were evaluated by quantifying the corresponding antibody-staining areas/intensities per unit area in each photograph using NIH ImageJ software, as described previously (Umeda *et al.*, 2014, 2015).

Biochemical analysis of amyloid and tau pathologies in model mice

Hippocampal tissues were dissected from mouse brains and homogenized by sonication in four volumes of TBS containing protease inhibitor cocktail (P8340). The homogenates were subjected to western blot with SVP-38 antibody for synaptophysin and anti-actin antibody. The remaining brain tissues, not including the cerebellum, were homogenized in four volumes of TBS containing P8340, along with phosphatase inhibitor cocktail (Nacalai Tesque) for tau609 mice, using a tissue grinder with Teflon pestle. The homogenates were subjected to western blot with C40 antibody and mouse monoclonal 6E10 antibody (Covance) for total and human APP, respectively; and rabbit polyclonal pool-2 antibody (Umeda *et al.*, 2013) and mouse monoclonal Tau12 antibody (Abcam) for total and human tau, respectively.

Brain homogenates from APP_{OSK} and Tg2576 mice were fractionated by three-step ultracentrifugation including TBS, 2% SDS, and 70% formic acid extraction, essentially as described previously (Tomiyama *et al.*, 2010). A portion of SDS and formic acid extracts were dialyzed against TBS at 4 °C overnight using Slide-A-Lyzer™ Dialysis Cassette G2 with 2K cut-off membrane (Thermo Scientific). The levels of amyloid- β_{40} , amyloid- β_{42} , and amyloid- β oligomers in each fraction were measured using the corresponding ELISA kits. Dialyzed SDS extracts were used only for oligomer ELISA, whereas dialyzed formic acid extracts were used for all ELISA. For the measurement of amyloid- β_{40} and amyloid- β_{42} in APP_{OSK} samples, synthetic peptides with the Osaka (E22 Δ) mutation (American Peptide Company) were used as standards. The levels of amyloid- β oligomers were also quantified by direct ELISA with 11A1 antibody (at 1 μ g/ml) according to the method of Castillo-Carranza *et al.* (2015), except that each fraction was diluted 2-fold in 0.05 M sodium bicarbonate and allowed to coat ELISA plates at 50 μ l/well. In contrast, brain homogenates from tau609 mice were fractionated by three-step ultracentrifugation including TBS, 1% *N*-lauroylsarcosinate (sarkosyl), and 4 M guanidine hydrochloride (GuHCl) extraction, essentially as described previously (Umeda *et al.*, 2013). GuHCl extracts were dialyzed against TBS at 4 °C overnight using Slide-A-Lyzer™ Dialysis Cassette G2 with 20K cut-off membrane (Thermo Scientific). The levels of human tau, pSer396-tau, and pSer199-tau in each fraction were measured using TAU (total) ELISA kit, Human; TAU (pS396) Human ELISA kit; and TAU (pS199) Phospho-ELISA kit, Human (all from Novex, Thermo Scientific), respectively. The levels of tau oligomers were quantified by direct ELISA with T22 antibody (1:250) according to the method of Castillo-Carranza *et al.* (2015), as described above.

Statistical analysis

Comparisons of means among more than two groups were performed using ANOVA or two-factor repeated measures ANOVA (for the behavioural tests), followed by Fisher's PLSD test. Differences with a *P*-value of <0.05 were considered significant.

Results

Effects of candidate compounds on intracellular amyloid- β oligomers in cultured cells

A recent study using induced pluripotent stem cells derived from patients with Alzheimer's disease suggests that Alzheimer's disease can be classified into two categories: extracellular amyloid- β type and intracellular amyloid- β type (Kondo *et al.*, 2013). This finding indicates that it is desirable for amyloid- β -targeting medicines to penetrate into cells. The Osaka mutation in APP has been shown to cause an intraneuronal accumulation of amyloid- β oligomers (Tomiyama *et al.*, 2008, 2010; Nishitsuji *et al.*, 2009; Umeda *et al.*, 2011), and thus provides a good model for study of the intracellular amyloid- β type. We examined the effects of five candidate compounds: rifampicin, curcumin, EGCG, myricetin, and scyllo-inositol, on the accumulation of intracellular amyloid- β oligomers in APP_{OSK}-expressing cells. COS-7 cells transfected with APP_{OSK} were cultured for 2 days in the presence of each compound at 100 μ M and stained with amyloid- β oligomer-selective NU-1 antibody. All compounds decreased NU-1-positive staining, with rifampicin having the highest activity and myricetin the lowest (Fig. 1A and B). Western blot with cell homogenates revealed that rifampicin significantly reduced the levels of intracellular amyloid- β dimers and possible trimers and tended to decrease amyloid- β monomers without affecting APP expression (Fig. 1C and D). Simultaneously, conditioned media of rifampicin-treated cells were collected and subjected to ELISA to measure amyloid- β_{40} , amyloid- β_{42} , and amyloid- β oligomers. Rifampicin significantly increased extracellular levels of amyloid- β_{40} and amyloid- β_{42} and significantly decreased amyloid- β oligomers (Fig. 1E–G). We observed that rifampicin did not affect amyloid- β production from full-length APP or its β -cut fragment in cultured cells (Supplementary Fig. 1). Taken together, these results suggest that rifampicin inhibits intracellular and extracellular amyloid- β (particularly amyloid- β_{42}) oligomerization and promotes the secretion of amyloid- β monomers from cells.

We also compared the protective effects of the five compounds against intracellular amyloid- β -induced cell damage. Transfected cells treated with each compound at 1–100 μ M for 2 days were harvested, and the level of cytochrome *c* released from the mitochondria into the cytoplasm was

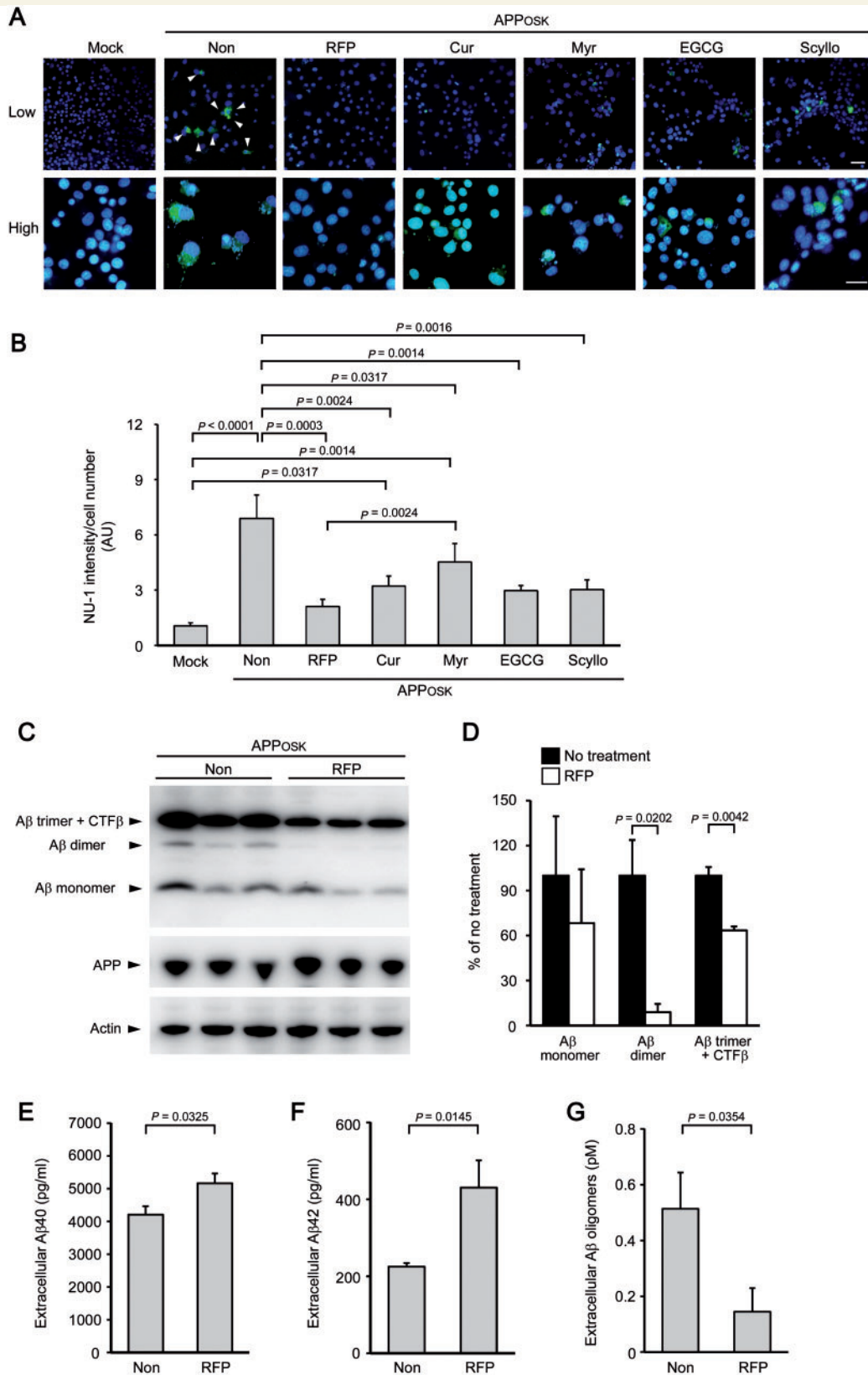


Figure 1 Effects of candidate compounds on intracellular accumulation of amyloid- β oligomers in cultured cells. **(A)** COS-7 cells transfected with APP_{OSK} were cultured for 2 days in the presence of rifampicin (RFP), curcumin (Cur), myricetin (Myr), EGCG, and scyllo-inositol (Scyllo) at 100 μ M and stained with amyloid- β oligomer-selective NU-1 antibody (green) and DAPI (blue) after fixation. Note that the number of cells was decreased due to cell death when the tested compound was not effective against amyloid- β oligomer accumulation. Scale bars = 50 μ m (top row) and 30 μ m (bottom row). **(B)** NU-1 fluorescence intensity in a constant field of the culture was quantified, and the number of DAPI-

(continued)

measured by western blot. All compounds reduced the level of cytosolic cytochrome *c* in a dose-dependent manner, with rifampicin and curcumin having the highest activity and scyllo-inositol the lowest (Supplementary Fig. 2). Thus, rifampicin showed the strongest protective effects on the accumulation and toxicity of intracellular amyloid- β oligomers. As rifampicin has long been used in humans as an antibiotic without serious adverse effects, we chose rifampicin as a favourable candidate for preventive medicine and subjected it to further evaluation.

Effects of rifampicin on protein oligomerization under cell-free conditions

We examined the effects of rifampicin on the oligomer formation of amyloid- β , tau, and α -synuclein under cell-free conditions. Synthetic amyloid- β_{40} and amyloid- β_{42} peptides were incubated in the presence of rifampicin at a molar ratio of 1:1 and 1:4 and cross-linked by the PICUP method. Rifampicin inhibited the oligomer formation of both amyloid- β_{40} and amyloid- β_{42} peptides in a dose-dependent manner with complete inhibition of amyloid- β_{40} oligomerization at the ratio 1:4 (Fig. 2A and B). Similar experiments were performed using recombinant tau-441 and α -synuclein proteins, with GST as the control. Again, rifampicin inhibited the oligomer formation of tau and α -synuclein in a dose-dependent manner, with almost complete inhibition at the ratio 1:4 (Fig. 2C and D). No effect was seen with GST (Fig. 2E). The result for amyloid- β_{42} was confirmed by ELISA using synthetic amyloid- β_{42} peptide with the Osaka mutation. Rifampicin decreased the levels of amyloid- β oligomers (Fig. 2F) and inversely increased the levels of amyloid- β_{42} (probably monomers) in the peptide solutions in a dose-dependent manner (Fig. 2G).

To investigate the mechanism by which rifampicin inhibits oligomer formation of neuropathogenic proteins but not GST, we analysed the interaction of rifampicin with amyloid- β_{42} , tau-441, α -synuclein, and GST by nuclear magnetic resonance (NMR) spectroscopy, as described previously (Ono *et al.*, 2012; Takahashi *et al.*, 2015). NMR spectroscopy cannot detect heterogeneous, minor species, such as oligomers in the protein solutions, and thus provides information primarily on monomers. When incubated with these proteins, rifampicin caused neither chemical shift changes nor line broadening (Supplementary Fig. 3), suggesting that rifampicin does not bind to the protein monomers. Amyloid- β , tau, and α -synuclein are known to take

the β -sheet structure during aggregation into fibrils (Tomiya *et al.*, 1996; Li *et al.*, 2004; von Bergen *et al.*, 2005). We considered whether rifampicin binds to only protein oligomers that grow to amyloid fibrils taking extended β -sheet structure, which would suggest GST oligomers do not. To test this theory, we performed thioflavin T assays, in which the fluorescence intensity reflects the levels of β -sheet structure primarily in high-order protein assemblies such as fibrils. Amyloid- β_{42} peptide at 25 μ M showed an increase in fluorescence intensity during incubation, whereas GST at the same concentration did not (Supplementary Fig. 4). Taken together with the findings in Figs 1C–G and 2F–G, these results suggest that rifampicin binds to a certain conformation (for example, extended β -sheet) of amyloidogenic protein oligomers that is not formed in physiological oligomers of non-amyloidogenic proteins, in order to promote their dissociation into monomers. Thus, rifampicin was shown to have anti-oligomerizing activity with a broad spectrum but specific to amyloidogenic proteins.

Effects of rifampicin on memory and amyloid- β oligomers in APP_{OSK} mice

APP_{OSK} mice are a transgenic mouse model of Alzheimer's disease that accumulate amyloid- β oligomers without forming amyloid plaques (Tomiya *et al.*, 2010). The mice exhibit intraneuronal accumulation of amyloid- β oligomers and subsequent tau hyperphosphorylation, synapse loss, and memory impairment at 8 months, microglial activation at 12 months, and neuronal loss at 24 months. Thus, APP_{OSK} mice are an ideal model for investigating the effects of rifampicin on amyloid- β oligomers *in vivo*. Rifampicin is usually prescribed to adult humans at 10 mg/kg daily (No authors, 2008). Assuming a mean body weight of adult mice of 30 g, the mean dose for mice corresponds to 0.3 mg daily. We decided the daily dose of rifampicin for mice as 0.5 mg. Rifampicin dissolved in 0.5% CMC was orally administered to 11–12-month-old APP_{OSK} mice (mean body weight 35.5 g) at 0.5 mg/shot every day for 1 month. The spatial reference memory of mice was examined using the Morris water maze. Compared with CMC-treated age-matched non-transgenic littermates, CMC-treated transgenic mice showed significantly impaired memory, whereas rifampicin-treated transgenic mice exhibited a significant improvement of memory to almost the same level (Fig. 3A and B). We repeated the experiment using older APP_{OSK} mice. Rifampicin was administered to

Figure 1 Continued

positive cells in the same field was counted. Each bar represents the mean fluorescence intensity/cell \pm standard error of the mean (SEM) ($n = 3$ for all groups). (C) Non-treated and rifampicin-treated cells were harvested and subjected to western blot with 82E1 antibody for amyloid- β , C40 antibody for APP, and antibody for actin. (D) The levels of amyloid- β monomers, dimers, and trimers + CTF β (carboxyl terminal fragment β of APP) in cell lysates were quantified. Each bar represents the mean \pm SEM ($n = 3$ for both groups). (E–G) Conditioned media were collected and subjected to ELISA to measure amyloid- β_{40} (E), amyloid- β_{42} (F), and amyloid- β oligomers (G). Each bar represents the mean \pm SEM ($n = 7$ for both groups).

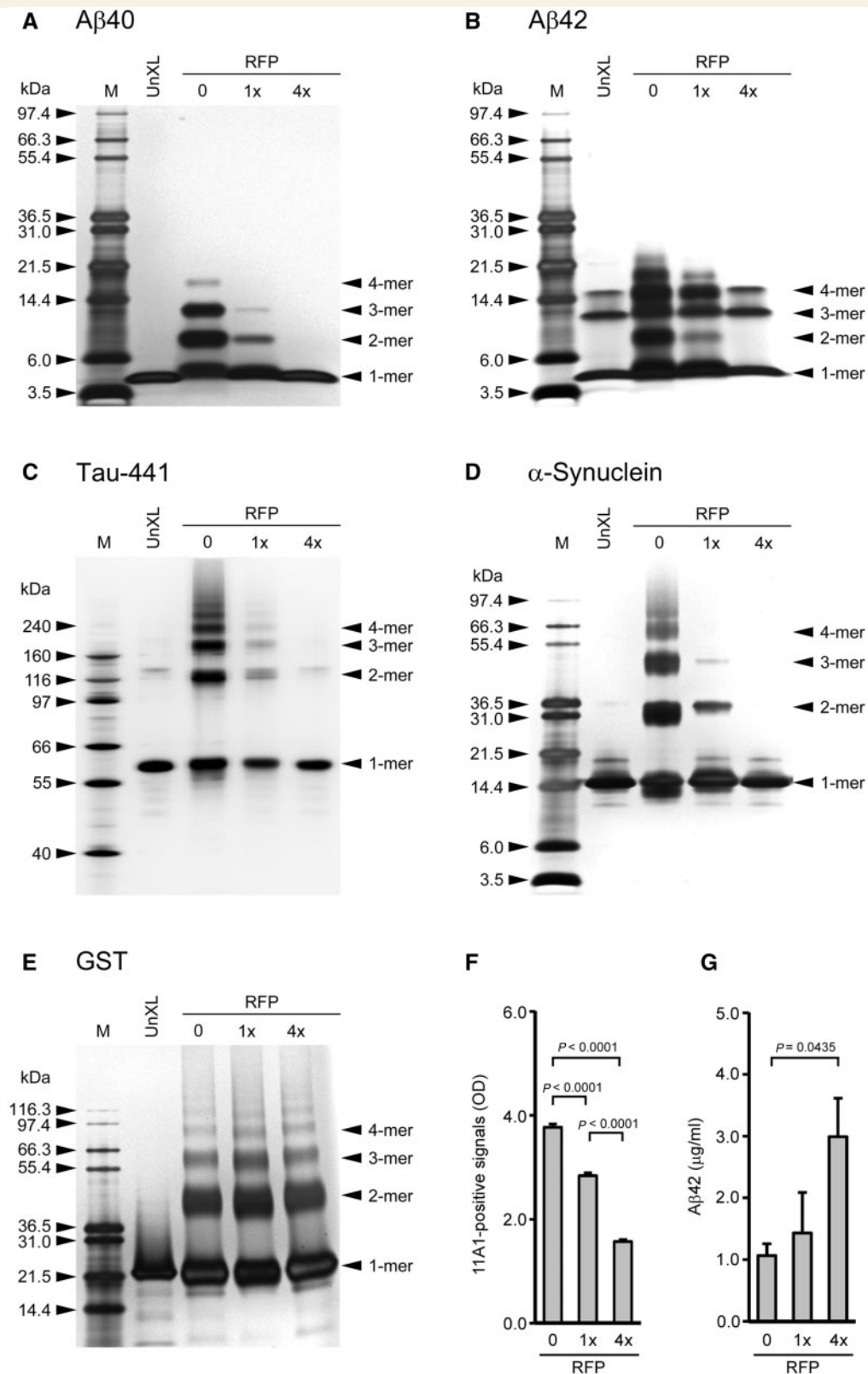


Figure 2 Effects of rifampicin on oligomer formation of amyloid- β , tau, and α -synuclein under cell-free conditions. Amyloid- β_{40} (A), amyloid- β_{42} (B), tau-441 (C), α -synuclein (D), and GST (E) were incubated at 25 μ M with rifampicin (RFP) at 25 μ M (molar ratio of 1:1) or 100 μ M (1:4) and cross-linked by PICUP. The samples were subjected to SDS-PAGE under reducing conditions, and the oligomer formation by each protein was visualized by silver staining. M = molecular size marker; UnXL = uncross-linked samples; 1x = RFP 25 μ M; 4x = RFP 100 μ M. (F and G) Amyloid- β_{42} peptide with the Osaka mutation was mixed at 25 μ M with rifampicin at 25 μ M or 100 μ M and subjected to direct oligomer ELISA (F) and sandwich amyloid- β_{42} ELISA (G). Each bar represents the mean \pm SEM ($n = 3$ for each group).

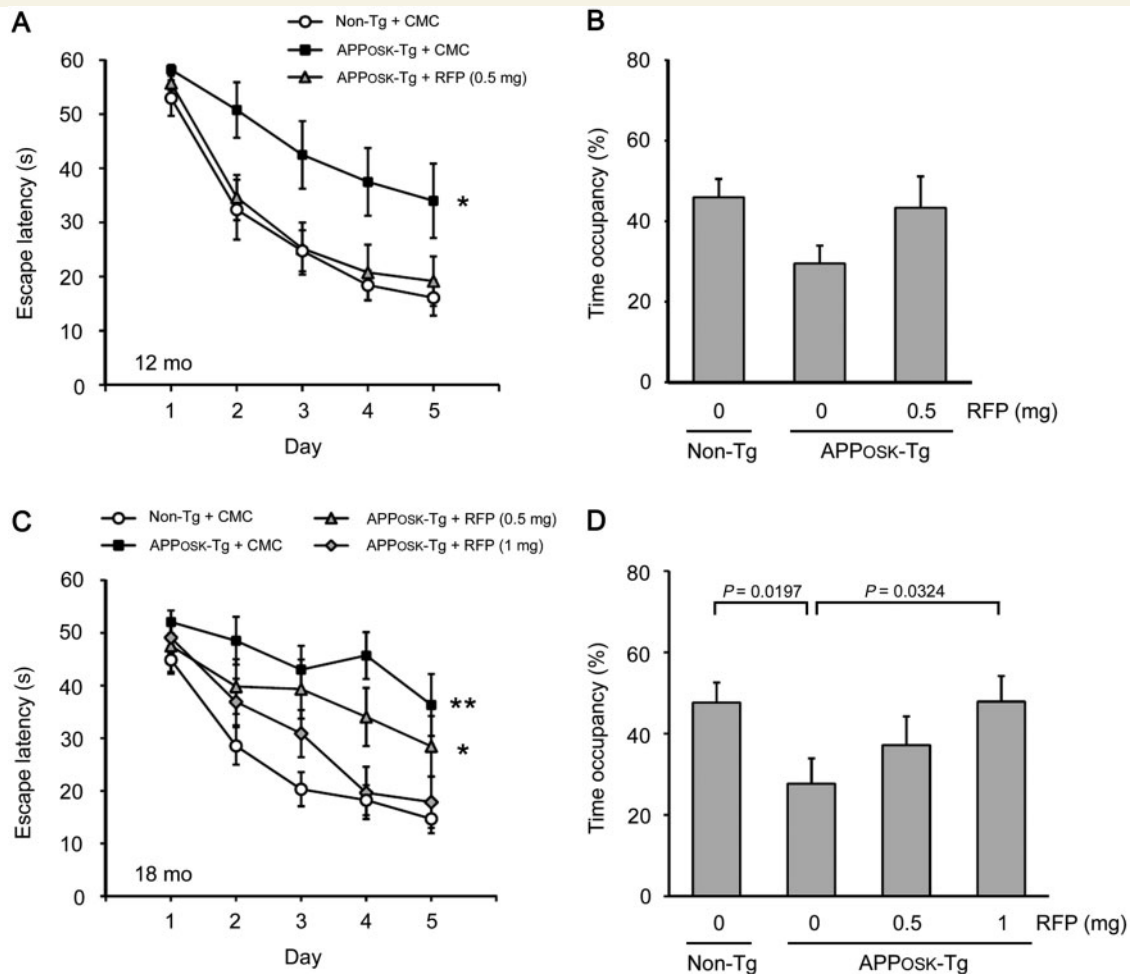


Figure 3 Effects of rifampicin on the memory of APP_{OSK} mice. (A) Rifampicin dissolved in 0.5% CMC was orally administered to 11–12-month-old APP_{OSK} mice at 0.5 mg/shot every day for 1 month. The spatial reference memory of mice was examined using the Morris water maze, in which mice were trained to swim to the hidden platform for five consecutive days. Each point represents the mean latency of five trials per day \pm SEM [$n = 10$ for non-transgenic (Non-Tg); $n = 9$ for each transgenic (Tg) group]. * $P = 0.0034$ versus non-transgenic and $= 0.0119$ versus RFP. (B) At Day 6, retention of memory was assessed by a probe trial for 60 s with the platform removed. Each bar represents the mean time occupancy \pm SEM in the target quadrant. (C) Rifampicin was administered to 17-month-old APP_{OSK} mice at 0.5 and 1 mg/shot every day for 1 month, and the Morris water maze was performed. $n = 16$ for non-transgenic; $n = 10$ for each transgenic group. ** $P < 0.0001$ versus non-transgenic and $= 0.0060$ versus 1 mg RFP; * $P = 0.0073$ versus non-transgenic. (D) Retention of memory was assessed by a probe trial.

17-month-old APP_{OSK} mice (mean body weight 37.8 g) at 0.5 and 1 mg/shot every day for 1 month. In the Morris water maze, rifampicin restored the memory of transgenic mice in a dose-dependent fashion. The memory of transgenic mice was markedly improved by 1 mg/shot of rifampicin to a level similar to that in non-transgenic littermates and significantly but to a lesser degree at 0.5 mg/shot (Fig. 3C and D).

Brain sections were prepared from 18-month-old rifampicin-treated mice and stained with amyloid- β oligomer-specific 11A1 antibody. Rifampicin significantly reduced 11A1-positive staining in the cerebral cortex and hippocampus in a dose-dependent manner (Fig. 4A, B and Supplementary Fig. 5A). Brain homogenates were prepared from 18-month-old rifampicin-treated mice (at 1 mg/day) and separated into TBS-, SDS-, and formic acid-soluble

fractions. Western blot with brain homogenates revealed no significant differences in the levels of total (C40-positive) and human APP (6E10-positive) between CMC- and rifampicin-treated transgenic mice (Supplementary Fig. 4B and C). In ELISA, rifampicin significantly reduced the levels of amyloid- β_{40} and amyloid- β_{42} in the TBS-soluble fraction and tended to decrease them in the SDS-soluble fraction, but showed no discernible effect in the formic acid-soluble fraction (Fig. 4C and D). Amyloid- β oligomers were reduced in the TBS- and SDS-soluble fractions and were undetectable in the formic acid-soluble fraction in both commercially available sandwich ELISA (Fig. 4E) and homemade direct ELISA with 11A1 antibody (Fig. 4F).

We also examined tau hyperphosphorylation, synapse loss, and microglial activation by staining brain sections with PHF-1 (anti-pSer396/404-tau), anti-synaptophysin,

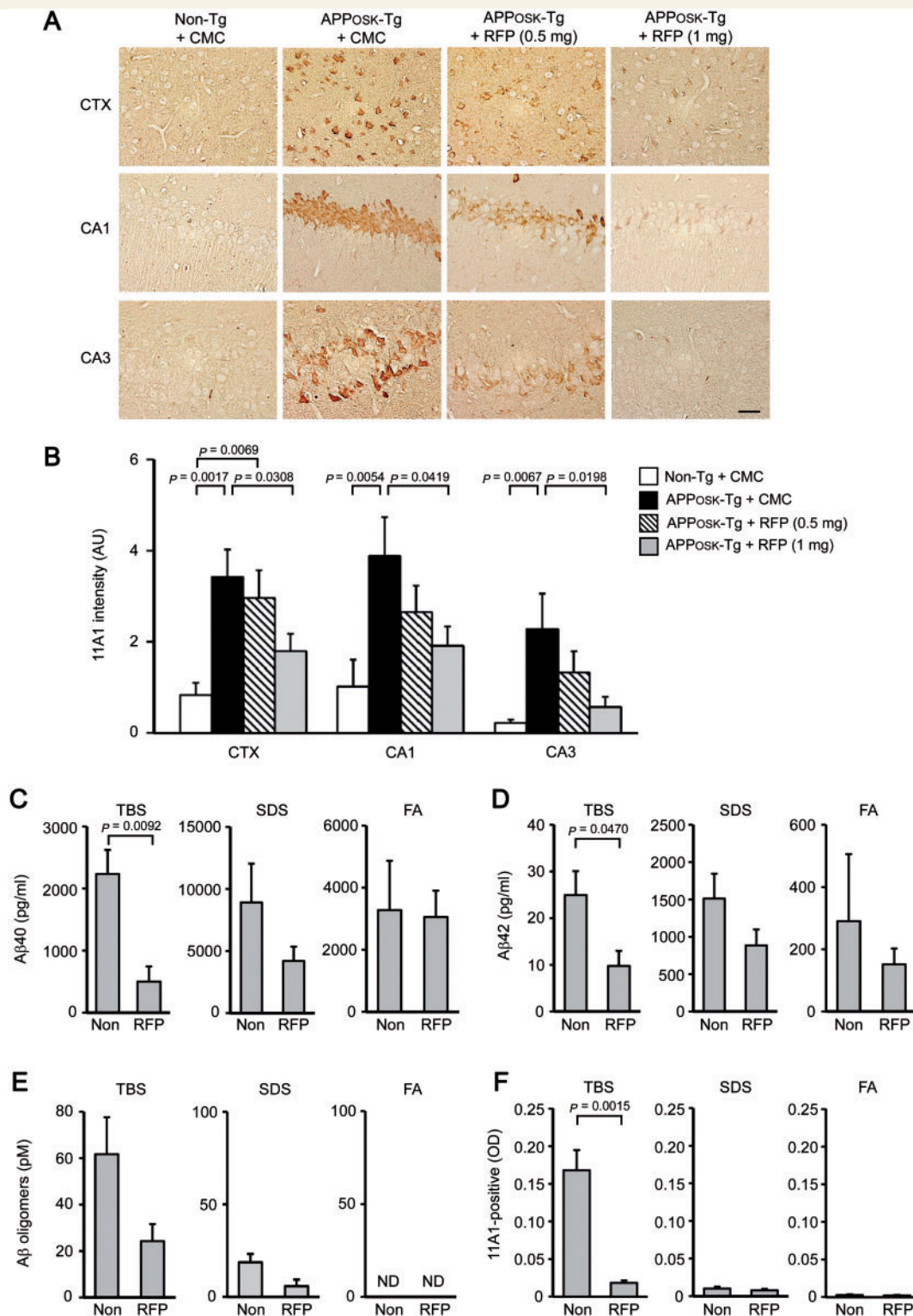


Figure 4 Effects of rifampicin on amyloid- β oligomer accumulation in APP^{OSK} mice. (A) Brain sections prepared from 18-month-old rifampicin (RFP)-treated mice were stained with amyloid- β oligomer-specific I1A1 antibody. Photographs were taken from the cerebral cortex (CTX) and hippocampal CA1 and CA3 regions. Scale bar = 30 μ m. (B) Amyloid- β oligomer accumulation was evaluated by quantifying I1A1-staining intensities in each photograph. Each bar represents the mean intensity \pm SEM ($n = 5$ for all groups). (C–F) Brain homogenates from 18-month-old rifampicin-treated mice (at 1 mg/day) were separated into TBS-, SDS-, and formic acid (FA)-soluble fractions and diluted 10-, 20-, and 100-fold, respectively. The levels of amyloid- β ₄₀ (C), amyloid- β ₄₂ (D), and amyloid- β oligomers (E) in each fraction were measured by ELISA. Each bar represents the mean \pm SEM ($n = 4$ for both groups). The original concentrations of each amyloid- β species before sample dilution were calculated and are shown. The levels of amyloid- β oligomers were also measured by direct ELISA with I1A1 antibody (F), where each fraction was diluted 2-fold and used to coat ELISA plates.

and anti-Iba-1 antibodies, respectively. Rifampicin successfully lowered PHF-1-positive staining in the hippocampal mossy fibres (Supplementary Fig. 5D and E), restored synaptophysin levels in the hippocampal CA3 region (Supplementary Fig. 5F and G), and inhibited microglial activation in the hippocampal CA3 region in a dose-dependent fashion (Supplementary Fig. 5H and I). Rifampicin-mediated recovery of synaptophysin levels was confirmed by western blot with hippocampal homogenates (Supplementary Fig. 5J and K).

We previously showed that APP_{OSK} mice suffered mitochondrial damage and apoptosis concomitantly with an intraneuronal accumulation of amyloid- β oligomers (Nishitsuji *et al.*, 2009; Umeda *et al.*, 2011). Thus, we examined whether rifampicin prevents amyloid- β -induced cytochrome *c* release from the mitochondria and caspase-3 activation, a measure of apoptosis, with mouse hippocampal homogenates. Rifampicin significantly reduced the levels of cytosolic cytochrome *c* (Supplementary Fig. 6A and B) and caspase 3 activation in transgenic mice to levels similar to or lower than those in CMC-treated non-transgenic littermates (Supplementary Fig. 6C and D). Thus, oral rifampicin was shown to improve the memory and amyloid pathology in amyloid- β oligomer model mice by reducing amyloid- β oligomers.

Effects of rifampicin on amyloid- β oligomers and amyloid deposition in Tg2576 mice

Based on the above results, we next investigated the effects of rifampicin on wild-type amyloid- β -induced amyloid pathology. Tg2576 mice are a well-known mouse model of Alzheimer's disease that produce abundant wild-type amyloid- β and starts amyloid deposition from 9–10 months of age (Hsiao *et al.*, 1996). Rifampicin was orally administered to 13-month-old Tg2576 mice (mean body weight 29.5 g) at 0.5 mg/shot every day for 1 month. Brain sections were prepared from 14-month-old rifampicin-treated mice and stained with β 001 antibody for amyloid deposition and 11A1 antibody for amyloid- β oligomer accumulation. Rifampicin significantly reduced 11A1-positive staining in the cerebral cortex and hippocampus, but did not clear β 001-positive staining; rather, it appeared to slightly increase amyloid deposition (Fig. 5A and B). Interestingly, amyloid- β oligomers were stained in the central area of amyloid plaques (Fig. 5A and Supplementary Fig. 7A). Brain homogenates and the TBS, SDS, and formic acid extracts were prepared and subjected to western blot and ELISA, respectively. Rifampicin did not affect the expression levels of total and human APP (Supplementary Fig. 7B and C). The levels of amyloid- β ₄₀ and amyloid- β ₄₂ were reduced in the TBS- and SDS-soluble fractions (Fig. 5C and D). In contrast, those in the formic acid-soluble fraction were increased. This result seems to be consistent with the immunohistochemical observation that

rifampicin slightly increased amyloid deposition (Fig. 5A and B). Amyloid- β oligomers were reduced in the TBS- and SDS-soluble fractions and undetected in the formic acid-soluble fraction in sandwich ELISA (Fig. 5E), whereas a significant reduction of amyloid- β oligomers was observed in all fractions in direct ELISA (Fig. 5F).

We also examined tau hyperphosphorylation, synapse loss, and microglial activation. PHF-1-positive staining in the hippocampal mossy fibres and in the dystrophic neurites around plaques was significantly decreased by rifampicin (Supplementary Fig. 7A, D and E). Rifampicin also prevented the accumulation of Iba-1-positive microglia around plaques (Supplementary Fig. 7A) and restored synaptophysin levels in the hippocampal mossy fibres (Supplementary Fig. 7F and G). The latter finding was confirmed by western blot with hippocampal homogenates (Supplementary Fig. 7H and I). Thus, oral rifampicin was shown to improve amyloid pathology in standard Alzheimer's disease model mice by reducing amyloid- β oligomers but not amyloid deposition.

Effects of rifampicin on memory and tau oligomers in tau609 mice

Tau609 mice are a transgenic mouse model of tauopathy that were designed to express both 3-repeat and 4-repeat human tau (Umeda *et al.*, 2013). Furthermore, they come to dominantly express 4-repeat human tau at adult age by the presence of FTDP-17-related *MAPT* (tau) intron mutation. This imbalanced expression of tau isoforms leads to tau hyperphosphorylation, tau oligomer formation, synapse loss, and memory impairment at 6 months, microglial activation at 12 months, and neurofibrillary tangle formation and neuronal loss at 15 months (Umeda *et al.*, 2013, 2015). Rifampicin was orally administered to 7–8-month-old tau609 mice (mean body weight 33.5 g) at 0.5 mg/shot every day for 1 month. Compared with age-matched non-transgenic littermates, CMC-treated transgenic mice showed significantly impaired memory, whereas rifampicin-treated transgenic mice exhibited a significant improvement of memory to a similar level (Fig. 6A and B). We repeated the experiment using older tau609 mice. Rifampicin was administered to 14–15-month-old tau609 mice (mean body weight 36.7 g) at 0.5 and 1 mg/shot every day for 1 month. Again, rifampicin recovered the memory of transgenic mice in a dose-dependent fashion: at 1 mg/shot, rifampicin improved the memory almost completely (Fig. 6E and F), whereas 0.5 mg/shot of rifampicin showed a significant, but not complete, recovery of memory (Fig. 6C and D).

Brain sections were prepared from 15–16-month-old rifampicin-treated mice and stained with tau oligomer-specific T22 antibody. Rifampicin significantly reduced T22-positive staining in hippocampal CA3 regions in a dose-dependent fashion (Fig. 7A and B, and Supplementary Fig. 8A). Brain homogenates were prepared

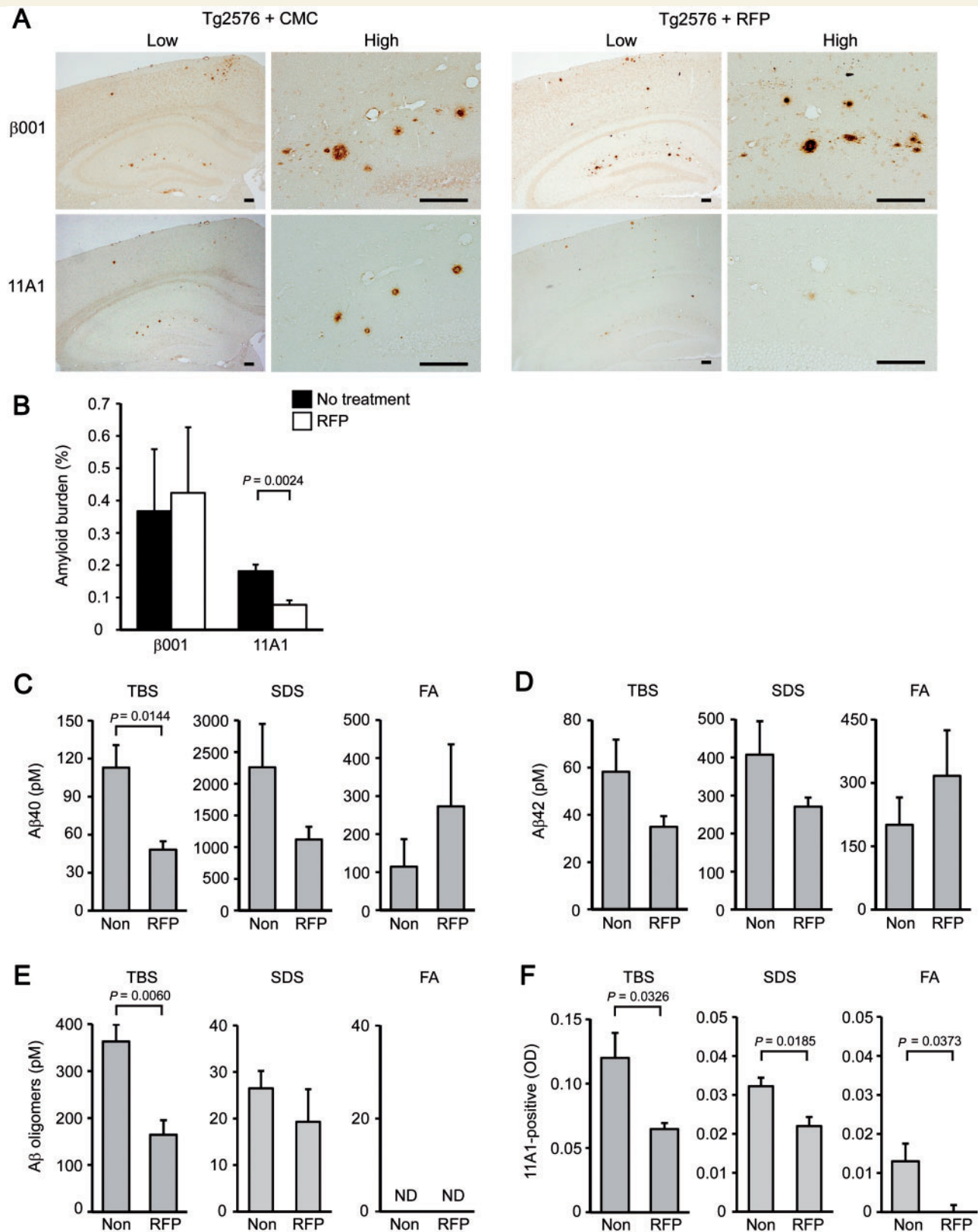


Figure 5 Effects of rifampicin on amyloid deposition and amyloid- β oligomer accumulation in Tg2576 mice. (A) Brain serial sections prepared from 14-month-old rifampicin-treated mice (at 0.5 mg/day) were stained with β 001 (anti-amyloid- β) and 11A1 (anti-amyloid- β oligomers) antibodies. Photographs were taken from the cerebrum including the hippocampus at low magnification and from the hippocampus at high magnification. Scale bars = 100 μ m. (B) Amyloid deposition and amyloid- β oligomer accumulation were evaluated by quantifying β 001- and 11A1-staining areas (% of the whole area) in each photograph of the hippocampus. Each bar represents the mean \pm SEM ($n = 4$ for CMC-treated transgenic and $n = 6$ for rifampicin-treated transgenic mice). (C–F) Brain homogenates were separated into TBS-, SDS-, and formic acid (FA)-

(continued)

from 15–16-month-old rifampicin-treated mice (at 1 mg/day) and separated into TBS-, sarkosyl-, and GuHCl-soluble fractions. Western blot with brain homogenates revealed no significant differences in the levels of total (pool 2-positive) and human tau (Tau12-positive) between CMC- and rifampicin-treated transgenic mice (Supplementary Fig. 8B and C). In direct ELISA with T22 antibody, rifampicin reduced the levels of tau oligomers significantly in the TBS- and sarkosyl-soluble fractions but not in the GuHCl-soluble fraction (Fig. 7C).

Tau hyperphosphorylation was examined with PHF-1, AT8 (anti-pSer202/Thr204-tau), and Ta1505 (anti-pSer413-tau) antibodies. Rifampicin significantly reduced PHF-1-, AT8-, and Ta1505-positive staining in hippocampal mossy fibres in a dose-dependent fashion (Fig. 8A–D and Supplementary Fig. 8A). In ELISA, no significant differences in the levels of human tau between CMC- and rifampicin-treated transgenic mice were detected in all brain fractions (Fig. 8E). Rifampicin reduced the levels of pSer396-tau and pSer199-tau in the TBS- and GuHCl-soluble fractions, but not in the sarkosyl-soluble fraction (Fig. 8F and G).

We also examined the effects of rifampicin on synapse loss and microglial activation. Rifampicin restored synaptophysin levels (Supplementary Fig. 8D and E) and inhibited microglial activation in the hippocampal CA3 region in a dose-dependent fashion (Supplementary Fig. 8F and G). Rifampicin-mediated recovery of synaptophysin levels was confirmed by western blot with hippocampal homogenates (Supplementary Fig. 8H and I).

Recent studies have suggested that intracellular tau aggregates are cleared, at least in part, by p62-dependent selective autophagy (Congdon *et al.*, 2012; Schaeffer *et al.*, 2012; Shimada *et al.*, 2012; Ozcelik *et al.*, 2013). p62, also known as sequestosome-1 (SQSTM1), is a ubiquitin- and LC3 (microtubule-associated protein light chain 3)-interacting protein (Komatsu and Ichimura, 2010). It binds to polyubiquitinated proteins and the resultant p62/protein complex is selectively sequestered in autophagosomes through the interaction between p62 and LC3. Because p62 is constantly degraded by the autophagy-lysosome system, ablation of autophagy leads to marked accumulation of p62, resulting in the formation of p62-positive inclusions (Komatsu and Ichimura, 2010). Thus, we examined the conversion of LC3-I to LC3-II, a marker of autophagy activation, and the level of p62 in 15–16-month-old rifampicin-treated mice (at 1 mg/day). Compared with non-transgenic littermates, CMC-treated tau609 mice showed increased levels of LC3 conversion and p62 (Supplementary Fig. 9), implying that autophagy

was activated in these mice but its function was impaired. Rifampicin significantly reduced the levels of p62 in transgenic mice without affecting LC3 conversion, suggesting that rifampicin helps the restoration of autophagy-lysosomal function.

To investigate whether rifampicin directly influences the autophagy-lysosomal function or restores it by reducing intracellular tau oligomers, we examined the levels of LC3 conversion and p62 in cultured cells. Rifampicin treatment at 100 μ M for 2 days resulted in a slight but significant reduction in LC3 conversion (i.e. suppression of autophagy) with no changes in p62 levels in IMR-32 cells (Supplementary Fig. 10A–D) and no significant changes in either LC3 conversion or p62 in COS-7 cells (Supplementary Fig. 10E–H). These results indicate that rifampicin does not directly activate autophagy but restores its function by preventing abnormal protein accumulation beyond the capacity of the protein-degrading system. Thus, oral rifampicin was shown to improve the memory and tau pathology in tauopathy model mice by reducing tau oligomers.

Discussion

Rifampicin is a well-known orally available antibiotic used in the treatment of mycobacterium infections, including tuberculosis and leprosy. Rifampicin is lipid-soluble, and following oral administration it is rapidly absorbed and highly distributed throughout the body tissues and body fluids, including the CSF (No authors, 2008). Rifampicin can penetrate into cells by passing through the cell membranes as well as into the brain by crossing the blood–brain barrier. These pharmacokinetic properties make rifampicin a suitable medicine to treat neurodegenerative diseases that show extracellular and intracellular protein aggregates in the CNS.

An epidemiological study revealed that a treated group of patients with leprosy in Japan had a significantly lower incidence of dementia compared with an untreated group (McGeer *et al.*, 1992). Subsequent histological analyses indicated that non-demented leprosy patients aged over 70 years in Japan showed significantly lower levels of senile plaques in the brain than age-matched non-demented non-leprosy subjects (Namba *et al.*, 1992; Chui *et al.*, 1994). These observations led us to speculate that certain medicine(s) prescribed to these patients may have anti-amyloid- β activities. Since rifampicin was always used in combination with dapsone and/or clofazimine in the aforementioned treatment of leprosy, we examined rifampicin

Figure 5 Continued

soluble fractions and diluted 10-, 20-, and 100-fold, respectively. The levels of amyloid- β_{40} (C), amyloid- β_{42} (D), and amyloid- β oligomers (E) in each fraction were measured by ELISA. Each bar represents the mean \pm SEM ($n = 4$ for both groups). The original concentrations of each amyloid- β species before sample dilution were calculated and are shown. The levels of amyloid- β oligomers were also measured by direct ELISA with 11A1 antibody (F), where each fraction was diluted 2-fold and used to coat ELISA plates.

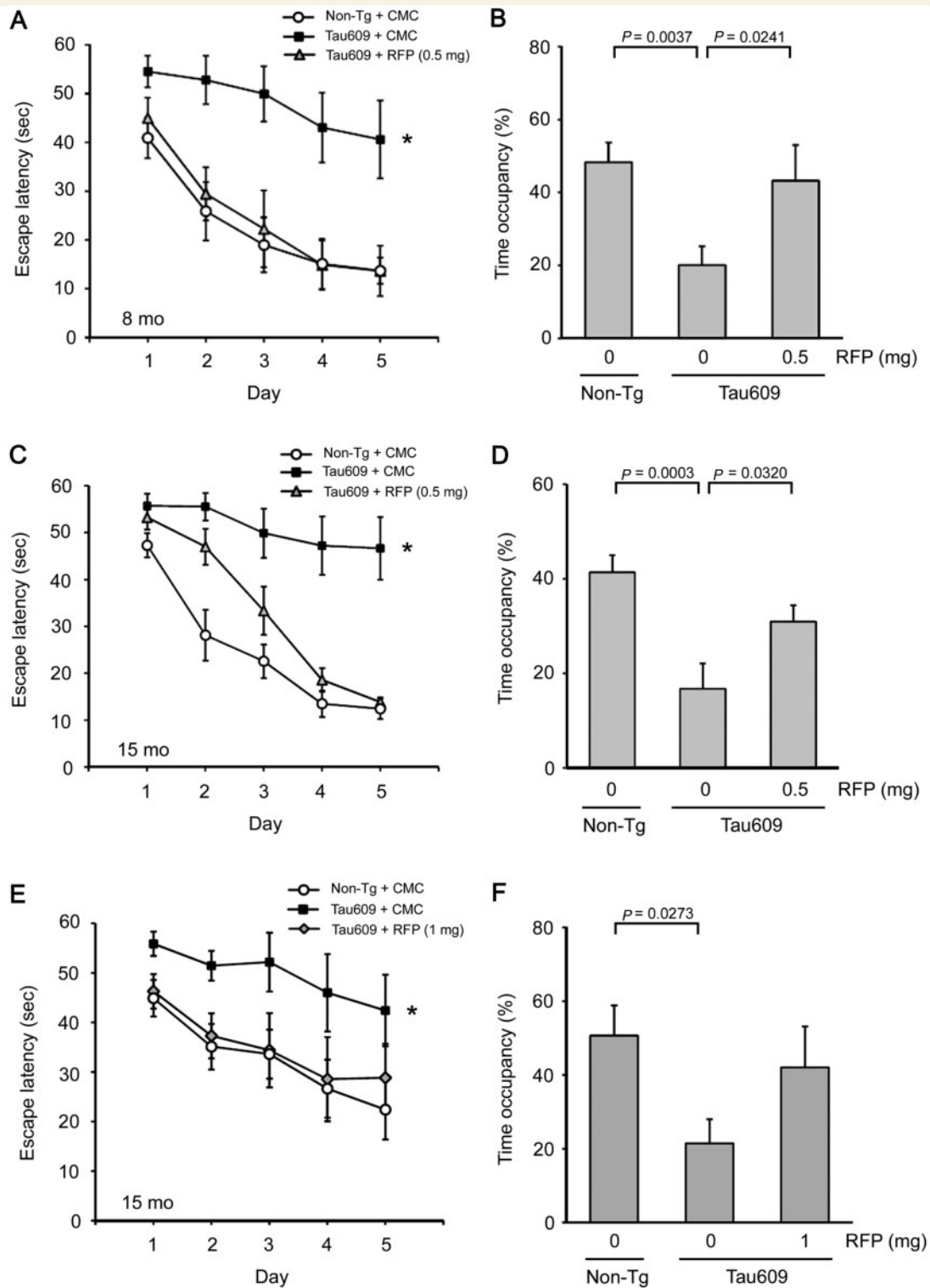


Figure 6 Effects of rifampicin on the memory of tau609 mice. (A) Rifampicin (RFP) was administered to 7–8-month-old tau609 mice at 0.5 mg/shot every day for 1 month, and the Morris water maze was performed. Each point represents the mean latency of five trials per day \pm SEM [$n = 10$ for non-transgenic (Non-Tg); $n = 9$ for CMC-treated transgenic (Tg); and $n = 8$ for rifampicin-treated transgenic mice]. * $P = 0.0009$ versus non-transgenic and $= 0.0117$ versus rifampicin. (C) Rifampicin was administered to 14–15-month-old tau609 mice at 0.5 mg/shot every day for 1 month, and the Morris water maze was performed. $n = 10$ for non-transgenic; $n = 9$ for CMC-treated transgenic; and $n = 8$ for rifampicin-treated transgenic mice. * $P < 0.0001$ versus non-transgenic and $= 0.0011$ versus RFP. (E) Rifampicin was administered to 14–15-month-old tau609 mice at 1 mg/shot every day for 1 month, and the Morris water maze was performed. $n = 11$ for non-transgenic; $n = 7$ for each transgenic group. * $P = 0.0150$ versus non-transgenic. (B, D and F) Retention of memory was assessed by a probe trial. Each bar represents the mean time occupancy \pm SEM in the target quadrant.

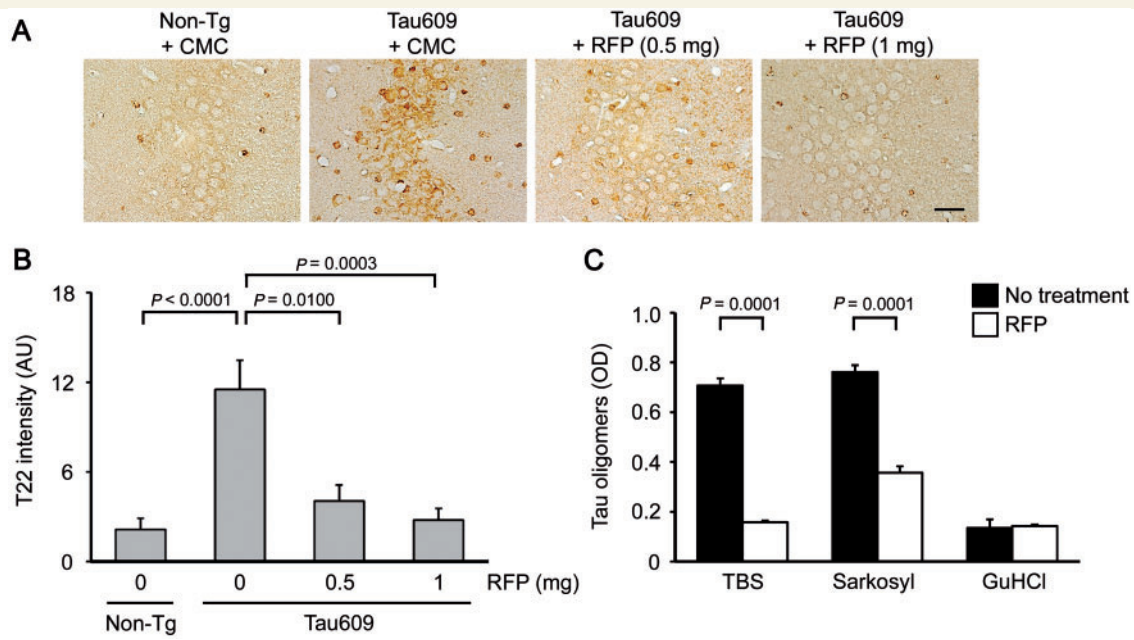


Figure 7 Effects of rifampicin on tau oligomer accumulation in tau609 mice. **(A)** Brain sections were prepared from 15–16-month-old rifampicin (RFP)-treated mice and stained with tau oligomer-specific T22 antibody. All photographs were taken from the hippocampal CA3 region. Scale bar = 50 μ m. **(B)** Tau oligomer accumulation was evaluated by quantifying T22-staining intensities in each photograph. Each bar represents the mean intensity \pm SEM [$n = 10$ for non-transgenic (Non-Tg); $n = 10$ for CMC-treated Tg; $n = 3$ for 0.5 mg rifampicin-treated transgenic (Tg); and $n = 6$ for 1 mg rifampicin-treated transgenic mice]. **(C)** Brain homogenates from 15–16-month-old rifampicin-treated mice (at 1 mg/day) were separated into TBS-, sarkosyl-, and GuHCl-soluble fractions. The levels of tau oligomers were measured by direct ELISA with T22 antibody, where each fraction was diluted 2-fold and used to coat ELISA plates. Each bar represents the mean \pm SEM ($n = 3$ for CMC-treated transgenic and $n = 5$ for rifampicin-treated transgenic mice).

and dapsone for effects on amyloid- β aggregation and toxicity *in vitro*, finding that rifampicin has inhibitory activities against amyloid- β fibrillization and toxicity (Tomiya *et al.*, 1994, 1996). Following our studies, researchers examined the action of rifampicin against other amyloidogenic proteins, such as α -synuclein. Rifampicin was found to inhibit α -synuclein fibrillization *in vitro* (Li *et al.*, 2004) and oligomerization within the cultured cells (Xu *et al.*, 2007). When administered intraperitoneally at 25 mg/kg for 3 months, rifampicin decreased α -synuclein accumulation and the associated neurodegeneration in a mouse model of multiple system atrophy (Ubhi *et al.*, 2008). In the present study, we showed that rifampicin inhibited the oligomerization of amyloid- β , tau, and α -synuclein *in vitro*, and when orally administered for 1 month, it attenuated amyloid and tau pathologies and improved memory in mouse models of Alzheimer's disease and tauopathy. These studies collectively indicate that rifampicin has a broad spectrum and can be used for various neurodegenerative diseases alone. On the other hand, recent clinical trial studies have shown that rifampicin failed to prevent disease progression in patients with mild-to-moderate Alzheimer's disease (Molloy *et al.*, 2013) and possible or probable multiple system atrophy (Low *et al.*, 2014). These reports support the notion that treatments after disease onset have little effect and

emphasize the importance of prevention in neurodegenerative diseases. Sperling *et al.* (2011) have proposed three stages for the prevention of Alzheimer's disease: primary (before amyloid deposition), secondary (before the onset of mild cognitive impairment), and tertiary prevention (before the onset of dementia). Here we showed that rifampicin is a good candidate for preventive medicine to be used in the secondary and tertiary prevention of Alzheimer's disease. Therefore, we suggest that rifampicin be evaluated at the early presymptomatic stage of neurodegenerative diseases in further studies.

Several mechanisms underlying the preventive actions of rifampicin are presumed. The primary effect of rifampicin is the inhibition of protein oligomerization. Protein oligomers are in a rapid equilibrium with monomers and higher-order assemblies, and the aggregation process of amyloidogenic proteins is thought to take the following pathway: monomers \rightarrow low- n oligomers \rightarrow high-molecular-weight oligomers \rightarrow protofibrils \rightarrow fibrils. Once fibrils are formed, monomers may come to directly participate in fibril growth. Our results suggest that rifampicin specifically binds to low- n oligomers of amyloidogenic proteins by recognizing a certain conformation that is not formed in physiological oligomers of non-amyloidogenic proteins, to dissociate them into monomers. Inhibition of extracellular amyloid- β oligomerization would result in reduced amyloid plaque formation, as seen in a

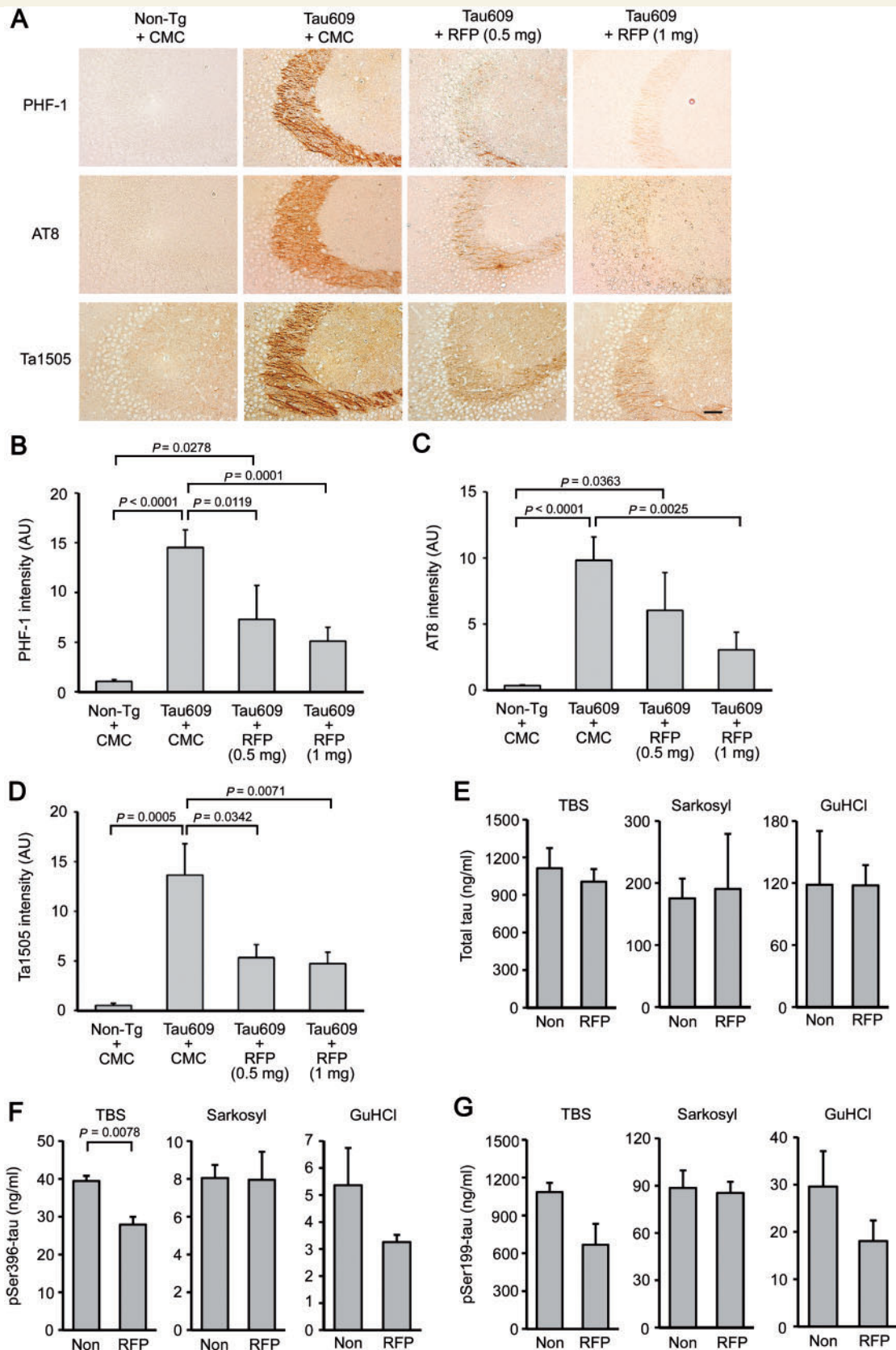


Figure 8 Effects of rifampicin on tau hyperphosphorylation in tau609 mice. (A) Brain sections prepared from 15–16-month-old rifampicin (RFP)-treated mice were stained with PHF-I, AT8, and Ta1505 antibodies. All photographs were taken from the hippocampal CA3 region. Scale bar = 50 μ m. (B–D) Tau phosphorylation was estimated by quantifying PHF-I (B), AT8 (C), and Ta1505 (D) staining intensities in the hippocampal mossy fibre field. Each bar represents the mean intensity \pm SEM [in PHF-I and AT8 staining, $n = 10$ for non-transgenic

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treated group of Japanese leprosy patients (Namba *et al.*, 1992; Chui *et al.*, 1994). Meanwhile, in the present study, rifampicin failed to decrease amyloid deposition in aged Tg2576 mice, despite it evidently clearing amyloid- β oligomers in the plaques. This result suggests that rifampicin does not affect preformed fibrils in amyloid plaques and rather promotes their growth by providing amyloid- β monomers to the fibrils. Recent evidence suggests that amyloid plaques are formed to sequester toxic amyloid- β oligomers and plaque itself is not toxic (Cheng *et al.*, 2007; Shankar *et al.*, 2008). Rifampicin may mediate the conversion of plaque amyloid- β from toxic oligomers to non-toxic fibrils via monomers.

By binding to oligomers, rifampicin would prevent a possible oligomer–membrane interaction. One of the toxic mechanisms of amyloid oligomers is the disruption of membrane impermeability, probably by forming amyloid pores in the membranes (Demuro *et al.*, 2005; Quist *et al.*, 2005). The oligomer-induced membrane damage allows an excess influx of Ca^{2+} into the cells, which causes mitochondrial dysfunction and subsequent reactive oxygen species (ROS) generation in the mitochondria (Mancuso *et al.*, 2006; De Moura *et al.*, 2010). We and others have shown that rifampicin can block the interaction of oligomers/aggregates of islet amyloid polypeptide (also known as amylin) with the cell membranes (Tomiyama *et al.*, 1996; Harroun *et al.*, 2001; Balali-Mood *et al.*, 2005). Even if neurotoxic oligomers happen to attack cells, rifampicin can act as a hydroxyl radical scavenger with its naphthohydroquinone structure (Tomiyama *et al.*, 1996). Free radical generation and the related oxidative stress have been implicated in the mitochondrial damage and cell death of many neurodegenerative diseases (Mancuso *et al.*, 2006; De Felice *et al.*, 2007; De Moura *et al.*, 2010; Nomura *et al.*, 2013). In the present study, rifampicin attenuated the mitochondrial damage and apoptosis in APP_{OSK} mice. This protective effect of rifampicin may be achieved by scavenging ROS as well as by inhibiting amyloid- β oligomerization and/or the oligomer–membrane interaction. Our results also indicate that the inhibition of intracellular protein oligomerization leads to the restoration of autophagy-lysosomal function. The autophagy-lysosome pathway, together with the ubiquitin-proteasome system, participates in the clearance of cytoplasmic abnormal proteins, and its impairment has been suggested in many neurodegenerative diseases (Ghavami *et al.*, 2014; Vidal *et al.*, 2014). Finally, rifampicin may promote the efflux of amyloidogenic proteins

from the brain into the periphery. Rifampicin has been shown to facilitate amyloid- β clearance by upregulating the expression of low density lipoprotein receptor-related protein-1 and P-glycoprotein at the blood–brain barrier (Qosa *et al.*, 2012), and such clearance may be more efficient for protein monomers than for oligomers. These mechanisms may synergistically work to protect neurons from toxic oligomers and represent the multifunctional property of rifampicin.

The primary adverse effect of rifampicin is hepatotoxicity (No authors, 2008). In many cases, the liver damage is cured by discontinuing the medication. If necessary, the medication can be resumed after liver function is restored. Meanwhile, rifampicin has been reported to have cytoprotective effects in the brain (Bi *et al.*, 2013). We observed that rifampicin treatment resulted in a suppression of microglial activation in three different model mice. Another problem of rifampicin is its drug–drug interactions. Rifampicin activates the nuclear pregnane X receptor in hepatocytes that in turn upregulates the expression of cytochrome P450 enzymes and P-glycoprotein: the former metabolizes many drugs and the latter mediates drug adsorption and efflux (Chen and Raymond, 2006). The rifampicin-induced alteration of drug metabolism leads to a decreased efficacy of drugs concomitantly administered with rifampicin. These adverse effects of rifampicin may cause serious problems when used for a long period. However, it is noteworthy that in the present study, only 1-month treatment of rifampicin demonstrated potent effects against oligomers. Similar effects may be achieved at lower doses if rifampicin treatment is continued for longer periods of time. In addition, our data indicate that the dose of rifampicin necessary for cognitive improvement was lower in younger mice than in older mice. This observation suggests that if rifampicin treatment is started at the early presymptomatic stage of the disease, the dose of rifampicin can be reduced to levels in which any adverse effects of rifampicin are minimized. Although our results indicate that rifampicin binds to only amyloidogenic protein oligomers, we cannot exclude the possibility that rifampicin interacts with physiological oligomers with the β -sheet structure to disturb their function. To avoid such undesired interaction and for developing more specific, safer preventive medicine, further studies are necessary on the interaction between rifampicin and protein oligomers.

To conclude, our results indicate that rifampicin has the properties required for preventive medicines against

Figure 8 Continued

(Non-Tg); $n = 10$ for CMC-treated transgenic (Tg); $n = 3$ for 0.5 mg rifampicin-treated Tg; and $n = 6$ for 1 mg rifampicin-treated Tg; in Ta1505-staining, $n = 5$ for Non-Tg; $n = 7$ for CMC-treated Tg; $n = 3$ for 0.5 mg rifampicin-treated Tg; and $n = 6$ for 1 mg rifampicin-treated Tg]. (E–G) TBS-, sarkosyl-, and GuHCl-soluble brain fractions prepared from 15–16-month-old rifampicin-treated mice (at 1 mg/day) were diluted 2000-, 200-, and 200-fold, respectively, for the measurement of total tau (E) and pSer199-tau (G); and 200-, 20-, and 20-fold, respectively, for pSer396-tau (F) by ELISA. Each bar represents the mean \pm SEM ($n = 3$ for CMC-treated transgenic and $n = 5$ for rifampicin-treated transgenic mice). The original concentrations of each tau species before sample dilution were calculated and are shown.

neurodegenerative diseases: i.e. orally available, little adverse effects, and good efficacy at reducing extracellular and intracellular neurotoxic oligomers with a broad spectrum. Thus, rifampicin could be a promising, ready-to-use medicine for the prevention of Alzheimer's disease and other neurodegenerative diseases.

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Supplementary material

Supplementary material is available at *Brain* online.

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