The effects of MLC901 on tau phosphorylation

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Tauopathies are neurodegenerative diseases that are characterized by the presence of hyperphosphorylated tau-containing neurofibrillary tangles (NFTs) in the brain and include Alzheimer's disease and frontotemporal dementia, which lack effective disease-modifying treatments. The presence of NFTs is known to correlate with cognition impairment, suggesting that targeting tau hyperphosphorylation may be therapeutically effective. MLC901 is a herbal formulation that is currently used in poststroke recovery and consists of nine herbal components. Previously, several components of MLC901 have been shown to have an effect on tau phosphorylation, but it remains unknown whether MLC901 itself has the same effect. The objective of this study was to assess the effects of MLC901 on ameliorating tau phosphorylation at epitopes associated with NFT formation. A stably transfected cell culture model expressing tau harboring the P301S mutation was generated and treated with various concentrations of MLC901 across different time points. Tau phosphorylation profiles and protein levels of enzymes associated with tau phosphorylation were assessed using western blotting. One-way analysis of variance with Bonferroni post-hoc analysis showed that MLC901 significantly reduced tau phosphorylation at epitopes recognized by the AT8, AT270, and PHF-13 antibodies. MLC901 also induced a significant increase in the s9 phosphorylation of glycogen synthase kinase 3β and a concurrent decrease in the activation of cyclin-dependent kinase 5, as measured by a significant decrease in the levels of p35/cyclin-dependent kinase 5. Our results provide supporting evidence to further study the effects of MLC901 on tau pathology and cognition using mouse models of tauopathy. *NeuroReport* **00:000–000**

NeuroReport 2017, **00**:000–000

Keywords: MLC901, NeuroAiD, tau phosphorylation

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Received 3 May 2017 **accepted** 3 August 2017

Introduction

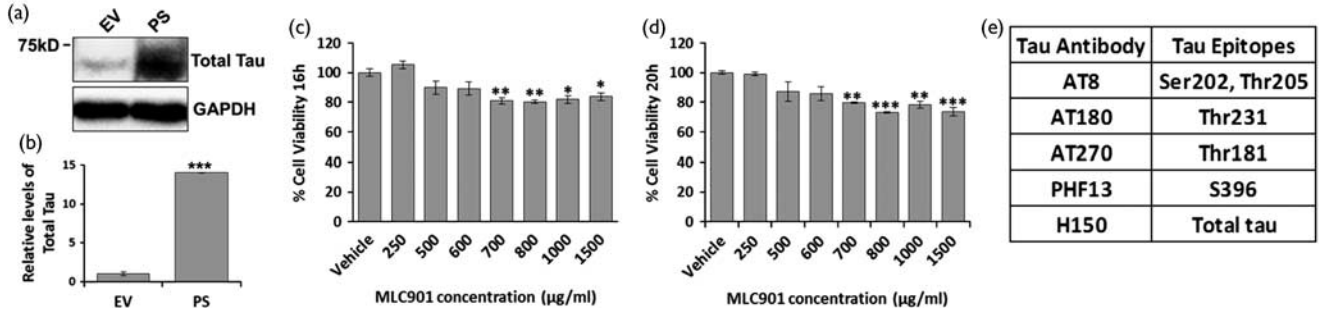
Tauopathies are neurodegenerative diseases that are characterized by the presence of tau-containing neurofibrillary tangles (NFTs) in the brain and include conditions such as Alzheimer's disease (AD) and frontotemporal dementia [1]. Tau is a microtubule-associated protein that is mainly expressed in neurons and its dysfunction contributes toward neuronal cell death [1]. Phosphorylation of tau is governed by a balance between the actions of kinases and phosphatases on tau [1]. In conditions such as AD, the activity and expression of key kinases such as glycogen synthase 3β (GSK3β) and cyclin-dependent kinase 5 (cdk5) are known to be increased [2,3], whereas the opposite is true for key phosphatases such as protein phosphatase-2A (PP2A), promoting the formation of NFTs [4]. It is known that NFT load is strongly associated with cognitive impairment [5], emphasizing the significant role of tau in cognition. This has resulted in the development of various strategies targeting tau that are currently undergoing clinical testing for the treatment of AD [6].

Recently, several clinical trials of various herbs/herbal formulations used in traditional Chinese medicine have been shown to be beneficial in AD patients [6,7], but their underlying mechanisms remain to be elucidated. MLC901 is a second-generation, herbal-only version of MLC601 that is also currently in use for poststroke recovery [8]. MLC901 is made up of nine herbal components in composition per capsule [9]: 0.57 g *Radix astragali*, 0.114 g *Radix salviae miltiorrhizae*, 0.114 g *Radix paeoniae rubra*, 0.114 g *Rhizoma chuanxiong*, 0.114 g *Radix angelicae sinensis*, 0.114 g *Carthamus tinctorius*, 0.114 g *Prunus persica*, 0.114 g *Radix polygalae*, and 0.114 g *Rhizoma acori tatarinowii* (Fig. 1f). MLC901 has previously been shown to exert neuroprotective and neuroregenerative effects in rodent models of stroke [10]. Recently, MLC901 has been shown to improve cognition in normal C57BL/6 mice by promoting hippocampal neurogenesis [9], raising the possibility that it may be useful against cognitive impairment in neurodegenerative diseases.

Previously, some of the components in MLC901 such as tanshinone IIA found in *Radix salviae miltiorrhiza* have been shown to significantly decrease tau hyperphosphorylation through GSK3β, cdk5, and PP2A [11,12]. These findings suggest that MLC901 may also exert an effect against tau hyperphosphorylation, but this remains to be confirmed. Hence, in this study, we assessed the effects of MLC901 on tau phosphorylation using a cell culture model of tauopathy. We hypothesize that MLC901 treatment will decrease tau phosphorylation at

DOI: 10.1097/WNR.0000000000000884

Fig. 1



Tau Antibody	Tau Epitopes
AT8	Ser202, Thr205
AT180	Thr231
AT270	Thr181
PHF13	S396
H150	Total tau

Generation of PS stably transfected cells and assessment of MLC901 concentrations to be used in the study. (a) Expression blot of tau comparing SH-SY5Y cells stably transfected with the shortest isoform of tau harboring the P301S mutation (PS) compared with the empty vector (EV)-transfected cells. (b) Densitometry analysis showed that the PS cells have a significantly increased level of tau higher than the EV-transfected cells. Results are from technical repeats $N = 3$, analysis of variance with Bonferroni post-hoc analysis, $^{***}P < 0.001$. (c) MTT result for 16 h treatment of PS cells with MLC901 shows that concentrations up to 600 µg/ml are not toxic to PS cells. (d) MTT result for 20 h treatment of PS cells with MLC901 shows that concentrations up to 700 µg/ml are not toxic to PS cells. (e) Table summarizing tau antibodies and the tau phosphorylation epitopes that they recognize. Values are normalized against their respective vehicle treatment groups and error bars represent SEM. $N = 3$ independent biological experiments, analysis of variance with Bonferroni post-hoc analysis. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)tetrazolium.

epitopes known to be associated with NFT formation through effects on associated enzymes.

Materials and methods

Cell culture

Human tau encoding full-length cDNA (1N4R isoform) harboring the P301S (PS) mutation was cloned into the TOPO gateway entry vectors (Thermo Fisher Scientific, Waltham, Massachusetts, USA) before sequencing was performed for confirmation. Then, the coding sequence was transferred into the pCDNA6.2/V5-DEST mammalian expression vector (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the LR clonase reaction according to the manufacturer's protocol. The resulting plasmid was transfected into SH-SY5Y human neuroblastoma cells using Lipofectamine LTX (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocols before the selection with 4 µg/ml blasticidin to generate the stably transfected PS cell line. PS cells were maintained in a 1 : 1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Waltham, Massachusetts, USA) and Ham's F12 nutrient mix (Life Technologies) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 100 U/ml penicillin/streptomycin (Life Technologies), and 2 µg/ml blasticidin as the selection antibiotic (Life Technologies).

MLC901 treatment preparation

MLC901 treatment stocks were prepared as described previously [13,14]. Briefly, MLC901 was added to double-distilled water to a final concentration of 50 mg/ml, vortexed for 1 min, and incubated for 1 h at 37°C, then centrifuged at 1000*g* for 3 min and filtered through a 0.22 µm filter just before cell treatment.

Cell viability assay

PS cells were treated with increasing concentrations of MLC901 across 16 and 20 h before a cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)tetrazolium (MTT) was performed as per the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland).

Cell lysis and protein extraction

Cell lysates were obtained by lysing treated cells in cold RIPA buffer (Sigma–Aldrich, Missouri, USA) supplemented with complete EDTA-free protease inhibitor and PhosSTOP (both from Roche, Basel, Switzerland) according to the manufacturer's protocols, sonicated for 10×1 s pulses using the Vibra-Cell VCX 130 ultrasonic processor (Sonics & Materials, Newton, Connecticut, USA), and centrifuged at 20 000*g* for 20 min at 4°C. The supernatant was retrieved and protein levels were determined using the DC protein assay reagent (Bio-Rad, California, USA).

Western blotting

20 µg of proteins was separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes, blocked with 5% BSA in TBS containing 0.1% Tween-20 at room temperature for 1 h before incubation with the respective primary antibodies overnight at 4°C. Primary antibodies against various tau phosphorylation epitopes known to occur in AD were all used at a 1 : 1000 dilution in 5% BSA and are as follows: AT8 that recognizes Ser202 and Thr205 ptau epitopes (#1020; Thermo Fisher Scientific, Waltham, Massachusetts, USA), AT180 that recognizes Thr231 ptau epitope (#MN1040; Thermo Fisher Scientific, Waltham, Massachusetts, USA), PHF-13 that recognizes S396 ptau epitope (#9632; Cell Signaling

Technology, Danvers, Massachusetts, USA), AT270 that recognizes Thr181 ptau epitope (#MN1050; Thermo Fisher Scientific, Waltham, Massachusetts, USA), H-150 for total tau (sc-5587 Santa Cruz Biotechnology, Santa Cruz, California, USA), GSK-3β (S9) (pS9 GSK-3β #9336; Cell Signaling Technology, Danvers, Massachusetts, USA), GSK-3β (Y216) (pY216 GSK-3β Ab75745 Abcam, Cambridge, UK), total GSK-3β (#9836; Cell Signaling Technology, Danvers, Massachusetts, USA), p35 (C-19) (sc-820; Santa Cruz Biotechnology, Santa Cruz, California, USA), and cdk5 (#2506; Cell Signaling Technology, Danvers, Massachusetts, USA) and PP2A α + β antibody (Y119) (Ab32141; Abcam, Cambridge, UK). Blots were washed and incubated with horseradish peroxidise-conjugated secondary antibodies, before visualizing with Luminata Horseradish Peroxidise Chemiluminescence Substrate (Millipore, Billerica, Massachusetts, USA), and bands were detected. Blots were then stripped in freshly prepared 0.2 M NaOH for 10 min at room temperature, before incubating with the GAPDH antibody (G8795; Sigma–Aldrich) as a loading control. Densitometry bands were quantified using ImageJ (NIH).

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was carried out using SPSS (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance with Bonferroni post-hoc correction was carried out and *P* values less than 0.05 were considered statistically significant.

Results and discussion

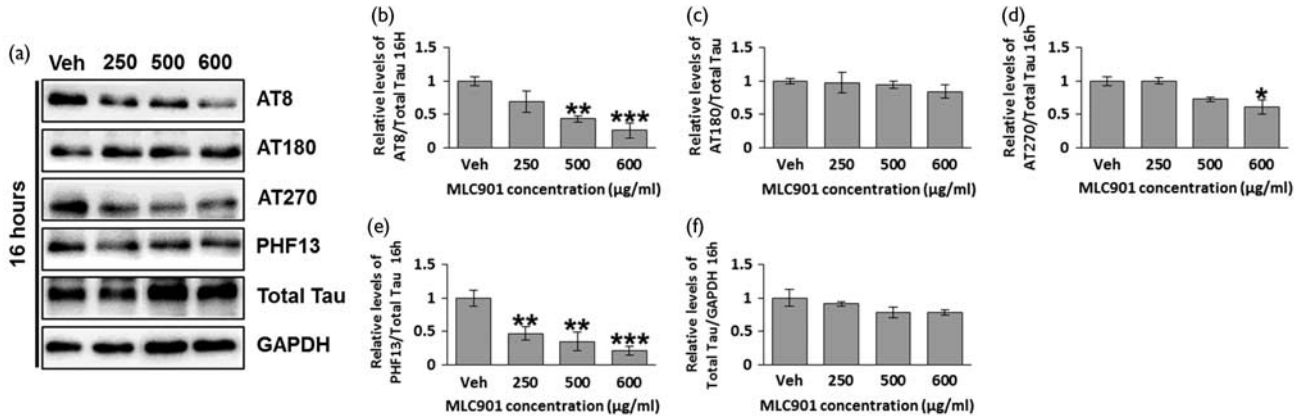
To study the effect of MLC901 on tau phosphorylation at epitopes known to be associated with NFT formation, we first generated stably transfected PS cells (Fig. 1a), which were found to significantly overexpress the P301S tau mutation (Fig. 1b). The overexpression of the P301S tau mutant is a widely used and well-established method to induce tau hyperphosphorylation in in-vitro and in-vivo models, and has been proposed as a robust system for screening treatments against tau phosphorylation at epitopes associated with NFT formation [15].

To determine the concentration range of MLC901 to be used in this study, we treated PS cells with a range of MLC901 concentrations and assessed their cell viabilities using the MTT assay at 16 (Fig. 1c) and 20 h time points (Fig. 1d). The time points in this study were chosen on the basis of previous in-vitro studies where components of MLC901 were found to exert neuroprotective effects [16]. Our results showed that MLC901 concentrations up to 600 μg/ml were not significantly different from the vehicle treatment at 16 h (Fig. 1c) as well as at 20 h (Fig. 1d). Therefore, the nontoxic concentrations of 250, 500, and 600 μg/ml were chosen for all subsequent experiments in this study to assess the effects of MLC901 on tau phosphorylation. PS cells were then treated with the selected concentrations of MLC901 for 16 (Fig. 2) and 20 h (Fig. 3) and analyzed using western blotting. The ranges of tau phosphorylation epitopes chosen for this study (those identified by AT180, AT270, AT8, and PHF-13) (Fig. 1e) have all previously been shown to be associated with NFT formation in AD brains [17].

MLC901 treatment for 16 h induced a significant decrease in the levels of tau phosphorylation at epitopes detected by AT8, AT270, and PHF-13 (Fig. 2a, b, d, e), but AT180 remained unchanged (Fig. 2c). Previously, phosphorylation of AT180 has been shown to be transient [18], and may explain why we did not detect any changes for AT180, but this remains to be investigated in future studies at earlier time points. Total tau levels also remained unchanged (Fig. 2f), indicating that MLC901 has no effect on tau protein expression. Treatment with MLC901 for 20 h (Fig. 3a) induced a significant sustained decrease in the phosphorylation levels at AT270 (Fig. 3d) and PHF-13 (Fig. 3e), whereas the other epitopes did not differ significantly from the vehicle treatment (Fig. 3b and c). Total tau levels also remained unaltered (Fig. 3f). We did not observe any tau oligomer formation in this study. We also did not observe any obvious differences in tau phosphorylation staining patterns or tau phosphorylation distribution even at the highest MLC901 concentrations at any time point assessed in this study. Collectively, the results indicate that MLC901 has effects on tau phosphorylation as the results from both time points assessed in this study indicate that tau treatment with MLC901 significantly decreased tau phosphorylation levels at the epitopes detected by AT8, AT270, and PHF-13, but had no effect on the epitope detected by AT180. Previously, different tau phosphorylation epitopes have been shown to be correlated with the severity of AD [17]. Specifically, AT180 was observed to appear in the pretangle stage, followed by AT270 in intraneuronal NFTs and then AT8 and PHF-13 in the extracellular NFTs in AD. In our study, MLC901 induced a sustained decrease in epitopes recognized by AT270 and PHF-13 across both time points assessed, but had no effect on the AT180 at any of the time points assessed. This indicates that MLC901 may have a more pronounced effect on 'later' tau phosphorylation epitopes, which has implications for its potential for use in tau-mediated cognitive decline.

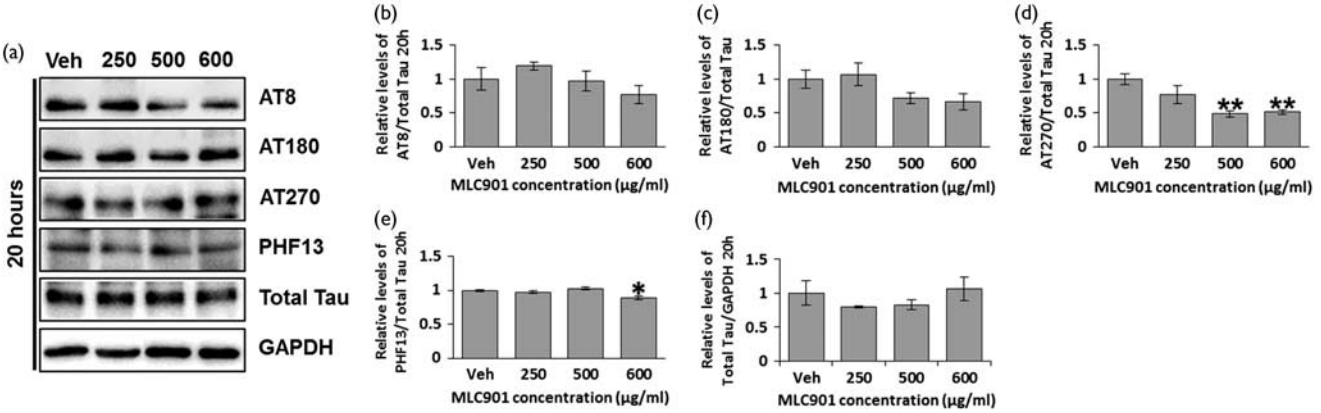
Previously, several components of MLC901 were found to induce GSK3β phosphorylation at the s9 epitope (thereby decreasing its activity), leading to a decrease in the levels of tau phosphorylation at Ser202 recognized by AT8 [19]. We found that MLC901 induced significant increases in the levels of s9 pGSK3β across both 16 (Fig. 4a and b) and 20 h (Fig. 5a and b), but did not alter the phosphorylation of Y216 of GSK3β (Figs 4c and 5c), confirming previous studies [19]. We observed a downregulation of total GSK3β levels at both time points, which is known to lead

Fig. 2



The effects of MLC901 on tau phosphorylation in PS cells treated with MLC901 for 16 h. (a) Representative blots of PS cells treated with MLC901 at 16 h. Quantification of western blots show that MLC901 induces a significant decrease in levels of tau phosphorylation recognized by (b) AT8, (d) AT270, and (e) PHF-13, but not (c) AT180, compared with their respective vehicle treatments. (f) Total tau remained unchanged. Values are normalized against their respective vehicle treatment groups and error bars represent SEM. $N=3$ independent biological experiments, analysis of variance with Bonferroni post-hoc analysis. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Cyclin-dependent kinase 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK, glycogen synthase kinase 3β; PP2A, protein phosphatase-2A.

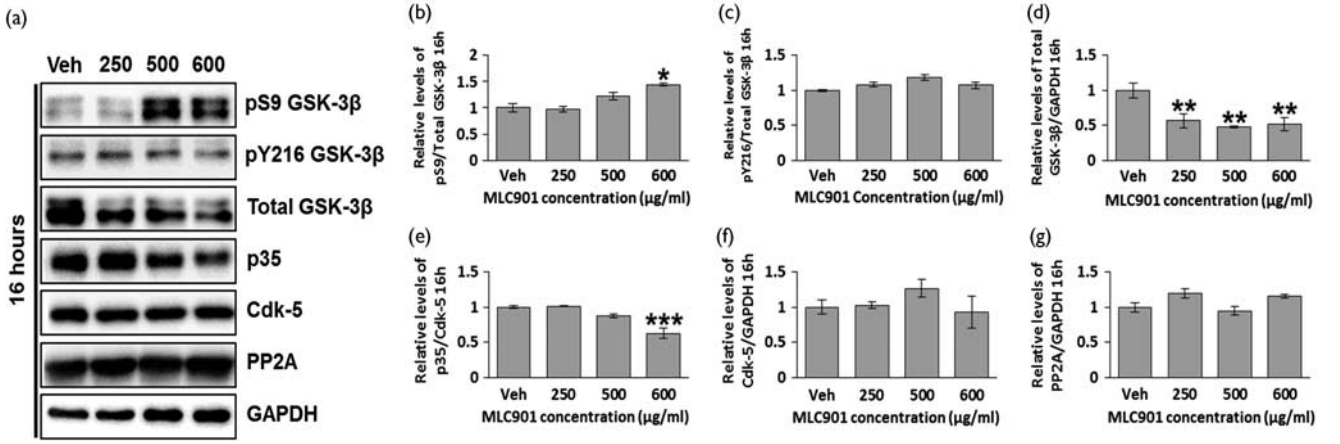
Fig. 3



The effects of MLC901 on tau phosphorylation in PS cells treated with MLC901 for 20 h. (a) Representative blots of PS cells treated with MLC901 at 20 h. Quantification of western blots show that MLC901 induces a significant decrease in the levels of tau phosphorylation recognized by (d) AT270 and (e) PHF-13, but not (b) AT8 or (c) AT180, compared with their respective vehicle treatments. (f) Total tau remained unchanged. Values are normalized against their respective vehicle treatment groups and error bars represent SEM. $N=3$ independent biological experiments, analysis of variance with Bonferroni post-hoc analysis. $^{*}P<0.05$; $^{**}P<0.01$. Cyclin-dependent kinase 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK, glycogen synthase kinase 3β; PP2A, protein phosphatase-2A.

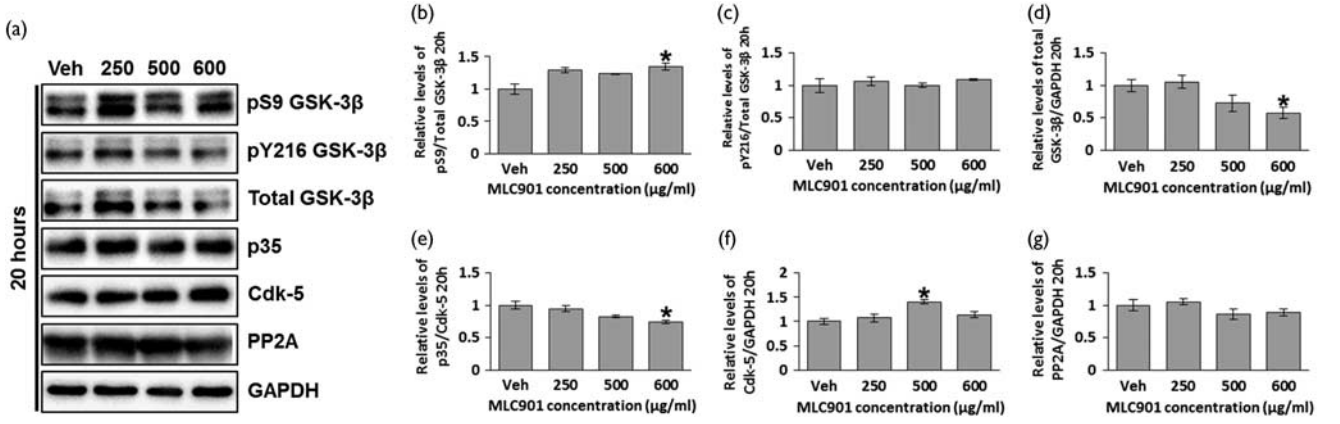
to a decrease in GSK3β activity during neuronal differentiation [20]and in line with the known neuroprotective properties of MLC901 [10]. Together, the results suggest that GSK3β is a target of MLC901. Cdk5 and PP2A have also been shown to play a role in the phosphorylation of tau epitopes recognized by AT8, AT270, and PHF-13 [21]. The activity of Cdk5 has been shown to be regulated by the levels of p35 and also its cleavage product p25 [22]. We observed that MLC901 induced significant decreases in the levels of p35/cdk5 (Figs 4e and 5e), but not total cdk5 levels, except for one treatment concentration at 20 h (Figs 4f and 5f). Decreasing levels of p35/cdk5 have been shown to promote neurogenesis [23] and supports the known neuroproliferative effects of MLC901 [10]. We did not observe an accumulation of p25 in our model and we postulate that this may be because of the short time points in our experimental setup. However, the relationship between Cdk5, p35, and p25 is known to be complex, and warrants investigation in future follow-up studies to ascertain the effects of MLC901 on the regulation of Cdk5 activity. Finally, the expression levels of PP2A alpha and beta catalytic (PP2AC) subunit isoforms were not altered

Fig. 4



The effect of MLC901 on GSK3β, cdk5 and PP2A in PS cells treated with MLC901 for 16 h. (a) Representative blots of PS cells treated with MLC901 for 16 h. Quantification of western blots show that MLC901 induced a significant increase in (b) s9 phosphorylation of GSK3β, and significant decreases in (d) total levels of GSK3β and (e) p35/cdk5. No significant changes were observed for (c) Y216 phosphorylation of GSK3β, (f) total levels of cdk5 and (g) levels of PP2A catalytic subunit. Values are normalized against their respective vehicle treatment groups and error bars represent SEM. $N=3$ independent biological experiments, analysis of variance with Bonferroni post-hoc analysis. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Cyclin-dependent kinase 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK, glycogen synthase kinase 3β; PP2A, protein phosphatase-2A.

Fig. 5



The effect of MLC901 on GSK3β, cdk5, and PP2A in PS cells treated with MLC901 for 20 h. (a) Representative blots of PS cells treated with MLC901 for 20 h. Quantification of western blots show that MLC901 induced a significant increase in (b) s9 phosphorylation of GSK3β and (f) total cdk5 levels, and significant decreases in (d) total levels of GSK3β and (e) p35/cdk5. No significant changes were observed for (c) Y216 phosphorylation of GSK3β and (g) levels of the PP2A catalytic subunit. Values are normalized against their respective vehicle treatment groups and error bars represent SEM. $N=3$ independent biological experiments, analysis of variance with Bonferroni post-hoc analysis. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Cyclin-dependent kinase 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK, glycogen synthase kinase 3β; PP2A, protein phosphatase-2A.

at both time points assessed in this study (Figs 4g and 5g). Hence, our results suggest that the effect of MLC901 on tau phosphorylation may be more dependent on its action on GSK3β and cdk5 rather than on PP2A, which should be assessed in future animal studies.

The results of this study indicate that MLC901 treatment significantly decreased tau phosphorylation and also inhibited kinase activities of GSK3β and cdk5, albeit at two time points, on the basis of previous in-vitro studies that assessed components found in MLC901 [16]. The time points chosen for this study are also in line with previous research showing that components of MLC901 that have effects on tau phosphorylation such as tanshinone IIA and Danshenshu have short half-lives and/or a short duration of action [24,25]. Our results nevertheless showed that MLC901 has an effect on tau phosphorylation, and raises the need to explore additional time points and dosing regimens.

As MLC901 is a mixture, it is also not clear which active compounds within MLC901 are mediating the tau changes in our study. Nonetheless, our results confirm previous studies that components of MLC901 exert anti-tau phosphorylation effects by inhibiting GSK3β [12] and cdk5 [11]. Hence, future studies could focus on those MLC901 components and their readouts on kinases as a starting point to assess efficacy with in-vivo models. The results from this study provide supporting data to assess kinases such as GSK3β and cdk5 in future follow-up studies to further investigate the effects of MLC901 on tau-mediated cognitive impairment in animal models of tauopathy.

Acknowledgements

This work was supported by grants from the National Medical Research Council to C.C.L.H. and L.Y.A. (NMRC/CG/NUHS/2010-R-184-005-184-511) and also from Moleac Pte Ltd to L.Y.A. The funders played no role in study design, data collection, and analysis, the decision to publish, or preparation of the manuscript. Moleac Pte Ltd is marketing NeuroAiD II and NeuroAiD II (MLC 901) for the treatment of stroke (*http://www.moleac.com/products/neuroaid-ii-mlc901/*).

Conflicts of interest

There are no conflicts of interest.

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