NeuroAiD® (MLC601) and Amyloid Precursor Protein Processing

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Key Words

Alzheimer's disease · Stroke · Amyloid precursor protein · MLC601 · NeuroAiD

Abstract

Background: Amyloid precursor protein (APP) undergoes cleavage under physiological conditions, predominantly by α- and γ-secretases, to form the nonpathogenic sAPPα and p3 fragments. By contrast, amyloid-beta (Aβ) is produced via proteolytic cleavage by β- and γ-secretases. In Alzheimer's disease (AD), APP is preferentially processed via the amyloidogenic pathway, producing large amounts of Aβ that form the major constituent of senile plaques and tau-containing neurofibrillary tangles. Similarly, stroke patients have a higher level of Aβ around the area of infarct, suggesting that Aβ may mediate at least some of the secondary neurotoxicity observed in stroke patients. **Methods:** To investigate the effects of MLC601 (NeuroAiD®) on regulation of APP processing, the human neuroblastoma cell line SH-SY5Y was used for all experiments. Stocks of MLC601 were prepared at a final concentration of 50 mg/ml. Cells were treated with different concentrations of MLC601 before assessing changes in the levels of released lactate dehydrogenase (LDH), full-length APP and secreted sAPPα. **Results:** Concentrations of MLC601 between 1 and 1,000 µg/ml significantly lowered the levels of LDH released into the media when compared to control cells. In contrast, MLC601 concentrations at 5,000 and 10,000 µg/ml resulted in a significant increase in the LDH release. Treatment with 100, 500 and 1,000 µg/ml of MLC601 significantly increases the levels of sAPPα secreted by SH-SY5Y into the media. Treatment with 1,000 µg/ml of MLC601 significantly decreased the levels of full-length APP. **Conclusion:** MLC601 is a possible modulator of APP processing and has implications as a putative therapeutic strategy for the treatment of poststroke dementia and AD.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that is characterized by the deposition of amyloid-beta (Aβ) and tau-containing neurofibrillary tangles. AD consists of the majority of all cases of dementia [1]. Currently, over 36 million people worldwide suffer from AD, with the associated annual cost at a staggering USD 604 billion [2]. Both figures are expected to increase exponentially with the increasing ageing global population, which presents a huge social and economic burden. Current treatments for AD provide limited temporary symptomatic relief while an effective disease-modifying treatment for this devastating disease remains to be discovered [1]. It is widely acknowledged that Aβ plays a central role in the pathogenesis of AD since the presence of Aβ plaques is necessary for the diagnosis of AD. In addition, Aβ has been shown in vitro [3] and in vivo [4] to exert toxic effects on a wide range of AD disease models via the hyper-

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Fig. 1. The regulated intramembrane proteolysis of β-APP. In the nonamyloidogenic pathway, APP is cleaved by α- and γ-secretase, generating a large soluble APP fragment (sAPPα), which is released extracellularly. The nonamyloidogenic pathway also generates the p3 and APP intracellular domain (AICD). In the amyloidogenic pathway, APP is cleaved by β- and γ-secretases, leading to the generation of the neurotoxic Aβ fragment, which is released extracellularly, and a small intracellular fragment (AICD).
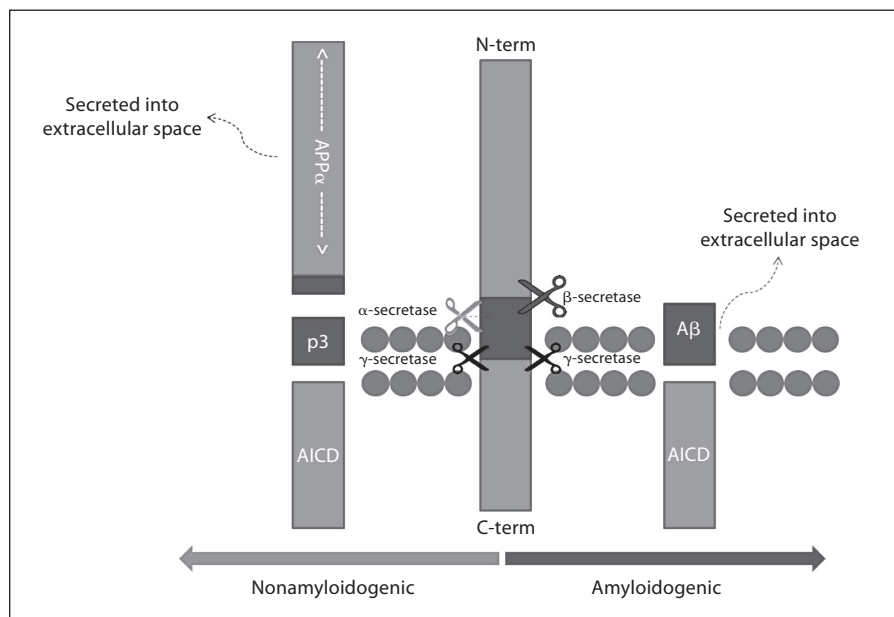
phosphorylation of tau to form neurofibrillary tangles, which have been found to correlate closely with disease progression [5]. However, the exact conditions precipitating Aβ accumulation and subsequent deposition in the brain remain largely unknown.

During AD, Aβ is cleaved from its precursor, amyloid precursor protein (APP), by β- and γ-secretases. APP is a transmembrane protein that undergoes cleavage by various secretases (fig. 1). It is known that under physiological conditions, APP is processed predominantly by α- and γ-secretases to form the neuroprotective sAPPα, which is secreted into the extracellular space [6]. However, for unknown reasons, the protein level expression and activity of β-secretases is significantly increased during AD, which leads to the increase in the production of toxic Aβ peptides [7]. This leads to a corresponding decrease of the levels of sAPPα, since total APP levels have not been observed to change significantly in AD [8]. Indeed, previous research has suggested that enhancing α-secretase activity may actually be a putative therapeutic strategy for the treatment of AD [8–10].

Recently, analysis of poststroke animal models showed that APP and Aβ are deposited at the border of the ischemic region [11], suggesting that stroke may promote the aggregation of APP and Aβ. In addition, stroke patients have been previously found to have a significantly higher risk of developing AD [12]. It is widely known that excitotoxicity due to an excessive cellular calcium influx is a major consequence of stroke [13]. Importantly, research

has shown that calcium accelerates Aβ aggregation [14, 15]. Given that Aβ is known to be neurotoxic, current research suggests that poststroke neurotoxicity may, at least in part, be mediated by Aβ.

NeuroAiD®, also known as MLC601, is a current treatment used to promote recovery and rehabilitation of stroke patients [16]. NeuroAiD is a standardized traditional Chinese medicine formulation remedy that contains 9 herbal and 5 animal extracts (fig. 2). MLC601 is currently given to stroke patients to promote poststroke recovery [17]. Research in various cell and animal models shows that MLC601 has neuroprotective properties and also seems to promote neurogenesis in rodent and human cells [18], suggesting that it may stimulate the brain to repair itself after injury.

Some of the components found within MLC601 have been previously tested to assess their effects on known events that occur along the amyloid cascade (fig. 3). So far, 13 of the components found within MLC601 have been found to have the ability to reverse and prevent events associated with Aβ-induced toxicity [19–45]. Specifically, radix salviae miltiorrhizae, radix paeoniae rubrae, radix angelicae sinensis, radix astragali, rhizoma chuanxiong and *Carthamus tinctorius* have all been shown to ameliorate critical toxic events within the amyloid cascade hypothesis (fig. 3). In contrast, 4 of the components within *Prunus persica* have been found to exacerbate Aβ-associated toxicity [19, 20] (fig. 3).
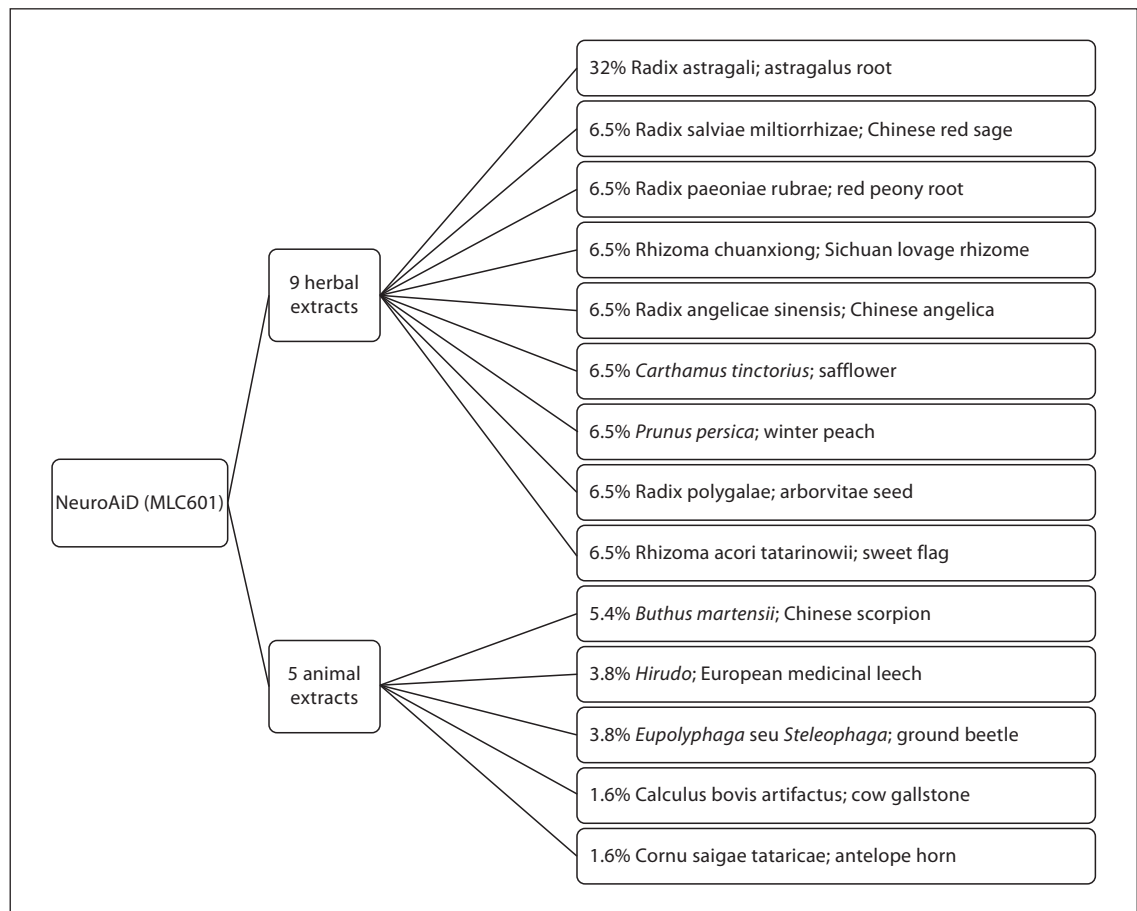
Fig. 2. Composition of NeuroAiD (MLC601). MLC601 contains 9 herbal and 5 animal extracts. Extracts are listed by their binomial name, followed by their common name.

Previously, levels of toxic Aβ peptides were found to be upregulated after stroke [46], suggesting that APP is preferentially processed in the amyloidogenic pathway to produce the toxic Aβ peptide, which may actively contribute to the propagation of brain injury initially induced by stroke. Given that the production of sAPPα is neuroprotective [47] and NeuroAiD is known to have neuroprotective properties, we therefore aimed to investigate the effects NeuroAiD may exert via the regulation of APP processing, which will have implications for the treatment of stroke and AD.

Materials and Methods

Cell Culture and Treatment
The human neuroblastoma cell line SH-SY5Y was used for all experiments. SH-SY5Y expresses wild-type APP and the α-secretase candidates ADAM10 and ADAM17 [48, 49] and is known to se-

crete the α-secretase product sAPPα under basal and stimulated conditions [48]. All cell culture reagents are from Gibco, Invitrogen unless otherwise stated. Cells were grown in growth media (1:1 DMEM/F12), supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine at 37°C, and 5% CO_2 until they were 70% confluent before treatment. Cells were washed once with prewarmed sterile PBS before treating with different concentrations of MLC601 in serum-free DMEM/F12.

Stocks of MLC601 were prepared as previously performed [18]. Briefly, MLC601 was dissolved in ddH$_2$O at a final concentration of 50 mg/ml and incubated at 37°C for 1 h before sterile filtering through a 0.2-μm filter. Aliquots were stored at –20°C until required.

Lactate Dehydrogenase Assay
The lactate dehydrogenase (LDH) assay was chosen to assess the suitable MLC601 nontoxic concentration treatment range to use within this system. Cell viability was assessed after the 12-hour MLC601 treatment using the Cytotoxicity Detection Kit PLUS (LDH) from Roche and performed according to the manufacturer's protocols. Briefly, cells were treated with different concentrations of MLC601 and their conditioned media was harvested for use to assess the levels of LDH released.
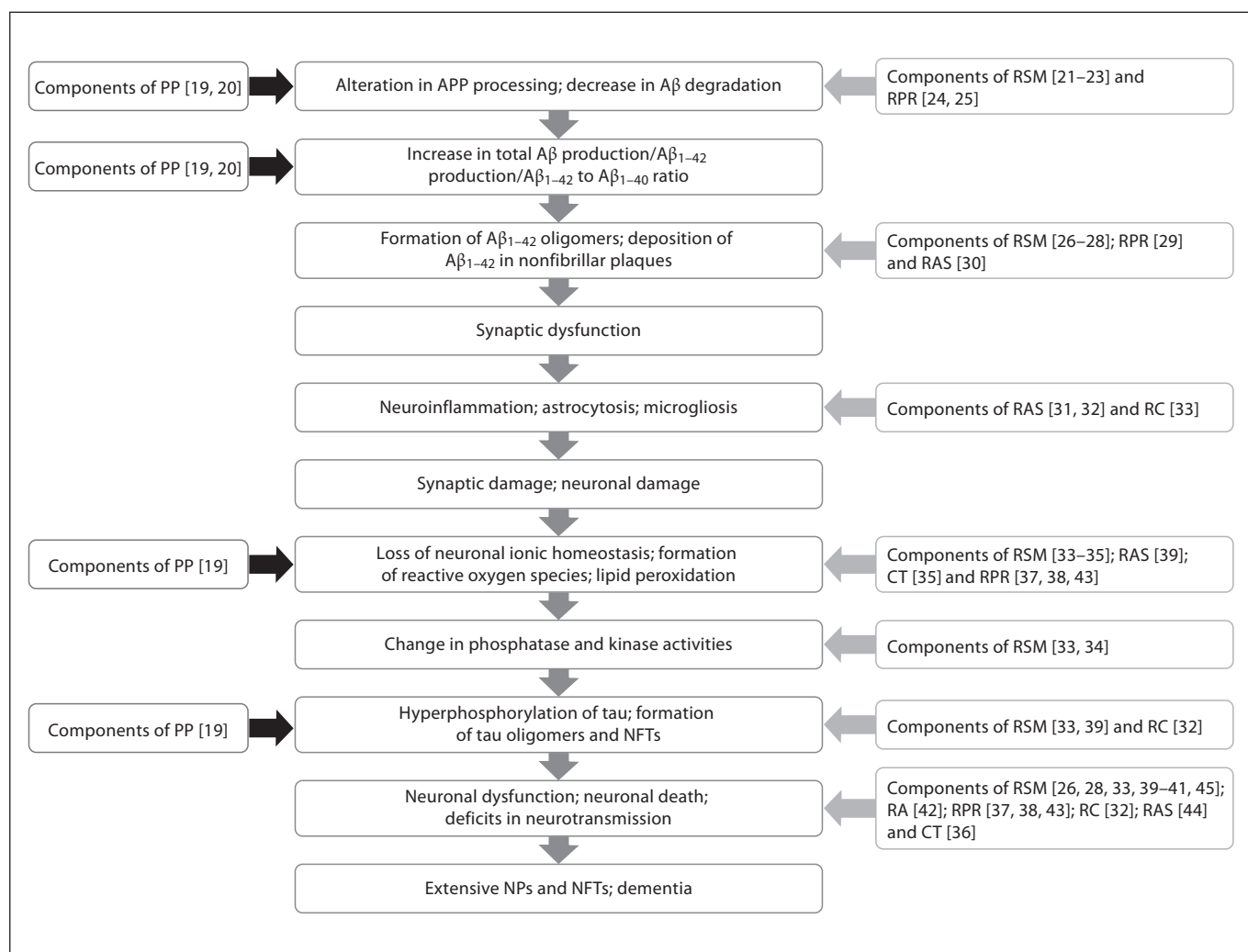
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| Components of PP [19, 20] | → | Alteration in APP processing; decrease in Aβ degradation | ← | Components of RSM [21–23] and RPR [24, 25] |

| Components of PP [19, 20] | → | Increase in total Aβ production/Aβ$_{1-42}$ production/Aβ$_{1-42}$ to Aβ$_{1-40}$ ratio |

Formation of Aβ$_{1-42}$ oligomers; deposition of Aβ$_{1-42}$ in nonfibrillar plaques ← Components of RSM [26–28]; RPR [29] and RAS [30]

Synaptic dysfunction

Neuroinflammation; astrocytosis; microgliosis ← Components of RAS [31, 32] and RC [33]

Synaptic damage; neuronal damage

Components of PP [19] → Loss of neuronal ionic homeostasis; formation of reactive oxygen species; lipid peroxidation ← Components of RSM [33–35]; RAS [39]; CT [35] and RPR [37, 38, 43]

Change in phosphatase and kinase activities ← Components of RSM [33, 34]

Components of PP [19] → Hyperphosphorylation of tau; formation of tau oligomers and NFTs ← Components of RSM [33, 39] and RC [32]

Neuronal dysfunction; neuronal death; deficits in neurotransmission ← Components of RSM [26, 28, 33, 39–41, 45]; RA [42]; RPR [37, 38, 43]; RC [32]; RAS [44] and CT [36]

Extensive NPs and NFTs; dementia

Fig. 3. Effect of MLC601 components on events in the Aβ cascade. Blue (color in online version only) boxes indicate the cascade of neurochemical, structural and functional changes that culminate in the characteristic neuropathological features and symptoms of AD [modified from 51]. Green boxes indicate beneficial effects that reverse or prevent Aβ-induced pathologies. Red boxes indicate adverse effects that induce AD-like pathologies. NFT = Neurofibrillary tangles; NPs = neuritic plaques; RSM = radix salviae miltiorrhizae; RPR = radix paeoniae rubrae; RAS = radix angelicae sinensis; PP = *Prunus persica;* RA = radix astragali; RC = rhizoma chuanxiong; CT = *Carthamus tinctorius* [19–45].

Protein Extraction

Media

For the detection of secreted sAPPα, the conditioned medium was collected and centrifuged at 2,000 *g* for 10 min at 4°C to pellet debris and detached cells. The supernatant for each treatment was transferred to a clean Vivaspin-500 centrifugal concentrator tube (Sartorius Stedim Biotech, Surrey, UK) and centrifuged at 15,000 *g* for 15 min at 4°C. The filtrate was discarded and residual fluid was washed with ddH$_2$O and centrifuged again at 15,000 *g* for 15 min at 4°C. The residual sample was mixed with 25 μl Laemmli sample buffer containing 2-mercaptoethanol and boiled for 5 min to reduce and denature proteins. 40 μl of supernatant aliquots were resolved on 10% glycine gels.

Cells

Cell monolayers were lysed with hot Laemmli sample buffer containing 2-mercaptoethanol (200 μl/well) and lysates were boiled for 5 min. Then, 25 μl of cell lysate per treatment group was loaded onto 10% glycine gels and proteins were transferred onto nitrocellulose membranes using the iBlot dry blotting system (Invitrogen).

Western Blot Analysis

Levels of sAPPα and full-length APP were assessed by Western blot analysis; β-actin was used as a loading control.

Blots were incubated for 1 h at room temperature in 5% nonfat dry milk in PBS containing 1% Tween-20 (PBST) before incubating with 22C11 (dilution 1:1,000; Millipore, Billerica, Mass.,
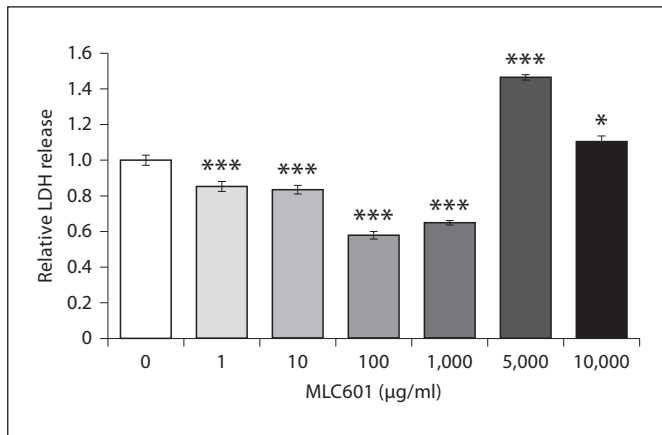
Fig. 4. Effect of MLC601 on LDH release in SH-SY5Y cells. SH-SY5Y cells were treated with increasing concentrations of MLC601 (0–10,000 µg/ml) for 12 h in serum-free DMEM/F12. ANOVA and Bonferroni post hoc analyses revealed that concentrations of MLC601 between 1 and 1,000 µg/ml significantly lowered the levels of LDH released into the media when compared to control cells (0 µg/ml), while MLC601 concentrations at 5,000 and 10,000 µg/ml resulted in a significant increase in the LDH release. Results were obtained from four independent experiments, performed in triplicate and are expressed as mean ± SEM. * $p < 0.05$; *** $p < 0.001$.

USA), which recognizes residues 66–81 of APP. Blots were then incubated for 1 h at room temperature with the relevant horseradish peroxidase-conjugated secondary antibodies (1:5,000; Jackson ImmunoResearch, Reston, Va., USA) diluted in 5% nonfat dry milk in PBST. Blots were extensively washed in PBST before and after antibody incubation to remove residual antibody. Luminata horseradish peroxidase substrate (Millipore) was added to blots and chemiluminescence was detected using the Alliance 4.7 digital imaging system (Uvitec, Cambridge, UK). Quantitative analysis was performed using UVIband software (Uvitec). Subsequently, blots were stripped for 15 min using Re-Blot Plus Strong Solution (Millipore) and reprobed with mAβ anti-β-actin (1:5,000; Sigma-Aldrich, St. Louis, Mo., USA) for normalization of protein loading. Data are expressed as a percentage of immunoreactivity in control groups, which was calculated as a ratio of the band densities of MLC601-treated groups to that of respective control groups.

Statistical Analyses

Results correspond to between three and five independent experiments. Data are expressed as mean ± SEM. Statistical analyses were performed using SPSS 13.0 software and graphs were generated using GraphPad Prism 3.0. One-way analysis of variance (ANOVA) was performed. Post hoc analyses were performed with the Bonferroni test. p values <0.05 were considered statistically significant.

Results

Concentrations of MLC601 up to 1,000 µg/ml Are Not Toxic to SH-SY5Y Cells

To determine the concentration range of MLC601 to use in our system, we assessed the putative effects on LDH release across different concentrations of MLC601 (fig. 4). ANOVA and Bonferroni post hoc analyses revealed that at 12 h of treatment, concentrations of MLC601 between 1 and 1,000 µg/ml significantly lowered the levels of LDH released into the media when compared to control cells (0 µg/ml), while MLC601 concentrations at 5,000 and 10,000 µg/ml resulted in a significant increase in the LDH release ($p < 0.05$ and $p < 0.001$, respectively).

Treatment with MLC601 Significantly Increases the Levels of sAPPα by SH-SY5Y into the Media

Upon determining the suitable concentrations as in the previous section, we proceeded to determine the putative effects that MLC601 may exert on the production of sAPPα, which is secreted into the media. Conditioned media was collected upon treatment of SH-SY5Y cells with various concentrations of MLC601 after 12-hour treatments and Western blotting was performed (fig. 5). We found that levels of secreted sAPPα were significantly increased after 12-hour treatments with 100, 500 and 1,000 µg/ml of MLC601 ($p < 0.01$ each).

Treatment with 1,000 µg/ml of MLC601 Significantly Decreased the Levels of Full-Length APP

Given that sAPPα is produced from full-length APP, there should be a corresponding change in the levels of full-length APP, since significant sAPPα levels were produced and secreted into the media upon exposure to MLC601 (fig. 5). To ascertain whether MLC601 elicits a significant change in levels of full-length APP, SH-SY5Y cells were harvested and proteins were extracted and analyzed by Western blotting (fig. 6). Full-length APP levels were found to be significantly decreased upon treatment with 1,000 µg/ml MLC601 at 12 h.

Discussion

AD is a devastating neurodegenerative disease that is characterized by the deposition of Aβ in the brain parenchyma of patients [1]. Current treatments only provide limited symptomatic treatment, and effective disease-modifying treatments are yet to be discovered. Given the rapidly ageing global population and the exorbitant costs
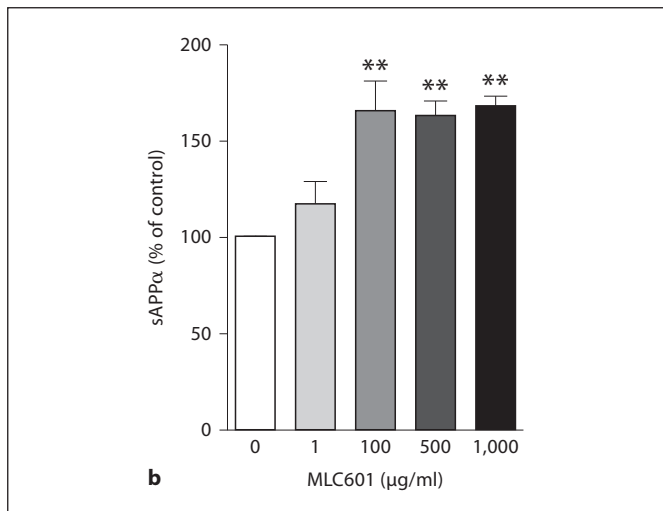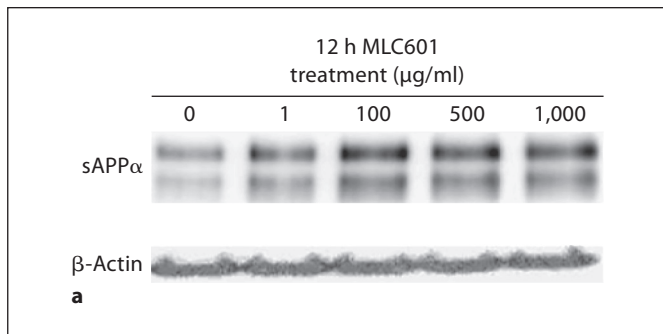
Fig. 5. Effect of MLC601 treatment on sAPPα release from SH-SY5Y cells. **a** Western blot for sAPPα in conditioned media after 12 h incubation with MLC601. Incubation of SH-SY5Y cells with MLC601 at 100, 500 and 1,000 µg/ml induced a significant increase in the levels of sAPPα when compared to control treatment (0 µg/ml MLC601) at 12 h. **b** Quantification of blots in **a**. ANOVA revealed a significant increase in MLC601-treated cells at 12 h ($F_{(4, 10)} = 11.858$, $p = 0.001$). The Bonferroni post hoc test revealed a statistically significant increase in sAPPα release at 12 h in cells treated with MLC601 concentrations of 100, 500 and 1,000 µg/ml compared to control. Data are expressed as a percentage of sAPPα released from control groups after normalization to the loading control β-actin. Results were obtained from three independent experiments and are expressed as mean ± SEM. ** $p < 0.01$.

Fig. 6. Effect of MLC601 treatment on levels of full-length APP (fl-APP) in SH-SY5Y cells. **a** Western blot for full length APP in cells exposed to various concentrations of MLC601 after 12 h of treatment. Proteins extracted from SH-SY5Y cells treated with various concentrations of MLC601 (1–1,000 µg/ml) for 12 or 24 h in serum-free DMEM/F12 were analyzed by Western blotting. **b** Quantification of blots in **a**. Incubation with 1,000 µg/ml of MLC601 significantly decreased the level of full-length APP in cell lysates of SH-SY5Y when compared to the control cells (0 µg/ml MLC601). Data are expressed as a percentage of fl-APP levels in control groups after normalization to the loading control β-actin. One-way ANOVA revealed a significant difference in fl-APP levels between MLC601-treated and control groups at 12 h ($F_{(4, 20)} = 4.354$, $p = 0.011$). Analysis with the Bonferroni post hoc test revealed that levels of fl-APP were significantly decreased in cells treated with 1,000 µg/ml MLC601 compared to control. Results were obtained from three independent experiments and are expressed as mean ± SEM. * $p < 0.05$.

associated with AD, there is an urgent need to develop novel treatments that address its underlying cause. The diagnosis of AD requires the presence of Aβ plaques, which are made up of mostly fibrillary forms of Aβ, which were historically thought to be responsible for the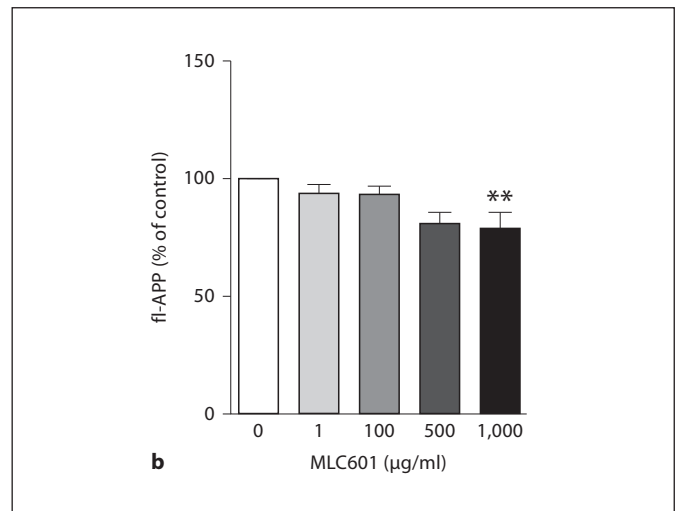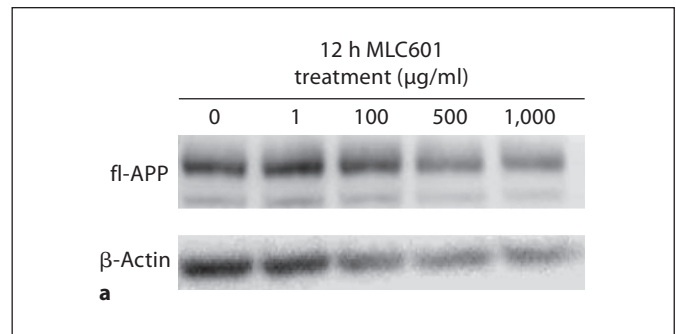 neurotoxicity observed in AD patients [50]. However, recent research has demonstrated that the soluble species of Aβ are the ones exerting significant toxicity using various AD models [50].

A possible approach to decrease the amount of soluble Aβ species is to preclude its formation. As mentioned before, APP can be processed via the nonamyloidogenic and

amyloidogenic pathways (fig. 1), and these pathways are mutually exclusive. The implication of this is that if the nonamyloidogenic pathway is enhanced, then less neurotoxic Aβ will be produced and this may help decrease Aβ-induced neurotoxicity observed in AD.

MLC601 is a traditional Chinese medicine that is currently prescribed to stroke patients and has been found to possess neuroprotective properties while seemingly promoting recovery in stroke patients [16]. It is known that neurotoxic Aβ levels are increased after stroke, suggesting that the presence of stroke may alter APP processing by promoting the amyloidogenic pathway [11]. Since MLC601 is neuroprotective, it is therefore possible that it may exert its neuroprotective effects via the regulation of APP processing.

We found that MLC601 at concentrations of up to 1,000 µg/ml significantly decreased the levels of LDH release (fig. 4). The absence of an increase in LDH levels indicates that the MLC601 concentrations used in this study are not toxic to SH-SY5Y cells. In addition, the observation that MLC601 actually decreased LDH release suggests that cells treated with MLC601 are actually more viable than control cells, which is in line with previous findings that found MLC601 to be neuroprotective [18]. However, the putative mechanisms by which MLC601 may stimulate cell survival remain to be investigated.

Using Western blot analyses, we found that MLC601 enhances sAPPα release (fig. 5), suggesting that MLC601 may promote the nonamyloidogenic processing of APP. It is unknown which component within MLC601 is re-sponsible for our observations, but future studies may be done to address that.

We also found that MLC601 decreases full-length APP levels (fig. 6). Interestingly, the MLC601-induced decrease in APP was concurrent with the enhanced sAPPα release at 12 h, suggesting that MLC601 may act through promoting the processing of APP via the nonamyloidogenic pathway. Since this pathway is governed by the activity of α-secretases, future studies should elucidate whether α-secretase activity is responsible for our current observations. Alternatively, MLC601 may directly alter the expression of APP, but that remains to be investigated. Furthermore, the relevance of the results arising from this preliminary study in cell culture to in vivo systems is unknown.

Conclusions

Our study presents MLC601 as a possible modulator of APP processing, which has implications for the treatment of AD. Further studies are needed to assess whether MLC601 is able to attenuate toxic mechanisms associated with AD in vitro and in vivo to present it as a possible treatment strategy for AD.

Disclosure Statement

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NeuroAiD (MLC601) and Amyloid
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