Novel beta-amyloid aggregate inhibitors for Alzheimer's disease

RMS Wong *, HW Li, DWJ Kwong, KKL Yung, Y Ke

KEY MESSAGES

1. Carbazole-based cyanines offer neuroprotection against amyloid-β-induced toxicity; thus, they have a potential role in the treatment of Alzheimer's disease.
2. We designed, synthesised, and screened more than 30 carbazole-based cyanines for effective and potent amyloid-β peptide oligomerisation and aggregation inhibitors.
3. Of these, six carbazole-based cyanines that were non-toxic, brain penetrable, and neuroprotective against amyloid-β-induced toxicity were identified as potential candidates for further clinical development.
4. Mice treated with one neuroprotective carbazole-based cyanine, SLM, showed improvement in cognitive functions, decrease in oligomeric amyloid-β contents and t-tau and p-tau proteins, particularly in the cerebral hippocampal region.

Hong Kong Med J 2019;25(Suppl 5):S26-9
HMRF project number: 01122066

[1] **RMS Wong,** [1] **HW Li,** [1] **DWJ Kwong,** [2] **KKL Yung,** [3] **Y Ke**

[1] *Department of Chemistry, Hong Kong Baptist University*
[2] *Department of Biology, Hong Kong Baptist University*
[3] *School of Biomedical Sciences, The Chinese University of Hong Kong*

* Principal applicant and corresponding author: mswong@hkbu.edu.hk

Introduction

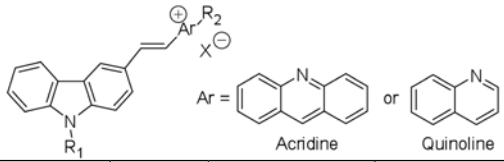
Alzheimer's disease (AD) is the most common form of dementia among older people and causes impairment of many cognitive functions such as memory, thinking, and recognition. More than 47 million people worldwide have AD according to the Alzheimer's Disease International. AD poses tremendous burdens on health care costs and social problems. Although some current AD treatments can improve symptoms, none can stop or reverse its progression. Several approaches that aim to inhibit AD progression have advanced to clinical trials. The most intensely investigated strategies are those targeting the production and clearance of the amyloid-β (Aβ) peptide, which is closely related to its pathogenesis.

One of the pathological hallmarks of AD is the accumulation of amyloid plaques in the brain. Aβ peptides, made up of 40 or 42 amino acids, are the major components of the Aβ plaques found in the brains of AD patients. Aβ peptides play a central role in the disease process, and their aggregates exert cytotoxic effect towards the neurons and initiate the pathogenic cascade, ie, the amyloid cascade hypothesis.[1] Oligomeric, prefibrillar, and diffusible assemblies of Aβ peptides are more neurotoxic.[2] Although there is no consensus on the mechanism for the pathogenic oligomeric assembly, finding brain-penetrating small molecules that can interfere the self-aggregation of Aβ monomers and thus inhibit the formation of the neurotoxic oligomers and the resulting Aβ plaques is an attractive approach to preventing/treating the disease.

In this study, we designed and synthesised a series of novel carbazole-based cyanines to investigate the structure-biological activity properties of these compounds (including bio-viability, blood-brain permeability, neuroprotection against Aβ-induced toxicity, and *in vivo* efficacy) via cell-based and animal model studies in the roles of AD treatment.

Results and discussion

More than 30 carbazole-based cyanines were designed, synthesised, and screened for effective and potent Aβ peptide oligomerisation and aggregation inhibitors as neuroprotective and therapeutic agents for AD treatment. Synthesis of the carbazole-based cyanines was carried out by the Knoevenagel reaction or the Wadsworth-Emmons reaction as the key step.[3] Some of the representative molecules are summarised in Fig 1. The screening of the potential Aβ aggregation inhibitors was performed by direct monitoring of the population and length of the resultant fibrils with total internal reflection fluorescence microscopy as well as SDS-PAGE coupled with the photoinduced crosslinking of unmodified proteins protocol. A general correlation between the binding affinity and the inhibitory potency was demonstrated. Acridinium-based cyanines were found to exhibit a stronger binding association with $A\beta_{(1-40)}$ fibrils (eg, K_d for SAM and SAOH were 11 and 2 μM, respectively), which also accounted for the pronounced and complete



	Ar	R_1	R_2
SAOH	Acridine	$(CH_2CH_2O)_2CH_3$	CH_2CH_2OH
SAM	Acridine	$(CH_2CH_2O)_2CH_3$	CH_3
SLG	quinoline	$(CH_2CH_2O)_2CH_3$	$(CH_2CH_2O)_2CH_3$
Me-SLG	quinoline	CH_3	$(CH_2CH_2O)_2CH_3$
SLNA	quinoline	$(CH_2CH_2O)_2CH_3$	$CH_2CH_2NHCOCH_3$
SLE	quinoline	$(CH_2CH_2O)_2CH_3$	CH_2CH_3
SLM	quinoline	$(CH_2CH_2O)_2CH_3$	CH_3
SLOH-Pr	quinoline	$(CH_2CH_2O)_2CH_3$	$CH_2CH_2CH_2OH$
SLOH	quinoline	$(CH_2CH_2O)_2CH_3$	CH_2CH_2OH
SLAD	quinoline	$(CH_2CH_2O)_2CH_3$	CH_2COONH_2
Me-SLM	quinoline	CH_3	CH_3

FIG 1. Molecular structures of representative carbazole-based cyanines

inhibition on the elongation of the $A\beta_{(1–40)}$ fibrils. This supported the important role of the hydrophobic and π-π stacking interactions being vital to this type of an association and inhibitory effect. The inhibitory mechanism was investigated by circular dichroism. Upon mixing of monomeric $A\beta_{(1–40)}$ with the cyanines, a substantial red spectral shift in the circular dichroism spectra was observed. Such shift indicated the conformational transition of the monomer in the presence cyanine and the formation of the cyanine-Aβ assembly. Hence, an addition of a cyanine to the Aβ peptide monomer could prevent its transition to the amyloidogenic β–sheet-rich conformer, thereby inhibiting its self-aggregation. So far, 18 newly synthesised cyanines have shown effective inhibitory effect on Aβ peptide aggregation.

The cytotoxicities of the new cyanines were evaluated by the MTT assay on human neuroblastoma (SH-SY5Y) cells at different concentrations (10 nM to 50 μM). Generally, the neuronal cells were highly susceptible to the minor change in the chemical structures of these compounds. The LC_{50} of some of the inhibitors were determined in the range of 2-133 μM. This confirmed that these inhibitors exhibited low cytotoxicity to neuronal cells. The neuroprotection effect of these non-toxic cyanines against $A\beta_{(1-40)}$- and $A\beta_{(1-42)}$-induced toxicity was also examined on SH-SY5Y and primary neuronal cells. Only SLE, SLM, SLOH, SLOH-Pr, SLAD, and Me-SLM showed significant neuroprotection in both types of cells, highlighting a potential for further development as neuroprotective and therapeutic agents for AD.

Reactive oxygen species (ROS) have been implicated in premature neuronal cell death in many neurodegenerative diseases. The ability of the inhibitors to reduce Aβ-induced ROS and oxidative stress in neurons was investigated. The ROS level induced by the presence of $A\beta_{(1-40)}$ or $A\beta_{(1-42)}$ in SH-SY5Y, primary cortical, and hippocampal cells was evaluated by the dichlorofluorescein assay. The results showed that the neuroprotective cyanine inhibitors could reduce the ROS-mediated toxicity induced by Aβ species. It is known that the neurotoxic Aβ oligomers can cause overloading of intracellular $[Ca^{2+}]$ leading to cell death. To explore the influence of the cyanines on the $A\beta_{(1-42)}$-induced calcium influx, the change in the calcium concentration in the primary hippocampal cells by an addition of $A\beta_{(1-42)}$ was monitored by fluo-4 AM indicator under confocal laser scanning microscopy. The results revealed that upon an addition of $A\beta_{(1-42)}$, a significant upregulation of $[Ca^{2+}]$ occurred. For primary cells pretreated with the selected cyanines, the uploading of $[Ca^{2+}]$ induced by $A\beta_{(1-42)}$ was readily suppressed. These demonstrated that these cyanines could protect the neuronal cells against Aβ-induced intracellular $[Ca^{2+}]$ influx.

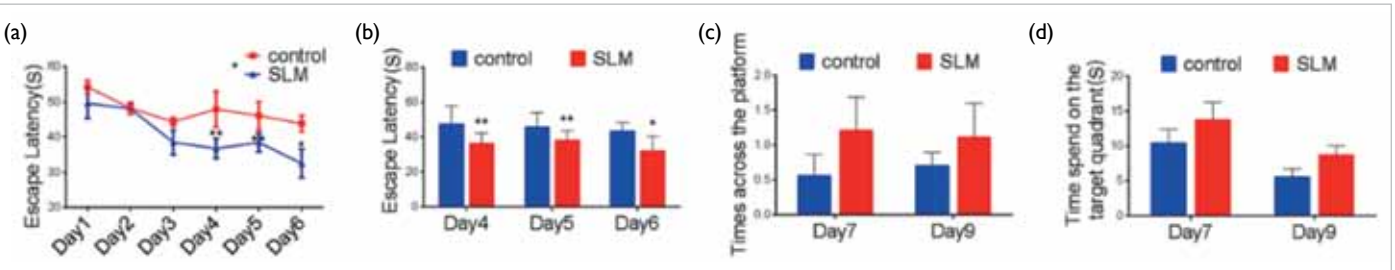


FIG 2. Cognitive improvement by a cyanine compound in the Morris water maze test: the 8-month-old 3×Tg mice expressing APPSwe, TauP301L, and PS1M146 received SLM for 45 d (2.25 mg/kg/d) and evaluated for cognitive function. (a and b) In hidden platform tests, the SLM-treated 3×Tg mice showed a shorter latency to escape onto the hidden platform on days 4-6 ($P<0.05$, $n=12$; $P<0.001$, $n=12$). This suggests that the spatial learning ability of Tg mice improved with SLM treatment. (c) In the probe trial on day 7 and day 9, the SLM-treated Tg mice travelled to the quadrant of the hidden platform significantly more frequently than did the controls. (d) The time spent in the previously learned platform quadrant was also longer for the treated mice on day 7 and day 9. These results suggest that the spatial impairment was attenuated by SLM treatment in Tg mice.

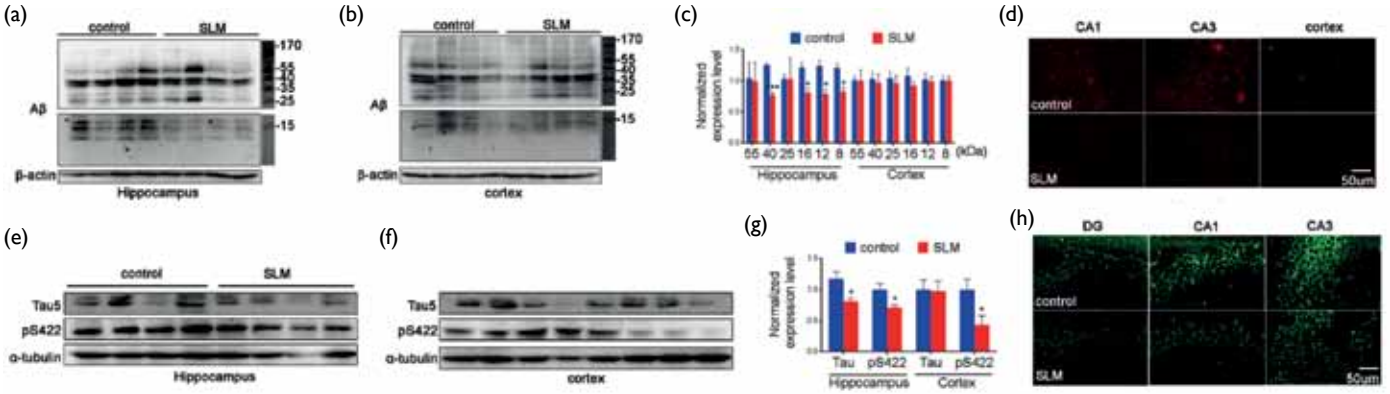


FIG 3. Levels of Aβ and tau in the hippocampal and cortical region of SLM-treated 3×Tg mice were analysed with the western blot and immunofluorescence. The mice were sacrificed. (a to c and e to g) The protein contents of the brain lysates were analysed with the western blot. (d) Brain slices of the hippocampus and the cortex were immunostained using mouse polyclonal antibodies against $A\beta_{(1-42)}$ followed by Alexa Fluor 488-conjugated Fab fragments of goat anti-mouse immunoglobulin. (h) For tau-5 immunofluorescent staining, the brain slices were labelled with a primary antibody specific for Tau5 followed by Alexa Fluor 488-conjugated Fab fragments of goat anti-mouse immunoglobulin. Aβ and tau clearly deposited in the cortex and hippocampus of the non-treated Tg mice, whereas the loading of this deposition was significantly reduced upon SLM-treatment, as demonstrated in the brain slices of the treated Tg mice, which correlated with the results of the western blot.

The blood-brain barrier permeability of these Aβ aggregate inhibitors is important for potential *in vivo* applications. Therefore, the tail-vein injection of the cyanine into normal and transgenic mice was conducted. These cyanines generally exhibited a strong fluorescence signal in the wavelength range of 600-650 nm in brain tissues; therefore, the blood-brain barrier permeability was readily confirmed by the confocal fluorescence images of the cyanine signals. Encouragingly, all the newly developed neuroprotective cyanine inhibitors were found to be blood-brain barrier penetrable.

In addition, the LD_{50} were determined to be in the range of 47 to >125 mg/kg in order to provide a safe dosage of these cyanines for test animals. These findings consistently confirmed that these cyanines are of low acute toxicity in the test animals.

To explore the potential of a neuroprotective cyanine, SLM, on halting or slowing the cognitive impairment, we conducted an animal model study on APPswe/TauP301L/PS1M146 transgenic mice. As these 3×Tg-AD mice showed intracellular Aβ in 3- to 6-month-old mice and cognitive impairment in 6-month-old mice, the 8-month-old 3×Tg-AD mice were treated with SLM for 45 days (2.25 mg/kg/d) intraperitoneally. After the 45-day SLM-treatment, the Morris water maze test was performed. The control group of 3×Tg-AD mice was included in the test for comparison. The treated and non-treated 3×Tg-AD mice were trained for 1 day (4 trials per day). Initially, the two groups showed similar escape latency and similar swimming speed on the first training day, indicating that SLM produced no effect on the vision and motility of the 3×Tg-AD mice. After the vision platform training, the mice were introduced to hidden platform tests for 5 days. In hidden platform tests, the SLM-treated 3×Tg-AD mice exhibited a significantly shorter latency to escape onto the hidden platform during days 4 to 6 than did those of the control group on days 1 to 6 (Fig 2). Furthermore, a probe trial was performed to assess the memory retention of the mice on day 7 and day 9. The SLM-treated 3×Tg mice travelled to the previously learned platform quadrant more frequently than the control ones. The time spent in the previously learned platform quadrant was also longer for the treated 3×Tg mice on day 7 and day 9. These results consistently indicated that the SLM-treated 3×Tg mice showed a significant cognitive improvement after 45 days of treatment, not only enhancing learning skills in the hidden platform test but also improving the spatial memory in the probe trial.

To determine the effect of SLM on Aβ and tau pathologies in 3×Tg AD mice after behavioural testing, the deposition of the Aβ and tau contents in the brain of the SLM-treated Tg mice were determined via multiple biochemical analyses, including the western blot analysis and immunofluorescence. Remarkably, the Aβ content substantially decreased, in particular those of toxic Aβ oligomers with MW of 8, 12, 16, 25, 40, and 55 kD, as did the total tau (tau-5) and phosphorylated-tau (ps422) proteins in hippocampal regions, as compared with those of the controls (Fig 3), suggesting that the cyanine inhibitor can ameliorate both Aβ and tau pathologies in the 3×Tg-AD mouse model. The size of neuritic plaques and the amount of Aβ and tau content were lower than those of the control 3×Tg-AD mice (Fig 3) as visualised by immunostaining in brain slices. In

addition, the level of inactive glycogen synthase kinase-3β (GSK3β) was significantly higher in the SLM-treated Tg mice, likely explaining for the decrease in the phosphorylated tau content.

Increasing evidence implicates that autophagy plays a crucial role in the pathogenesis of AD. Thus, we investigated whether SLM treatment would modulate autophagic pathway by western blot of Tg mouse brains after the aforementioned behavioural studies. It was found that SLM treatment significantly reduced the level of a key gatekeeper of autophagy, mammalian target of rapamycin (mTOR), which was down-regulated by the substantial decrease in an upstream effector, Akt and an increase in the proautophagic protein, mammalian orthologue of yeast Atg6 (Beclin 1) level. In addition, there was a marked reduction in microtubule-associated protein light chain 3-II (LC3-II) level and the LC3-associated protein p62 (sequestosome 1), a marker of autophagic flux also showed a decreasing trend. Meanwhile, the level of the lysosomal protease Cathepsin D (CatD) that mediates the degradation in autophagolysosomes was significantly increased. All these results consistently support that autophagic flux is induced in 3×Tg-AD mice upon SLM treatment resulting in the reduction in amyloid deposits and tau contents as well as improvement of cognitive deficits in the AD mouse model.

Conclusions

The present study represents the first *in vivo* evidence that the carbazole-based cyanines can ameliorate both Aβ and tau pathologies together with reduction in the levels of toxic Aβ oligomers and p-tau protein, representing an important advancement in AD drug development.

Acknowledgements

This work was supported by the Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (#01122066) and Interdisciplinary Research Matching Scheme of Hong Kong Baptist University (RC-IRMS/12-13/01). We thank the support of the National Natural Sciences Foundation of China (No. 81400847), the Young Scientists Fund from the National Science Foundation of China (21205006), and the University Grants Council, Hong Kong SAR (GRF/HKBU201612). The Institute of Molecular Functional Materials was supported by a grant from the University Grants Committee, Hong Kong, Areas of Excellence Scheme (AoE/P-03/08).

References

1. Makin OS, Serpell LC. Structures for amyloid fibrils. FEBS J 2005;272:5950-61.
2. Cohen FE, Kelly JW. Therapeutic approaches to protein-misfolding diseases. Nature 2003;426:905-9.
3. Yang W, Wong Y, Ng OT, et al. Inhibition of beta-amyloid peptide aggregation by multifunctional carbazole-based fluorophores. Angew Chem Int Ed Engl 2012;51:1804-10.