Levosimendan prevents tau pathology by inhibiting disulfide-linked tau oligomerization posing as a promising anti-tau therapeutics

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Abstract

Tau oligomers play critical roles in tau pathology, responsible for neuronal cell death and transmitting the disease in the brain. Accordingly, preventing tau oligomerization becomes an important therapeutic strategy to treat tauopathies including Alzheimer's disease, however progress has been slow due to difficulties of detecting tau oligomers in cellular context. Toward tau-targeted drug discovery, our group have developed a tau-BiFC platform to monitor and quantify tau oligomerization. By using the tau-BiFC platform, we screened 1,018 compounds in FDA-approved & Passed Phase I drug library, and identified levosimendan as a potent anti-tau agent inhibiting tau oligomerization. ^{14}C -isotope labeling of levosimendan identified that levosimendan covalently bound to tau cysteines, directly inhibiting disulfide-linked tau oligomerization. In addition, levosimendan was able to disassemble tau oligomers into monomers, and rescuing neurons from aggregation states. In comparison, the well-known anti-tau agents, methylene blue (MB) and LMTM, failed to protect neurons from tau-mediated toxicity, generating high-molecular weight tau oligomers. The administration of levosimendan also suppressed tau pathology in the brain, preventing cognitive declines in Tau^{P301L}-BiFC transgenic mice. Although careful validation is required, here we present the potential of levosimendan as a disease modifying therapy for tauopathies targeting tau oligomerization.

Introduction

Naturally, tau is an extremely soluble protein containing a number of positively charged lysine residues¹. The lysine residues play a role for binding negatively charged microtubules². Upon binding, tau stabilizes microtubule and promotes microtubule assembly, which is critical for axonal outgrowth^{3,4}. Under pathological condition, the charge balance between tau and microtubule is disrupted, and tau dissociates from microtubules⁵. The dissociated tau become a susceptible substrate of cytosolic enzymes that modify the states of tau phosphorylation, acetylation, or proteolytic cleavage^{6,7}. Chemically and structurally modified tau becomes aggregated and accumulated in neurons. Intraneuronal deposits of tau fibrils (PHFs and NFTs) are the pathological hallmark in a group of neurodegenerative diseases, called tauopathies. Tauopathies include not only Alzheimer's disease (AD), but also Fronto-Temporal Dementia (FTD), progressive supranuclear palsy, corticobasal degeneration, and chronic traumatic encephalopathy^{8,9}. Due to the implication of tau in neurodegenerative disorders, tau has become an important therapeutic target¹⁰. However, progress has been slow due to the lack of understanding tau pathology.

Until recently, neurotoxic tau species were presumed to be the filamentous tau (PHFs and NFTs), accumulated in the brain of AD patients. However, mounting evidence suggests that soluble tau oligomer, rather than fibrils, is neurotoxic species responsible for neuronal cell death^{11,12}. Tau oligomers are soluble forms of tau aggregates ranging from dimers to prefibrillar aggregates¹³. In AD brains, tau oligomers are detected in the early stage of pathogenic cascades, and the level of tau oligomers correlated with synaptic dysfunction and neuronal cell loss, rather than the levels of NFTs¹⁴⁻¹⁶. Evidence also suggested

that tau oligomers are transmittable between neurons, spreading the disease in the brain^{17,18}. Due to its relatively small size, tau oligomers are able to penetrate into cells and initiate nascent tau aggregation, providing structural seeds for aggregation¹⁹. The formation of insoluble filament (PHFs and NFTs) might be a survival mechanism for neurons to quarantine toxic tau species from cytosol²⁰. Accordingly, prevention of tau oligomerization or elimination of oligomers became an important therapeutic strategy for AD drug development.

The most studied tau aggregation inhibitor is methylene blue (MB), a blue dye, which has been widely used in various industrial and research settings. In 2008, Wischik et al. firstly reported the anti-tau activity of MB²¹. In their study, MB disrupted the stability of NFTs isolated from AD patients. In 2013, Zweckstetter et al. reported the inhibitory mechanism of MB on tau aggregation. In their study, MB inhibits tau aggregation through the oxidation of tau cysteine residues, converting tau into aggregation-incompetent monomer²². However, there are also conflicting data showing that MB increased tau dimers through the same cysteine-oxidation mechanism²³. Full length tau contains two cysteine residues (C291 and C322) in the microtubule-binding domain. Intra-molecular disulfide bond formation generates a compact monomer, resistant to tau aggregation, and inter-molecular disulfide bonds facilitate the aggregation cascade by generating structurally stable tau oligomers²⁴. In 2019, Takashima et al. reported that MB inhibited the formation of tau fibrils, but not tau oligomers²⁵. While the mechanism of action on tau pathology is still unclear, the reduced form of MB, LMTM (also known as TauRx0237 or LMT-X), has completed Phase III clinical trials for the treatment of Alzheimer's disease. In first Phase III trial, LMTM failed to ameliorate cognitive decline in patients with mild to moderate Alzheimer's disease^{26,27}. Although failed in AD treatment, LMTM is still on clinical trials for the treatment of FTDs.

Tau aggregation is a complicated multi-step process, which is controlled by a number of cellular enzymes. Therefore, in many cases, tau aggregation inhibitors which show strong inhibitory effects in *in vitro* tau aggregation assays, are not effective in cellular system. Therefore, the action-mechanism of tau-targeted drugs should be evaluated in cellular system, not in test tubes using purified tau. In intracellular space, diverse tau modifications occur simultaneously, changing its physiological properties; tau phosphorylation reducing its microtubule binding affinity^{28,29}, tau acetylation preventing ubiquitin-mediated degradation³⁰, and dityrosine or disulfide-bond formation promoting tau aggregation by generating covalently linked tau oligomers^{24,31}. In this regard, our group has developed tau-BiFC platform to monitor tau oligomerization in living cells and in the brain of mice^{32,33}. In tau-BiFC system, non-fluorescent N- and C-terminal compartments of Venus protein are fused to tau and Venus fluorescence turns on only when tau assembles together (Fig. 1a). By using tau-BiFC cell model, we have characterized prion-like tau oligomers¹⁹, and investigated pathological tau modification³⁴⁻³⁶.

In this study, by using tau-BiFC platform, we have screened FDA-approved & Passed Phase I Drug Library, and identified levosimendan as a new anti-tau agent.

Results

FDA-approved & Passed Phase I Drug Library screening identifies levosimendan as a new anti-tau oligomerization agent

To identify a new drug candidate inhibiting tau aggregation, we screened 1,018 compounds in the FDA-approved & Passed Phase I Drug Library on tau-BiFC cells. For tau-BiFC screening, tau-BiFC cells were plated on 384-well and cells were co-treated with each library drug with Forskolin (FK), which induces tau hyperphosphorylation through the activation of Protein kinase A³⁷ (Fig. 1a). MB and LMTM (3 mM) were used as a positive control. After 46 hours, FK treatment induced tau aggregation by showing 3.0 ± 0.4 -fold increased BiFC fluorescence intensity (Fig. 1a-d). FK treatment also induced cell cytotoxicity by showing $26.6 \pm 0.2\%$ decreased number of nuclei (Fig. 1b, c, and e). Among tested, only one drug, levosimendan suppressed FK-induced tau aggregation almost completely to the level of basal without showing any cytotoxic effects ($EC_{50} = 1.9 \pm 0.1$ mM, $GI_{50} = 124.0 \pm 0.3$ mM) (Fig. 1f and Supplementary Fig. 1). More interestingly, FK-induced cell toxicity was rescued to the level of basal by the treatment of levosimendan. In comparison, MB and LMTM decreased cell viability seriously near at the effective concentration (MB; $EC_{50} = 2.8 \pm 0.2$ mM, $GI_{50} = 6.2 \pm 0.4$ mM, and LMTM; $EC_{50} = 2.2 \pm 0.2$ mM, $GI_{50} = 6.4 \pm 0.3$ mM) (Fig. 1f and Supplementary Fig. 1).

Tau-BiFC cell screening identified that levosimendan is an effective drug in preventing FK-induced tau aggregation and also FK-induced cellular toxicity. However, FK activates not only tau kinases, but also other cellular processes activated with the increased level of cAMP³⁸. The inhibitory effect of levosimendan could be the result of regulating the upstream cAMP pathway, rather than tau pathology. To scrutinize the drug effect on tau pathology, tauK18^{P301L} was used to activate tau aggregation. TauK18 fragment containing P301L mutation, tauK18^{P301L}, activates tau aggregation as a prion-like seed¹⁹. Again, levosimendan prevented tauK18^{P301L}-induced tau aggregation and cellular toxicity almost completely ($EC_{50} = 2.2 \pm 0.1$ mM, $GI_{50} = 101.3 \pm 0.4$ mM) (Fig. 1g and Supplementary Fig. 2). MB and LMTM showed serious cell toxicity near at effective concentration (MB; $EC_{50} = 3.1 \pm 0.3$ mM, $GI_{50} = 7.1 \pm 0.3$ mM, and LMTM; $EC_{50} = 2.7 \pm 0.2$ mM, $GI_{50} = 6.6 \pm 0.3$ mM) (Fig. 1g and Supplementary Fig. 2).

Next, flow cytometry analysis was conducted to investigate drug effect on single-cell level. Since MB and LMTM caused substantial cell death at effective concentration, flow cytometry analysis was necessary to validate the drug effect on the remaining intact cells. For flow cytometry analysis, tau-BiFC cells were treated with the most effective concentration of each drugs. Upon the treatment of tauK18^{P301L}, 65.4% of total cells were selected as an intact cell population on forward and side scatter pulse area (FSC-A and SSC-A) (Fig. 1h). In case of MB and LMTM (5 mM), 13.7% and 14.5% of total cells were selected as an intact cell population. In comparison, 69.0% of levosimendan (15 mM) treated cells were selected as intact cells. The selected populations were analyzed for BiFC fluorescence intensity. Tau-BiFC cells are mixture of BiFC-null cells and BiFC-responding cells (Fig. 1i). In the basal condition, BiFC-responding cells showed low and widespread BiFC fluorescence profile (BiFC^{OFF} cells; Median = 332 ± 110). Upon tauK18^{P301L} activation, the fluorescence intensities of BiFC-responding cells increased significantly generating a distinctive population peak (BiFC^{ON} cells; Median = 717 ± 62), while BiFC-null cells still

remained. The fluorescence intensity of BiFC^{ON} cells dropped significantly by the treatment of levosimendan (Levo; Median = 249±59), proving the effectiveness of levosimendan in inhibiting cellular tau aggregation. In comparison, MB or LMTM reduced tau-BiFC intensity only slightly (MB; Median = 648±49, and LMTM; Median = 596±57). High magnification images show the representative BiFC fluorescence images of each group (Fig. 1j). Upon the treatment of tauK18^{P301L}, intracellular tau aggregation increased significantly showing thread-like phenotype, and the increased BiFC fluorescence was suppressed almost completely by the treatment of levosimendan. As shown in flow cytometry analysis, MB and LMTM was not effective in reducing intracellular tau aggregation.

Levosimendan inhibits intracellular disulfide-linked tau oligomerization while MB and LMTM increase disulfide-linked tau oligomers.

Next, immunoblot analysis was followed to evaluate tau phosphorylation and total tau levels in tau-BiFC cells. To investigate dose-dependency, tau-BiFC cells were treated with increasing concentrations of each drug in the presence of tauK18^{P301L} (MB and LMTM; 0.5, 1.5, 5 mM, and levosimendan; 5, 15, 45 mM). Upon the activation by tauK18^{P301L}, the levels of tau phosphorylation and total tau were increased by 1.9±0.1-fold at T205, 1.6±0.1-fold at S199, 1.4±0.1-fold at S396 and 2.1±0.1-fold, compared to that of basal (Fig. 2a-c and Supplementary Fig. 3). The increased tau phosphorylation was reduced by the treatment of levosimendan, and at the highest concentration (45 mM), the levels of tau phosphorylation was decreased almost to the level of basal, showing 0.9±0.1-fold at T205, 1.0±0.1-fold at S199 and 0.9±0.1-fold at S396 (Fig. 2a, b and Supplementary Fig. 3). Upon the treatment of MB and LMTM, the level of tau monomers was dose-dependently decreased, but instead, high-molecular weight oligomers were observed. In case of MB (5 mM), the level of pT205-, pS199-, or pS396-positive monomers was decreased to 0.1±0.9-, 0.2±0.1-, or 0.2±0.1-fold and the level of corresponding oligomers was increased to 0.7±0.3-, 0.4±0.1-, 0.5±0.2-fold, respectively, compared to that of basal. In case of LMTM (5 mM), the level of pT205-, pS199-, or pS396-positive monomers was decreased to 0.2±0.9-, 0.3±0.0-, or 0.2±0.1-fold and the level of oligomers was increased to 0.6±0.2-, 0.4±0.1-, or 0.4±0.1-fold, respectively. This result indicates that MB and LMTM increase unbreakable tau oligomers, which are resistant to SDS- and also b-mercaptoethanol. Also, the size of oligomers was increased dose-dependently in MB and LMTM treated cell lysates.

Upon the treatment of tauK18^{P301L}, the amount of total tau protein was increased to 2.0±0.1-fold compared to that of basal (Fig. 2c). TauK18^{P301L}-induced tau accumulation corresponds to the previous report showing that tau is metabolized at much slower rates when pathologically activated³⁹.

Levosimendan treatment effectively suppressed tauK18^{P301L}-induced tau accumulation in tau-BiFC cells, keeping total tau level at 1.0±0.2-fold, which is comparable to the level of basal. Next, we investigated the formation of disulfide-dependent tau oligomers on non-reducing SDS-PAGE gels. Substantial amount of disulfide-linked tau oligomers were detected even at basal condition and the level of disulfide-linked tau oligomers increased to 2.4±0.4-fold by the treatment of tauK18^{P301L} (Fig. 2d). Levosimendan treatment decreased the amount of disulfide-linked tau oligomers, comparable to the basal level (0.8±0.2-fold at 45

mM). In contrast, MB and LMTM increased the level of disulfide-linked tau oligomerization intensely (MB; 1.6 ± 0.3 -fold and LMTM; 1.9 ± 0.3 -fold at 5 mM), presenting highly enriched bands above 245 kDa (Fig. 2d). Non-reducing gel analysis indicates that levosimendan is effective in inhibiting tau-BiFC responses through the suppression of disulfide-linked tau oligomerization. In comparison, MB and LMTM did not inhibit tau-BiFC responses and promoted the formation of structurally stable tau oligomers that are SDS- and reducing agent resistant. However, careful validation was necessary since it was possible that MB and LMTM might act on BiFC complementation process instead of tau oligomerization.

Next, we evaluated the drug effect on tau oligomerization in primary neuron culture. Primary hippocampal neurons were isolated from day18 rat embryos. After 10 days of *in vitro* culture, the hippocampal neurons were treated with MB, LMTM (3 mM) or levosimendan (10 mM) in the presence of tauK18^{P301L}. In case of MB and LMTM, severe neuronal cell death was observed at higher concentrations over 10 mM, similar to those toxicity on tau-BiFC cells (data not shown). The neuronal cell lysates were subjected for immunoblot analysis in both reducing- and non-reducing- condition. Upon the treatment of tauK18^{P301L}, tau phosphorylation increased 2.0 ± 0.2 -fold at S396 and 1.7 ± 0.1 -fold at S199, indicating activated tau pathology in primary neuron (Fig. 2e, f). Again, levosimendan treatment suppressed tauK18^{P301L}-induced tau phosphorylation comparable to the basal level, showing 0.9 ± 0.2 -fold at S396 and 0.9 ± 0.1 -fold at S199. Also, in the primary neurons, the treatment of MB and LMTM increased the level of high-molecular weight tau oligomers resistant to SDS- and b-mercaptoethanol, while decreasing the level of monomers (Fig. 2e, f). Upon the treatment of tauK18^{P301L}, the level of total tau increased to 1.5 ± 0.1 -fold compared to that of basal, indicating the accumulation of pathological tau species in neurons (Fig. 2g). Levosimendan treatment also suppressed tau accumulation in neurons, maintaining total tau level at 1.0 ± 0.1 -fold comparable to that of basal.

Non-reducing SDS-PAGE analysis also indicated that, substantial amount of tau exists as disulfide-linked tau oligomers in primary neurons (Fig. 2h). In basal condition, the level of disulfide-linked oligomers was 2.4 ± 0.7 -fold, compared to the level of monomer. Upon tauK18^{P301L} activation, the amount of disulfide-linked tau oligomers was increased by 1.8 ± 0.3 -fold compared to that of basal. Levosimendan efficiently suppressed the formation of disulfide-linked oligomers, decreasing the level of oligomers to 0.5 ± 0.1 -fold, which is even lower than that of basal. In case of MB and LMTM, the level of oligomers was increased to 2.3 ± 0.5 - and 2.4 ± 0.3 -fold, respectively, and monomers were almost disappeared (Fig. 2h). Our results clearly indicated that while MB and LMTM increases the formation of tau oligomers in primary neurons, levosimendan prevented tau phosphorylation, inhibiting disulfide-linked tau oligomerization.

Non-reducing SDS-PAGE analysis indicated that significant amount of tau exists as disulfide-linked oligomers upon the activation of tau pathology. Full-length human tau has two cysteine residues (C291 and C322) that can form intra- and inter-molecular disulfide bonds⁴⁰. While intramolecular disulfide bonds lead to the formation of compact monomers that cannot form extended structure, intermolecular disulfide-bonds produce covalently linked oligomers. The disulfide-linked oligomers serve as “nuclei” for further tau aggregation²⁴ (Fig. 2i). Tau aggregation may occur in the absence of disulfide-bond

formation, but disulfide-linked tau oligomers could facilitate tau aggregation, serving as a structural seed for tau aggregation. Our result indicates that the treatment of MB and LMTM increased the level of disulfide-linked tau oligomers and levosimendan prevented the formation of disulfide-linked tau oligomers.

Next, we evaluated the effects of tau oligomers on neuronal integrity. At DIV10, hippocampal neurons were treated with MB, LMTM (3 mM) or levosimendan (10 mM) followed by tauK18^{P301L}. After 48 hrs of incubation, neurons were stained with NeuO, a neuron specific fluorescence dye⁴¹ (Fig. 2j). Then, fluorescence images were acquired and analyzed to evaluate the length of neurites (Fig. 2k). TauK18^{P301L}-induced neuronal degeneration was observed significantly by showing 142.8 ± 17.8 mm shortened neurites compared to that of basal ($p < 0.0001$). Levosimendan inhibited tauK18^{P301L}-induced neuronal degeneration by recovering neurite length to 346.8 ± 20.7 mm. In contrast, MB did not show significant effect on neuronal integrity and LMTM protected neuronal degeneration slightly by showing 151.1 ± 24.4 mm neurite length. This result clearly indicates that levosimendan prevented tauK18^{P301L}-induced toxicity not only in tau-BiFC cells, but also in primary neurons, via inhibiting the formation of disulfide-dependent tau oligomers.

Levosimendan not only prevents tau oligomerization, but also disaggregates filamentous tau.

Next, we validated the direct effect of each drug on tau oligomerization and tau fibrilization in *in vitro* conditions using purified tauK18 fragment. For tau oligomerization analysis, tau pre-aggregates were incubated with each drug at various concentrations (10, 30, and 100 mM) for 5 hours. Then, tau oligomers in the mixtures were separated into reducing and non-reducing SDS-PAGE (Fig. 3a, b). In DMSO-treated control, $50 \pm 1.1\%$ of total tau exists as disulfide-linked oligomers, which were formed spontaneously in phosphate-buffered saline (pH 7.4). Upon the treatment of levosimendan, the levels of disulfide-linked tau oligomers was decreased to $15.0 \pm 6.0\%$ at 10 mM, $15.7 \pm 7.8\%$ at 30 mM, and $12.3 \pm 15.0\%$ at 100 mM, increasing the level of monomers. This result indicates that levosimendan directly inhibits disulfide-linked tau oligomerization. In case of MB and LMTM, at 100 mM concentration, the level of disulfide-linked tau oligomers was increased to $77.6 \pm 2.0\%$ and $79.2 \pm 0.8\%$, respectively. MB- and LMTM-induced tau oligomers are not easily dissociable into monomers even in reducing condition, showing noticeable bands of dimers and trimers, resistant to SDS- & b-mercaptoethanol on a reducing SDS-PAGE gel (Fig. 3b). In vitro tau oligomerization assay indicates that levosimendan prevents disulfide-linked tau oligomerization by direct interaction with tau protein, while MB and LMTM increase disulfide-linked tau oligomerization.

Then, we investigated the inhibitory effect on tau aggregation. For tau aggregation assay, tau aggregation was induced by the treatment of heparin to purified tauK18 in the presence of each drug at various concentrations for 5 days. The formation of β -sheet aggregates was evaluated with Thioflavin S (ThS) assay. All drugs inhibited the formation of ThS-positive aggregates at micromolar concentration (MB; $EC_{50} = 3.1 \pm 0.1$ mM, LMTM; $EC_{50} = 1.9 \pm 0.2$ mM, and levosimendan; $EC_{50} = 2.6 \pm 0.1$ mM) (Fig. 3c). Then, the structures of tau aggregates were evaluated under transmission electron microscopy (TEM) (Fig. 3d).

TEM image of DMSO control shows the formation of long and straight filaments of tau. In comparison, tau filaments are not observed in levosimendan-treated mixture, indicating its effectiveness in inhibiting tau aggregation. In case of MB and LMTM, a few thread-like filaments were observed. We also investigated the activity of drugs on reversing tau aggregation. For disaggregation assay, tau aggregates were treated with each drug at various concentrations for 4 days. ThS assay indicated that all three drugs were effective in reducing ThS-positive tau aggregates (MB; $EC_{50}=3.8\pm0.1$ mM, LMTM; $EC_{50}=3.4\pm0.2$ mM, and levosimendan; $EC_{50}=3.7\pm0.2$ mM) (Fig. 3e). However, ThS results of MB and LMTM conflicted with TEM images showing thick bundles of tau filaments in MB- and LMTM-treated mixture (Fig. 3f). The result corresponds with a previous study showing, upon treated to tau filaments, MB did not reduced the tau filaments shown under electron microscope⁴². Our result implies that the treatment of MB or LMTM prevents the interaction between ThS and tau filaments, generating ThS-negative tau filaments. In case of levosimendan, ThS response correlated with high-resolution TEM image, which shows a bunch of short tau fragments. The lengths of tau filaments are ranging from 8.8 nm to 139.3 nm (n=403). The *in vitro* tau aggregation and disaggregation assay show the effectiveness of levosimendan as an anti-tau oligomerization agent, which not only inhibits tau aggregation, but also disassembles tau filaments.

To further confirm the effectiveness of levosimendan on tau disassembly, levosimendan was treated at diverse time-points of tau aggregation processes *in vitro* and in tau-BiFC cells. For *in vitro* assay, tauK18 aggregation was induced by heparin, and the level of tau aggregation was monitored with ThS over 5 days. Levosimendan was treated to an aliquot of the aggregation mixture at 0, 25, 44, and 75 hours after heparin activation (Fig. 3g). ThS-response curve shows that tau aggregation followed nucleation–elongation mechanism. Levosimendan treatment, no matter when it was treated, decreased ThS-positive tau aggregates (Fig. 3h). For tau-BiFC assay, tau pathology was activated by the treatment of tauK18^{P301L}, and tau-BiFC fluorescence intensities were monitored over three days. Tau-BiFC cells were treated with levosimendan at 1, 12, 24, and 36 hours after tauK18^{P301L} activation (Fig. 3i). Again, levosimendan treatment, no matter when it was treated, decreased tau-BiFC fluorescence responses, indicating the effectiveness as an anti-tau oligomerization agent (Fig. 3j and Supplementary Fig. 3).

Levosimendan inhibits disulfide-crosslinked tau oligomerization *in vitro*

Next we investigated the molecular mechanism of levosimendan in inhibiting oligomerization. We hypothesized that levosimendan inhibits tau-disulfide bond formation by blocking tau cysteine residues, since its nitrile group is able to form a thioimide bond with cysteine. To verify its covalent modification to tau, one of two nitrile groups of levosimendan was labelled with ¹⁴C-radioisotope (Fig. 4a and Supplementary materials). ¹⁴C-levosimendan was treated to tau pre-aggregates that contains disulfide-linked tau oligomers for 2 hrs. Then, disulfide-linked tau oligomers were separated on a non-reducing SDS-PAGE gel (Fig. 4b, c). ¹⁴C-radiography showed tau monomers and dimers labelled with ¹⁴C-levosimendan, indicating its covalent attachment to tau (Fig. 4c). Then, the relative intensities of Coomassie blue-stained bands of monomer, dimer, trimer, and tetramer were compared (Fig. 4c, d). Corresponding to Figure 3e, the treatment of ¹⁴C-levosimendan disassembled disulfide-linked tau

oligomers, increasing the level of monomers as levosimendan did (Fig. 4c, d). In addition, high-resolution TEM images were acquired to validate the size of tau oligomers in DMSO-treated control and levosimendan-treated mixture. Heterogeneous tau particles with diameters of 13.7 ± 5.2 nm were observed in DMSO-treated control ($n = 60$) (Fig. 4e, f). The diameters of tauK18 oligomers matches with a previous report showing that tauK18 formed spherical tau oligomers in size with diameters of 10~20 nm⁴³. In comparison, comparably small tau particles with diameters of 8.8 ± 2.0 nm were observed in levosimendan treated mixture ($n = 69$), supporting the disassembly of tau oligomers by levosimendan. To validate levosimendan-binding to tau cysteine residues, each repeat domain of tauK18 was synthesized and incubated with levosimendan (Fig. 4g). MALDI-mass spectrometry indicated that levosimendan covalently attached to R2 and R3 domains containing a cysteine residue.

Next, we confirmed the importance of the nitrile moiety of levosimendan in inhibiting tau aggregation by using its metabolites. In human, 5% of levosimendan is metabolized to OR-1855 and OR-1896 in which the nitrile moiety is absent⁴⁴ (Fig. 4h). In particular, OR-1896 is a pharmacologically active metabolite of levosimendan, acting as a powerful inodilator⁴⁵. The metabolite's effects on tau aggregation were evaluated *in vitro* and in tau-BiFC cells. For *in vitro* assay, tau aggregation was induced by heparin in the presence of each metabolite for 5 days. ThS responses indicated that OR-1855 and OR-1896 did not inhibit tau aggregation *in vitro*, while levosimendan showed strong anti-tau aggregation activity (Fig. 4i). For tau-BiFC assay, tau-BiFC cells were treated with each metabolite for 48 hrs in the presence of tauK18^{P301L}. Again, even at 100 mM concentration, OR-1855 and OR-1896 did not inhibit the tau-BiFC fluorescence increase (Fig. 4j). This result indicated that the nitrile group is critical in inhibiting tau aggregation through the modification of tau cysteine residues. Interestingly, OR-1896, which is a pharmacologically active metabolite acting as an inodilator, did not show any effect on tau. This result strongly suggests that the anti-tau activity is a new mode of action of levosimendan, distinguished from its known action as an inodilator.

Levosimendan rescues cognitive deficit and tau pathology in Tau^{P301L}-BiFC mice.

Next, we evaluated *in vivo* efficacy of levosimendan in Tau^{P301L}-BiFC mice³³. Tau^{P301L}-BiFC mouse model expresses human mutant (P301L) tau labeled by BiFC compartments (Fig. 5a). In the brain of Tau^{P301L}-BiFC mouse, tau oligomerization occurs from 3 months and neuronal degeneration occurs from 9 months, leading to cognitive deficits at 12 months of age. To evaluate the therapeutic effect of levosimendan in preventing neuronal degeneration and cognitive impairment, Tau^{P301L}-BiFC mice were received intraperitoneal administration of levosimendan or LMTM (5 mg/kg, three times per week) from 9 months to 12 months (Fig. 5a). After 4 month of drug administration, mice were subjected to behavioral tests to monitor cognitive function; novel objective test (NOR), Y-maze test, and passive avoidance test. For comparison, age-matched wild-type (WT) littermates were also subjected to the tests. In the novel objective recognition test, cognitive abilities were determined by the recognition index (RI) for a novel object. Vehicle-treated transgenic (TG) mice showed a significant decrease in the recognition index, with a RI value of 0.49 ($p < 0.0001$, compare to WT), while WT mice exhibited 0.74 of a RI value for a novel

object. Levosimendan-treatment significantly improved recognition memory performance with a RI value of 0.69 ($p < 0.0001$). LMTM-treatment also attenuated the recognition deficit of TG mice by showing a RI value of 0.59 ($p < 0.01$) (Fig. 5b). In Y-maze test, cognitive ability was determined by the percent alternation. Each levosimendan- and LMTM- treated group showed significant increase in the alternation ($70.4 \pm 4.2\%$, $p < 0.05$ and $68.9 \pm 3.5\%$, $p < 0.05$ compared to vehicle-treated, respectively), compared with the vehicle-treated group ($52.8 \pm 2.6\%$, $p < 0.01$ compared to WT) (Fig. 5c). Further, passive avoidance test was followed to assess emotion-associated learning ability. Learning abilities were determined with the latency of entering the dark compartment, where the mice received an electrical shock a day before. Most mice in the vehicle-treated group entered the dark chamber without hesitation ($p < 0.05$), indicating the impairment of fear memory. Levosimendan-treated mice exhibited improved memory showing delayed latency or not entering to the chamber compared with the vehicle-treated TG controls ($p < 0.05$) (Fig. 5d). Interestingly, the LMTM-treated group, did not show significant improvement similar to vehicle-treated TG mice. All these results indicate that administration of levosimendan ameliorates tauopathy-induced memory deficits of aged, symptomatic tau TG mice.

Next, brains were extracted from mice, and brain tissue sections were prepared to evaluate tau pathology. Tau-BiFC fluorescence images indicate the level of tau assembly including tau oligomers and aggregates and AT8-immunostain indicates the level of tau phosphorylation associated with late-state of tau aggregation. Tau-BiFC intensities decreased both in LMTM and levosimendan-treated groups (Fig. 5e, g). In levosimendan treated group, tau-BiFC intensities decreased to 0.6 ± 0.1 -fold in cortex and 0.4 ± 0.2 -fold in hippocampus, compared to those of vehicle-treated group. In LMTM-treated group, tau-BiFC intensities decreased to 0.7 ± 0.2 -fold in cortex and 0.6 ± 0.1 -fold in hippocampus. AT8-immunoreactivity also decreased in both LMTM and levosimendan treated groups (Fig. 5f, g). In levosimendan-treated group, AT8-immunoreactivity decreased to 0.4 ± 0.1 -fold in cortex and 0.4 ± 0.2 -fold in hippocampus, compared to those of vehicle-treated group. In LMTM-treated group, AT8-immunoreactivity decreased to 0.6 ± 0.2 -fold in cortex and 0.5 ± 0.2 -fold in hippocampus.

Next, tau immunoblot assay was followed to evaluate drug effects on tau oligomerization and aggregation, using RIPA-soluble and RIPA-insoluble brain lysates. On tau immunoblots, mouse tau is indicated with black arrow (50 kDa) and human tau-BiFC compartments are indicated with two green arrows (76 and 85 kDa). In RIPA soluble brain lysates, tau phosphorylation levels were significantly decreased in LMTM- and levosimendan-treated groups (Fig. 5h). In levosimendan-treated group, the levels of tau phosphorylation decreased 0.5 ± 0.0 -fold at S199 and 0.5 ± 0.1 -fold at S396, compared to that of vehicle-treated group. In LMTM-treated group, the levels of tau phosphorylation decreased 0.6 ± 0.1 -fold at S199 and 0.8 ± 0.2 -fold at S396. Different from the previous results showing the increased level of SDS- and b-mercaptoethanol resistant tau oligomers, LMTM-treated group did not show any high-molecular weight oligomers in the brain lysates (Fig. 5h). Also, the level of total tau decreased slightly in levosimendan treated group, although the decrease was not statically significant. Corresponding to AT8-immunofluorescence images in Figure 5f, the amount of insoluble tau decreased 0.5 ± 0.2 -fold in levosimendan-treated mice (Fig. 5i). In case of LMTM, the amount of insoluble tau decreased 0.7 ± 0.1 -fold. Collectively, our results indicated that levosimendan is a new drug candidate targeting tau

oligomerization, suppressing tau phosphorylation and aggregation in Tau^{P301L}-BiFC mice. Corresponding to AT8-immunofluorescence images in Figure 5f, the amount of insoluble tau decreased 0.5±0.2-fold in levosimendan-treated mice (Fig. 5i). In case of LMTM, the amount of insoluble tau decreased 0.7±0.1-fold.

Next, the level of disulfide-linked tau oligomers was evaluated by non-reducing SDS-PAGE analysis. Similar to the result of primary neuron cultures, disulfide-linked tau oligomers were observed in the brain lysates of wild type mice (Supplementary Fig. 4). In Tau^{P301L}-BiFC mice, most of human and murine tau exists as the form of disulfide-linked oligomers (> 245 kDa) (Fig. 5j and Supplementary Fig. 4). Levosimendan-treatment significantly decreased the high-molecular weight oligomers and increased soluble monomeric tau level. This result is consistent with previous data showing that levosimendan inhibits the formation of disulfide-cross linkage between tau. In contrast, LMTM-treatment did not increase nor decrease the level of disulfide-linked tau oligomers in the brain (Fig. 5j). The LMTM effect in the animal was contrasted with the effect when LMTM interacts with tau directly in test tubes. This result implies that when intraperitoneally administrated to mice, LMTM might not have direct interaction with tau as shown *in vitro* test (Fig. 3a, b). The anti-tau activity of LMTM shown in the brain of Tau^{P301L}-BiFC mice could be the result of indirect regulation of tau pathway, such as decreasing tau phosphorylation or increasing autophagic clearance of aggregated proteins^{7,46}. Collectively, our results indicated that levosimendan is a new drug candidate targeting tau oligomerization, suppressing tau phosphorylation and aggregation in Tau^{P301L}-BiFC mice.

Discussion

Levosimendan was identified 20 year ago and has been treated to patients with acute heart failure⁴⁷. As an inodilator, levosimendan possesses both positive inotropic and vasodilator actions; (i) as a calcium sensitizer, it enhances the sensitivity of contractile proteins to calcium through covalent binding to troponin C⁴⁸. (ii) as a vasodilator, levosimendan inhibits phosphodiesterase III and opens ATP-dependent K⁺ channels in smooth muscle cells, leading to arteriolar and venous dilation⁴⁹. In addition to heart, ATP-dependent K⁺ channels present in a number of tissues including brain⁵⁰. In 2010, Roehl et al reported neuroprotective effect of levosimendan *in vitro* model of traumatic brain injury, which might be associated with the activation of neuronal ATP-dependent K⁺ channels^{51,52}. There are also evidences indicating that the neuroprotective effect of levosimendan is associated with its action as a vasodilator. In 2015, Levijoki et al reported that orally administered levosimendan increased blood volume of the cerebral vessels, reducing mortality and morbidity in rat models of primary and secondary stroke⁵³. In 2015, Matti Kivikko et al reported a pilot study showing that low dose oral administration of levosimendan increased cerebral blood flow velocities in patients with an earlier ischemic cerebrovascular event, supporting the vasodilatory effect in the brain circulation⁵⁴.

Here our study shows a new molecular mechanism of levosimendan as an anti-tau agent. By modifying tau cysteine residues, levosimendan inhibits disulfide-linked tau oligomerization. Different from LMTM,

which showed conflicting effects on tau, levosimendan exhibited consistent, inhibitory effect on tau oligomerization *in vitro*, in cells, and in the brain of mice, suppressing tau aggregation. In tau-BiFC mice, 4 month-administration of levosimendan not only suppressed tau pathology, but also prevented memory deficits in aged Tau^{P301L}-BiFC mice. To apply levosimendan as AD therapeutics, safety issue should be clarified, due to its action as an inodilator. There is strong association between dementia and cardiovascular disease. Cardiovascular insufficiency impairs the function of diverse organs, including the brain, which can worsen a pathology related to dementia^{55,56}. For an example, phosphodiesterase III is known to be upregulated in cerebral blood vessels of AD patients due to vascular amyloid burden, and phosphodiesterase III inhibitors have shown protective effects in AD model^{57,58}. In that case, the vasodilator-action of levosimendan would be beneficial in improving AD brain function.

Drug repositioning is an attractive drug discovery strategy, which generates new therapeutic value from existing drugs⁵⁹. MB is one of the representative cases of drug repositioning. In 1891, MB was firstly approved to treat malaria with its activity as a chloroquine sensitizer⁶⁰. Then, MB had been approved to treat clinical pain syndromes, psychotic disorders, cyanide poisoning and urinary tract infections⁶¹. In 2016, MB was approved to treat Methemoglobinemia⁶². In addition to the known medical actions of MB, many studies have compiled wide variety of biological activities of MB, which i) inhibits the activity of monoamine oxidase A⁶³, nitric oxide synthase⁶⁴, and guanylate cyclase⁶⁵, ii) increases the release of neurotransmitters, such as serotonin and norepinephrine^{66,67}, iii) increases cholinergic transmission⁶⁸, iv) inhibits GSK3b and microtubule-affinity regulating kinases^{69,70}, and v) promotes autophagic clearance of b-amyloid⁷¹ and tau⁷². The action of methylene blue on multiple targets in the brain justifies its symptom relieving effects on Tau^{P301L}-BiFC mice; improving learning and cognitive abilities and reducing tau phosphorylation and aggregates. However, our *in vitro* and cell-based data clearly demonstrated that tau oligomer is not the direct therapeutic target of MB or LMTM.

In AD patients, tau oligomers are detected at the early stages of pathogenic cascades^{14,15,73}. Therefore, preventing tau oligomerization is an important therapeutic strategy to prevent neuronal loss and memory deficits in AD patients. However, it has been difficult to establish therapeutic strategy preventing tau oligomerization, since tau pathology is linked with a number of cellular processes, which are closely linked to each other. For example, if GSK3b a major tau kinase, is inhibited, other tau kinases activate tau pathology^{5,74}. Therefore, direct modification of tau would be more effective a strategy to prevent tau oligomerization without altering other cellular processes. Our results showed that levosimendan covalently binds to tau cysteine residues and inhibits tau oligomerization preventing tau pathology. Moreover, levosimendan could disassemble tau-tau interactions regardless of its aggregation states. The binding mode between levosimendan and tau should be clarified in future study; how levosimendan dissociates disulfide-linked tau oligomers into monomers; whether levosimendan could distinguish cytosolic tau from microtubule-bound tau. Once levosimendan disassembles tau oligomers into monomers, the monomeric tau would be degraded by proteasome complexes, reducing soluble tau burden⁷⁵. Our results supported this by showing the decreased level of tau in levosimendan-treated tau-

BiFC cells and primary neurons, together with the reduced level of tau oligomers. Levosimendan displayed robust potency against tau oligomerization and rescued tauopathy-induced cognitive declines in Tau^{P301L}-BiFC mouse model. Although careful validation is required, our data present the potential of levosimendan as a disease modifying drug for AD.

Materials And Methods

Source of chemicals

FDA-approved & Passed Phase I Drug Library was purchased from Selleckchem. Forskolin and MB were purchased from Sigma, and leucomethylene blue mesylate (LMTM) was purchased from MedChemExpress. Levosimendan and its metabolites, OR-1855 and OR-1896 were purchased from Toronto Research Chemicals Inc. Synthesis and characterization of ¹⁴C-levosimendan were conducted by Curachem, Inc. according to previously described protocol⁷⁶. The characterization of ¹⁴C-levosimendan is provided in the Supplementary Materials.

Preparation of tauK18 fragments

TauK18 and tauK18^{P301L} fragments were expressed and purified according to previously described protocols^{19,77}. 6xHis-tagged tauK18 and tauK18^{P301L} were expressed in *E.coli* BL21 (DE3) and purified by using Ni-NTA (QIAGEN) column chromatography. The purified protein was dissolved in PBS (pH 7.4). Peptide-synthesis and characterization of each repeat domain (R1-R4) were conducted by GL Biochem. ESI-MS characterization of the peptides is provided in the Supplementary materials.

Tau-BiFC cell culture and the treatment of FK or tauK18^{P301L}

HEK293 Tau-BiFC cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL Geneticin (G418) in humidified atmosphere containing 5% CO₂ at 37°C. Tau-BiFC cells were activated by the treatment of FK (30 mM) or tauK18^{P301L} (5 mg/mL).

1018 FDA-approved & Passed Phase I Drug Library screening and high-contents image analysis

For screening of FDA-approved & Passed Phase I Drug Library, tau-BiFC cells were plated on µ-clear 384-well plates. The next day, the cells were co-treated with forskolin and 1,018 library compounds at 10 µM concentration. MB and LMTM (3 µM) were used as a positive control. After 46 hrs, nuclei were counterstained with Hoechst (Invitrogen). BiFC (λ_{ex} = 460-490 nm and λ_{em} = 500-550 nm) and Hoechst (λ_{ex} = 355-385 nm and λ_{em} = 430-500 nm) fluorescence images were acquired automatically by using Operetta® (PerkinElmer). BiFC-fluorescence intensity and the number of nuclei were quantified using the Harmony 3.1 software (PerkinElmer). Integrated tau-BiFC intensity represents Area of selected cell X Mean fluorescence intensity. The means and standard deviations (S.D.) of BiFC fluorescence intensities and the number of nuclei were plotted using GraphPad Prism.

Flow cytometry analysis

Tau-BiFC cells, grown in 6-well plates, were treated with MB, LMTM (5 μ M), or levosimendan (15 μ M), followed by the treatment of tauK18^{P301L} (5 μ g/mL). After 24 hrs, BiFC cells were collected and subjected to flow cytometry analysis, using BD FACSLyticTM cytometer (BD Biosciences). A total of 50,000 events were acquired per sample. All samples were gated using the same gating tree and gate positions; side scatter area (SSC-A) vs. forward scatter area (FSC-A) for viable and singlet cell populations. BiFC fluorescence was excited by 488 nm wave laser and collected through a 527/32 band-pass filter.

Preparation of Tau-BiFC cell lysates

Tau-BiFC cells, grown in 6-well plates, were treated with MB, LMTM (0.5, 1.5, 5 μ M), or levosimendan (5, 15, 45 μ M) followed by tauK18^{P301L} (5 μ g/mL) activation. After 36 hrs, tau-BiFC cells were washed with PBS and lysed in CellLytic M lysis reagent (Sigma) containing protease/phosphatase inhibitor cocktail (Sigma).

Tau-immunoblot analysis of reducing and non-reducing SDS-PAGE gels

For reducing SDS-PAGE analysis, cell or brain lysates were mixed with 4x Laemmli buffer containing 10% β -mercaptoethanol (BME) and boiled at 97°C for 5 min. For non-reducing SDS-PAGE analysis, cell or brain lysates were mixed with 4x Laemmli buffer without BME. For immunoblot analysis, 10 μ g of each lysate was separated into 10% SDS-PAGE gel and transferred to PVDF membrane. The levels of total tau and phosphorylated tau were detected by anti-tau antibody; 2B11 (IBL), Tau5 (Abcam), pSer199 (Abcam), pSer396 (Abcam), and pThr205 (Abcam). Band intensity was quantified using Image J software (NIH).

Primary neuron culture and preparation of the lysates

Primary hippocampal neurons were isolated from day 18 embryonic Sprague-Dawley rat brains as described previously⁷⁸. The neurons were seeded at a density of 3.5×10^5 cells/well on a poly-D-lysine-coated 6-well plate and maintained in the neurobasal medium at 37°C in a humidified atmosphere of 5% CO₂. The neurobasal medium contains 2% B27 supplement, 0.5 mM glutamax, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Every 3 days, 50% of the medium was replaced with the fresh neurobasal medium. At DIV10, neurons were incubated with MB, LMTM (3 μ M), or levosimendan (10 μ M) in the presence of tauK18^{P301L} (10 μ g/mL) for 48 hrs. Then, the neurons were washed with PBS and lysed in CellLytic M lysis reagent (Sigma) containing protease/phosphatase inhibitor cocktail (Sigma).

NeuO stain and analysis of neurite lengths

At DIV10, neurons grown on a 96-well plate were treated with MB, LMTM (3 μ M), or levosimendan (10 μ M) followed by tauK18^{P301L} activation. After 48 hrs, the neurons were stained with NeuO (50 nM), a neuron selective probe⁴¹. NeuO-fluorescence (λ_{ex} = 460-490 nm and λ_{em} = 518-580 nm) images were

acquired by using Operetta® (PerkinElmer). The maximal length of neurites was quantified by Harmony 3.1 software (PerkinElmer).

TauK18 oligomerization assay *in vitro*

To induce tau oligomerization, tauK18 (0.5 mg/mL dissolved in PBS, pH7.4) was incubated with each drug in the presence of DTT (100 μ M) and heparin (0.1 mg/mL) at RT for 5 hrs with vigorous shaking. Then, tau oligomers were separated on 4-20% SDS-PAGE gels under reducing and non-reducing condition and visualized by Coomassie blue stain.

***In vitro* tau aggregation and disaggregation assays.**

To evaluate the inhibitory effect of drugs on tau aggregation, tauK18 protein (0.5 mg/mL) was incubated with each drug at various concentration in the presence of DTT (100 μ M) and heparin (0.1 mg/mL) at 37°C for 5 days, with vigorous shaking (220 rpm). For tau disaggregation assay, the preformed aggregation-mixture of tauK18 was incubated with each drug at 37°C for 4 days, with vigorous shaking (220 rpm). At the final day of the incubation, the level of β -sheet aggregates was evaluated by thioflavin S (ThS) assay. For ThS assay, 5 μ L of each mixture was transferred to a black 384-well plate with 45 μ L of PBS containing 10 μ M ThS. ThS fluorescence (λ_{ex} = 430 nm, and λ_{em} = 500 nm) was measured by using a Flexstation2 spectrophotometer (Molecular Devices).

Transmission electron microscopy (TEM)

Samples were placed onto carbon-coated copper electron microscopy grids, and then negatively stained with 2 % (w/v) aqueous uranyl acetate for 1 min. For imaging of tau filaments (Fig. 3D), the grids were observed using a JEM-1011 transmission electron microscope (JEOL) at the acceleration voltage of 80 kV. For imaging of tau filaments and oligomers (Fig. 3F and Fig. 4E), the grids were observed using Tecnai G2 F20 transmission electron microscope (FEI) at the acceleration voltage of 120 kV. Eight to twelve random images from each experimental condition were captured by the operator as a blind observer.

Autoradiography of ¹⁴C-levosimendan

TauK18 (1mg/mL, 72 μ M) was incubated with ¹⁴C-levosimendan (720 μ M) in PBS containing 5% of DMSO at RT for 2 hrs. Then, the mixture was separated on 15% SDS-PAGE gel under non-reducing condition and stained with Coomassie blue. For autoradiography, the SDS-PAGE gel was transferred to PVDF membrane and scanned by Typhoon FLA 7000 IP (GE Healthcare).

MALDI-TOF analysis of R1-R4 peptides

Each repeat domain (R1- R4, 150 mM) was incubated with levosimendan (molar ratio 1:10 of a tau repeat domain: levosimendan) at RT for 37 hrs. The samples were mixed with matrix solution (10 mg/mL of Sinapinic acid in 0.1 % (v/v) trifluoroacetic acid/CAN) at a ratio 1:1. The mixtures were directly spotted onto the MALDI target and dried. Mass spectra were acquired in reflection/linear positive ion mode in the

m/z range of 2,000-100,000 using an Ultraflex III TOF/TOF mass spectrometer controlled by Flex Control 3.0 (Bruker Daltonics). Default operating conditions are as follows: ion source 1, 25.0 kV; ion source 2, 23.0 kV; lens voltage, 6.0 kV; laser repetition rate = 100 Hz. All spectra were generated automatically in the instrument software and based on averaging 1000 shots from 10 non-overlapping positions (100 shots/position).

Animal studies

Tau^{P301L}-BiFC mice

Tau^{P301L}-BiFC mice were bred and maintained as described previously³³. Tau^{P301L}-BiFC mice were bred with C57BL/6N mice and maintained in pathogen-free facilities. Their heterogeneous offspring and wild-type littermates were used in this study. All mice were allocated randomly for experiments, but groups were counterbalanced for animal sex and group average body weight. Animal protocols followed the principles and practices outlined in the approved guidelines by the Institutional Animal Care and Use Committee of the Korea Institute of Science and Technology. All animal experiments were approved by the Korea Institute of Science and Technology.

Drug administration to Tau^{P301L}-BiFC mice

For drug administration, LMTM and levosimendan were dissolved in PBS containing 40% polyethylene glycol (PEG; Sigma). 9 month-old Tau^{P301L}-BiFC mice were intraperitoneally administered with each drug for 4 months, three times a week, with 5 mg/kg dosage (n=11 per group). Vehicle-treated Tau^{P301L}-BiFC mice and age-matched wild type mice were used as control groups. Behavioral assessments were conducted as described below for one month from the end of drug administration. All mice were sacrificed at the age of 14 months for pathological analysis.

Behavioral tests

Novel object recognition. Novel object recognition test was performed as described previously⁷⁹. Briefly, for the habituation, mice (n=9 per group) were individually placed in the center of an open field arena (40 × 40 cm) and allowed to freely explore for 15 min. Next day, in the training trial (familiarization phase), mice were allowed to explore two identical objects in the open field for 10 min. In the testing trial (recognition phase) performed 24 hours later, one familiar object was changed for a novel object which was different in color and shape. Mice were allowed to explore the objects for 10 min. The exploration time for the familiar (old) or the new (novel) object during the recognition phase was recorded using Noldus EthoVision XT video tracking system. Memory was operationally defined by the recognition index calculated by dividing the time an animal spent exploring the novel object or old object by the total time spent exploring objects in the testing period. The exploration time was measured when the mouse was pointing towards the object in the vicinity of the object.

Y-maze. Mice (n=11 per group) were tested for spontaneous alternation behavior in a Y-shaped maze (60 cm in length of each arm, 20 cm in depth) using standard protocol⁸⁰. Spontaneous alternation for 8 mins was calculated as the proportion of alternations. Alteration (%) was defined as consecutive entries in three different arms (ABC), divided by the number of possible alterations (total arm entries minus 2)⁸¹.

Passive avoidance. Passive avoidance test was performed as described previously⁸² with the following modifications. Mice (n=8 per group) were adapted in the passive avoidance chamber (Gemini) for 10 min, and then were returned to their home cages. The chamber is composed of a light compartment and a dark compartment separated by a connecting gate. The following day, mice were placed in the light compartment, and the gate was opened after 30 seconds. When mice entered the dark compartment, the gate was closed and an electrical foot shock (3 mA) was delivered for 2 sec. Mice were left in the dark compartment for 30 sec after the foot shock to enable association of the environment with the aversive stimulus. Mice were then returned to their home cages. The following day, mice were placed in the light compartment again, and the gate was opened after 30 seconds. The step-through latency, the time required for mice to enter the dark compartment, was measured up to 540 sec.

Preparation of brain tissue slices

Mice were perfused with 0.9% saline and fixed with PBS (pH 7.4) containing 4% paraformaldehyde (Sigma). Brains were extracted and fixed in PBS containing 4% paraformaldehyde at 4°C for 48~72 hrs. For cryoprotection, the brains were transferred to PBS containing 30% sucrose solution and incubated at 4°C until they sunk. For cryosectioning, the brains were embedded with O.C.T (Tissue-TEK), and cut serially using a cryostat (CM1860UV, Leica). 30- μ m thick tissue slices were maintained in PBS containing 0.05% sodium azide at 4°C.

Sudan Black B stain and BiFC fluorescence imaging

Brain tissue section (n=8 per group) were mounted onto slides. To reduce autofluorescence, brain tissue sections were stained with Sudan Black B solution (70% ethanol containing 0.05% Sudan Black B) for 10 min, washed three times with PBS containing 0.1% Triton X-100 (Sigma), and then washed with distilled water. For counter-staining of nuclei, brain tissues were stained with Hoechst (0.5 μ g/mL) for 30 min. BiFC fluorescence (λ_{ex} = 460-490 nm and λ_{em} = 500-550 nm) images were acquired using Axio Scan. Z1 (ZEISS). For quantitative analysis, Mean BiFC-fluorescence intensity was measured in somatosensory cortex (layer V) and hippocampal CA1 from AP;-1.82. To normalize the background fluorescence between the brain samples, habenular region was used as an internal control. Using image J software (NIH), the region of interest was masked, and the fluorescence intensity value was calculated. Data were shown as the average and standard deviation of Mean fluorescence intensities of brain images from six to eight animals.

Immunofluorescence analysis of brain tissue slices

For immunofluorescence image analysis, brain tissue slices (n=6~8 per group) were stained with AT8 (pS202/T205) antibody (1:200, Invitrogen). The primary antibody was detected using Alexa Fluor 633-conjugated anti-mouse antibody (1:500, abcam). Fluorescence images (λ_{ex} = 620-640 nm and λ_{em} = 650-700 nm) were acquired using Axio Scan. Z1 (ZEISS).

Preparation of RIPA-soluble and insoluble brain lysates

Brains were weighed and suspended in RIPA lysis buffer (Sigma) containing protease and phosphatase inhibitor cocktails. Then, tissues were mechanically disrupted using a cordless mortar and pestle (Sigma), and incubated at 4°C for 2 hrs, with shaking. The homogenates were centrifuged at 20,000 g at 4°C for 20 min. The supernatants were collected as RIPA-soluble fractions and stored at -80°C. To prepare RIPA-insoluble fractions, the remaining pellets were washed once with RIPA lysis buffer containing 1 M sucrose, and then resuspended in 2% SDS solution (1 mL per gram of tissue) and incubated at RT for 1 hr. The mixtures were centrifuged at 20,000 g for 1 min at RT. The supernatant was collected as RIPA-insoluble fractions and preserved at -80 °C.

Statistics

All data in quantitative analysis were presented as mean $\pm$ S.D. or S.E.M.. Unpaired *t*-test was performed when two groups were compared. One-way ANOVA or two-way ANOVA was performed when multiple groups were compared, depending on the number of independent variables. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software).

Declarations

Author contributions

Y.K.K. designed the study and provided theoretical guidance; S.L., S.S., H.E.L., J.Y.S., Y.S., G.H.L., H.A., N.L., K.H.K., and H.J., performed the experiments; S.L., S.S., and Y.K.K. interpreted the data and wrote the manuscript; and S.L., S.S., B.Y.Y., S.M.L., J.S.L., A.N.P., and Y.K.K. analyzed the data and revised the manuscript. All authors reviewed the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

All data associated with this study are present in the paper or the Supplementary Materials.

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Figures

Figure 1

FDA-approved & Passed Phase I Drug Library screen identifies levosimendan as a new anti-tau agent. a Schematic representation of tau-BiFC screening of FDA-approved & Passed Phase I Drug Library. For tau-BiFC screening, Tau-BiFC cells were treated with each 1,018 library compounds (10 μ M) in the presence of forskolin (FK). MB and LMTM (3 μ M) were treated as a positive control. b Scatterplot of 1,018 FDA-approved drug screening. Quadruplicate results of a single drug were plotted with the BiFC intensity on the Y-axis and the number of nuclei on the X-axis. Crosses indicate BiFCOFF-basal, and green dots indicate BiFCON-control. One selective drug, levosimendan (yellow) was plotted with MB (blue) and

LMTM (cyan). c BiFC fluorescence images of tau-BiFC cells treated with MB, LMTM, or levosimendan in the presence of FK. Nuclei were counterstained with Hoechst and indicated in red color. Scale bar, 100 μm . d, e Quantification of tau-BiFC responses (d) and cell viabilities (e). One-way ANOVA with Dunnett's multiple-comparisons test were performed; ** $p < 0.01$, *** $p < 0.001$, compared with control. f, g Dose-response curves of MB, LMTM, and levosimendan with EC50 and GI50 values. In the presence of FK (f) or tauK18P301L (g), each drug was treated to tau-BiFC cells at various concentrations. After 48 hours of treatment, tau-BiFC responses and the number of nuclei were analyzed. A Prism's non-linear regression analysis was used to measure the EC50 and GI50 values. h, i Flow cytometry analysis of tau-BiFC cells co-treated with MB, LMTM (5 μM), or levosimendan (15 μM) with TauK18P301L. h P1 population indicates mononuclear cells gated with red line based on the level of side scatter (SSC) and forward scatter (FSC), excluding cell debris or dead cells. The percentage of P1 indicates the ratio of healthy, mononuclear cells out of total cells. i BiFC fluorescence histograms of P1 cell populations. j High magnification images of a representative tau-BiFC cell treated with MB, LMTM, and levosimendan. Scale bar, 20 μm .

Tau oligomers in Tau-BiFC cells

Tau oligomers in primary neurons

Figure 2

Levosimendan inhibits tau oligomerization in tau-BiFC and primary neuronal cells while MB and LMTM increased tau oligomerization. a-c Immunoblot analysis of total and phospho-tau. For the immunoblot analysis, tau-BiFC cells were treated with MB, LMTM (0.5, 1.5, 5 μM), or levosimendan (5, 15, 45 μM) in the presence of tauK18P301L. Green arrows indicate monomer bands of hTau-VN173 and hTau-VC155. Bands above 100 kDa indicates tau oligomers. Relative amounts of monomeric tau (white bars) and

oligomeric tau (grey bars) were quantified by Image J. d Immunoblot analysis of total tau on non-reducing condition. Relative amounts of monomeric tau (white bars) and oligomeric tau (red bars) were quantified by Image J. e-g Immunoblot analysis of total and phospho-tau in the lysates of primary neurons. Relative amounts of monomeric and oligomeric tau were quantified by Image J. h Immunoblot analysis of total tau on non-reducing condition. a-h Data represent the mean $\pm$ S.D. of three independent experiments. To indicate significance of the results, Two-way ANOVA with Dunnett's multiple-comparisons test was performed; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared with the level of monomer in basal. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$, compared with the level of oligomers in basal. n.s., non-significant. i Schematic diagram of tau aggregation process and the effect of MB, LMTM, or levosimendan on neurotoxic tau oligomerization. j Representative images of primary hippocampal neurons treated with MB, LMTM, or levosimendan. At day 10 in vitro, hippocampal neurons were treated with MB, LMTM (3 μ M), or Levosimendan (10 μ M), in the presence of TauK18P301L. After 48hrs, hippocampal neurons were stained with NeuO, a neuron selective probe and imaged using Ti microscope. k Quantification of neurite lengths in NeuO-stained neurons. Scale bar, 50 μ m. The maximal length of neurites was quantified by Image J. Data represent the mean $\pm$ S.D. of three independent experiments. One-way ANOVA with Dunnett's multiple-comparisons test was performed; * $p < 0.05$, **** $p < 0.0001$, compared with control.

Figure 3

Levosimendan inhibits disulfide-dependent tau oligomerization in vitro. a, b Non-reducing (A) and reducing (B) SDS-PAGE analysis of tau oligomerization in vitro. Tau pre-aggregates were incubated with MB, LMTM, or levosimendan (10, 30, 100 μM) for 5 hrs at RT, and tau proteins were separated on an SDS-PAGE gel (4-20%) under reducing or non-reducing condition. Tau oligomers on SDS-PAGE gels were visualized with Coomassie blue stain. Black arrow indicates tau monomer (14 kDa). Bands above 25 kDa indicate tau oligomers. Relative amounts of tau monomers and oligomers were quantified by Image J. The percentages of SDS-resistant tau oligomers were indicated as red bars (a) and the percentages of SDS- and β -mercaptoethanol-resistant oligomers were indicated as grey bars (b). Data represent the

mean $\pm$ S.D. of two independent experiments. n.s., non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control. c EC50 curves of MB, LMTM, or levosimendan on inhibiting tau aggregation. TauK18 aggregation was induced by heparin and DTT in the presence of each drug for 5 days. The level of β -sheet aggregation was determined with thioflavin S (ThS). Data represent the mean $\pm$ S.D. of three independent experiments. A Prism's non-linear regression analysis was used to measure the EC50 values. d Representative images of transmission electron microscopy indicating the inhibitory effect of MB, LMTM, and levosimendan at 30 μ M. Scale bar, 300 nm. e EC50 curves of MB, LMTM, or levosimendan on disaggregating tau filaments. For disaggregation assay, tau aggregates were incubated with each drug at various concentration for 4 days. The level of β -sheet aggregation was determined with thioflavin S (ThS). A Prism's non-linear regression analysis was used to measure the EC50 values. Data represent the mean $\pm$ S.D. of three independent experiments. f Representative images of transmission electron microscopy indicating drug' effects on tau disaggregation at 30 μ M. Scale bar, 100 nm. g, h Schematic diagram of the treatment of levosimendan at diverse time points of tau aggregation in vitro (g). Levosimendan (10 μ M) was treated to an aliquot of the aggregation mixture at 0, 25, 44, and 75hr after heparin treatment. The level of tau aggregation was determined by ThS. (h) ThS-response curves indicating anti-tau aggregation effect of levosimendan in vitro. i, j Schematic diagram of the treatment of levosimendan at various time points of tau-BiFC aggregation (i). Levosimendan was treated to tau-BiFC cells at 1, 12, 24, and 36 hrs after the treatment of tauK18P301L. Tau-BiFC fluorescence images were acquired at various time points and the fluorescence intensities were quantified. (j) Tau-BiFC responses indicating the inhibitory effect of levosimendan on cellular tau aggregation. Data represent the mean $\pm$ S.D. of four repeated measurements.

Figure 4

Levosimendan inhibits disulfide-linked tau oligomerization by capping tau cysteine residues a, c

Schematic diagram of a thioimide bond formation of ^{14}C -levosimendan and tau cysteine (a). TauK18 pre-aggregates were incubated with DMSO control or ^{14}C -levosimendan for 2 hrs at RT. The mixtures were separated on a non-reducing SDS-PAGE gel (15%) for further analysis. Coomassie blue stain indicates disulfide-linked tau oligomers on the SDS-PAGE gel (b). The SDS-PAGE gel was transferred onto

a PVDF membrane, and ¹⁴C-labelled tau proteins were visualized by autoradiography ¹⁴C autoradiograph (c). d The relative levels of tau monomer and oligomers on the Coomassie Blue stained SDS-PAGE gel. Two independent experiments were performed. e Representative images of transmission electron microscopy indicating disassembly of tau oligomers. A representative tau particle from each group was indicated with a yellow arrow, with its magnified image indicating its width. Scale bar, 50nm. f Quantification of the widths of tau particles on TEM images. The width of tau particles was measured by Image J. Unpaired t-test was performed; ****p < 0.0001. g MALDI-TOF analysis of tau repeat domains (R1-R4) in the absence and in the presence of levosimendan. Each repeat domain was incubated with levosimendan for 36 hrs at RT (molar ratio 1:10 of a repeat domain: levosimendan). h Structures of OR-1855 and OR-1896, which are not thiol-reactive. i Evaluation of the inhibitory effect of OR-1855 and OR-1896 on tau aggregation in vitro by ThS. Data represent the mean ± S.D. of three repeated experiments. Unpaired t-test was performed; ***p < 0.001, n.s., non-significant. j Evaluation of the inhibitory effect OR-1855 and OR-1896 on tau-BiFC aggregation. Data represent the mean ± S.D. of three repeated experiments. Unpaired t-test was performed; ***p < 0.001, n.s., non-significant.

Figure 5

Levosimendan suppresses tau pathology and cognitive declines in aged TauP301L-BiFC mice. a Diagram of TauP301L-BiFC mouse model and experimental design for administration of LMTM and levosimendan. TauP301L-BiFC mice express human tau mutant (P301L) fused with two non-fluorescent compartments of Venus protein. TauP301L-BiFC mice intraperitoneally received LMTM or levosimendan (5 mg/kg) from 9-month-old for 4 months, three times a week (n=11). b Recognition index (RI) in a novel object recognition test (n=9). RI was defined as the ratio between the times spent exploring the familiar object (Old) or novel object (Novel) divided by total exploratory time. The RI close to 0.5 (dashed line) indicates the mice could not discriminate novel and familiar objects. Two-way ANOVA with Sidak's multiple-comparisons test was performed; **p < 0.01, ****p < 0.0001. c Percent alteration of y-maze test (n=11). A spontaneous alteration was calculated by dividing a number of mouse entering to different arm of the maze in each of 3 consecutive arm entries by the total number of arm entries. One-way ANOVA with Tukey's multiple-comparisons test was performed; *p < 0.05, **p < 0.01. d Latency to enter the dark compartment during the retention test of passive avoidance test (n=8). During training test, each mouse received a foot shock (3 mA, 2 s) upon entry into the dark compartment. Retention test was performed 24 h after training. One-way ANOVA with Tukey's multiple-comparisons test was performed; *p < 0.05. e, f Representative images of BiFC fluorescence (e) and AT8-immunofluorescence (f) in cortex and hippocampus of TauP301L-BiFC mouse. Scale bar, 50 μ m. g Quantification of the fluorescence intensities of tau-BiFC and AT8-immunofluorescence (n=6~8). The mean fluorescence intensities were measured in layer V of somatosensory cortex and CA1 of hippocampus. The values were normalized to that of vehicle-treated group. One-way ANOVA with Tukey's multiple-comparisons test was performed; *p < 0.05, **p < 0.01, ***p < 0.001. h Immunoblot analysis of total tau and phosphorylated tau with Tau5, anti-p-TauS199, and anti-TauS396 antibodies in RIPA-soluble fractions of TauP301L-BiFC brain lysates (n=6). Green arrows indicate hTau-VN173 and hTau-VC155. Black arrows indicate endogenous murine tau. Relative amounts of human tau (hTau, green bars), murine tau (mTau, white bars) monomers, and total tau (grey bars) were quantified by Image J. i Immunoblot analysis of total tau in RIPA-insoluble fractions. Total tau (grey bars) were quantified by Image J. j Immunoblot analysis of soluble tau oligomers in the brain lysates. To evaluate disulfide-linked tau oligomers, RIPA-soluble brain lysates were separated on a non-reducing SDS-PAGE gel (vehicle, LMTM, levosimendan; n=10). Relative amounts of hTau (green bars), mTau (white bars) monomers, and oligomers (red bars) were quantified by Image J. h-j To indicate significance of the results, Two-way ANOVA with Tukey's multiple-comparisons test was performed; *p < 0.05, ***p < 0.001, ****p < 0.0001, compared with mTau of the vehicle-treated; ##p < 0.01, ###p < 0.001, ####p < 0.0001, compared with hTau of the vehicle-treated; §§§§p < 0.0001, compared with oligomer of the vehicle-treated; n.s., nonsignificant.

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