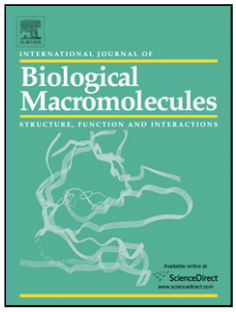

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4- Substituted Sampangine Derivatives: Novel Acetylcholinesterase and β-myloid Aggregation Inhibitors

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Research highlights

- The new sampangine derivatives exhibited high AChE inhibitory activity *in intro*.
- Most of the derivatives exhibited a significant *in vitro* inhibitory activity toward the self-induced Aβ aggregation and Aβ secretion levels of SH-SY5Y cells overexpressing the Swedish mutant form of human β-amyloid precursor protein (APPsw).
- Most of the synthetic sampangine derivatives were predicted to be able to cross the blood-brain barrier to reach their targets in the central nervous system.

Abstract:

A series of 4- substituted sampangine derivatives (4-aminoalkylaminosampangine Ar–NH$(CH_2)_nNR_1R_2$) has been designed, synthesized, and tested for their ability to inhibit acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and β-myloid (Aβ)

aggregation. The synthetic compounds exhibited high AChE inhibitory activity and a significant *in vitro* inhibitory potency toward the self-induced Aβ aggregation. While, treatment of SH-SY5Y cells overexpressing the Swedish mutant form of human β-amyloid precursor protein (APPsw) with derivatives was associated with significant reduction of Aβ42 secretion levels. Moreover, most of the synthetic compounds were predicted to be able to cross the blood-brain barrier (BBB) to reach their targets in the central nervous system (CNS) according to a parallel artificial membrane permeation assay for BBB. The result encourages us to study this class of compounds thoroughly and systematically.

Keywords: Sampangine derivatives; Acetylcholinesterase inhibitors; β-Amyloid aggregation

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disease of the brain that leads to the irreversible loss of neurons and dementia. Over the last few decades, a plethora of targets has been suggested in the attempt to identify the causative factors of neurodegeneration. Cholinesterases (ChEs) were the earliest research target. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), regulate cholinergic neurotransmission, the deterioration of which is responsible for the decline in memory and cognition in patients suffering from AD[1]. Amyloid-β (Aβ) was another vital target. According to the "amyloid hypothesis," one of the major neuropathological hallmarks of AD is the altered production, aggregation, and deposition of Aβ, which results in amyloid plaque formation[2-5]. Therefore, much effort was directed at

developing drugs to inhibit the production, aggregation, or neurotoxicity of Aβ. Indeed, AChE and Aβ aggregation is retained as one of the major pathogenic mechanisms in AD, and since it occurs early in the pathogenesis, it represents an ideal target for intervention[6].

The copyrine alkaloid sampangine (Fig.1) belongs to the aporphine family of alkaloids, which was widely distributed in Annonaceae plants, such as Canangaodorata[7], Cleistophathis patens[8], Duguetia hadrantha[9] and Duguetia hadrantha[10]. Plant-derived sampangine alkaloid shows broad and potent antibacterial[11-14], antifungal[8, 15-19], antimalaria[9], anti-inflammatory[20], antiparasitic[21] and antitumor[10, 22-24] activities. Several A, B-ring substituted and hetero analogues of sampangine were reported in order to enhance their biological activity[14, 19, 25]. In the paper, alkylamino were introduced to sampangine on 4-substituents. A series of 4-aminoalkylaminosampangine derivatives was synthesized. The inhibitory potency toward cholinesterase and Aβ aggregation of these sampangine derivatives were first reported.

2. Experimental methods

2.1 Chemistry

Target compounds **5–12** were synthesized as shown in Scheme1. Preparation of sampangine **3** was carried out by a reported method[11]. Cleistopholine **2** was obtained through the hetero Diels-Alder reaction of quinone **1** with hydrazine. The condensation of **2** with dimethylformamide dimethyl acetal provided sampangine **3**. The electrophilic

halogenation of **3** with pyridinium bromide perbromide in chloroform produced 4-bromosampangine **4** in 51 % yield. Subjection of **4** to a refluxing methanolic solution of sodium methoxide afforded the known 4-methorysampangine **13** in high yield. Finally, target compounds **5-12** were prepared by amination of **13** with corresponding diamine in 1-pentanol with moderate yields. Reaction conditions and compound characterisations are given in Supplementary Material.

2.2 *In vitro* inhibition studies on AChE and BChE

All the assays were under 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 8.0, using a PerkinElmer LAMBDA 45 Spectrophotometer. AChE from *Electrophorus electricus* (Sigma) were prepared to give 2.0 units/ml in 2 ml aliquots. The assay medium contained phosphate buffer, pH 8.0 (1 mL), 50 μL of 0.01 M DTNB, 10 μL of enzyme, and 50 μL of 0.01 M substrate (acetylthiocholine chloride). The substrate was added to the assay medium containing enzyme, buffer, and DTNB with inhibitor after 15 min of incubation time. The activity was determined by measuring the increase in absorbance at 412 nm for 1 min interval at 37 ^{o}C. Calculations were performed according to the method of the equation in Ellman et al[26]. In vitro BChE (from *equine serum*, Sigma) assay uses the similar method described above.

2.3 MTT assay

Cell viability was measured in 96-well plates by MTT assay. Briefly, after cells were treated using vehicle or compounds for indicated times, 500 μg/mL MTT (final concentration) was added into the medium and the mixture was then incubated at 37 °C

for 3h. The MTT solution was then removed and the colored formazan crystal was dissolved in dimethyl sulfoxide. The absorbance at 480 nm was measured using an iMark Microplate Absorbance Reader (Bio-Rad). The cell viability was expressed as the ratio of the signal obtained from the treatment group to that of the control group.

2.4 ELISA assay

$A\beta_{1-42}$ secretion was measured in 96-well plates by ELISA assay. Briefly, after cells were treated using vehicle or compounds for indicated times, the medium was removed, and the human $A\beta_{1-42}$ ELISA assay kit was used according to the procedures given in the manufacturer's instruction.

2.5 Determination of the inhibitory effect on the self-mediated Aβ(1-42) aggregation

The thioflavin-T fluorescence method was used[27-30], and Aβ(1-42) peptide (Anaspec Inc) was dissolved in phosphate buffer (pH 7.4, 0.01 M) to give a 40 μM solution. Compounds were firstly prepared in DMSO at a concentration of 10 mM. The final concentration of Aβ (1-42) and inhibitors were 20 μM and 10 μM, respectively. After incubating at 37 ^{o}C for 48 h, thioflavin-T (5 μM in 50 mM glycine-NaOH buffer, pH 8.0) was added. Fluorescence was measured at 450 nm (λ_{ex}) and 485 nm (λ_{em}). Each inhibitor was examined in triplicate. The fluorescence intensities were recorded, and the percentage of inhibition on aggregation was calculated with the following equation: $(1–IF_i/IF_c) \times 100\%$. IF_i and IF_c were the fluorescence intensities obtained in the presence and absence of inhibitors, respectively, after subtracting the fluorescence of corresponding blanks.

2.6 PAMPA-BBB procedure

Brain penetration of new compounds was evaluated using a parallel artificial membrane permeation assay (PAMPA), in a similar manner as described by Di et al[31]. Commercial drugs were purchased from Sigma and Aladdin (china). The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm) and the acceptor microplate was an indented 96-well plate, both from Millipore. The 96-well UV plate (COSTAR®) was from Corning Incorporated. The acceptor 96-well microplate was filled with 300 μL of Phosphate Buffered Saline (PBS) : ethanol (9:1) and The filter membrane was coated with 4 μL of porcine brain lipid (PBL) in dodecane (20 mg mL^{-1}). Test compounds were dissolved in DMSO at 5 mg mL^{-1}. Then the compound solution was diluted 200-fold in PBS : ethanol (9:1) (final concentration 25 μg mL^{-1}) to make secondary stock solution. 300 μL of the secondary stock solution were added to the donor wells. The acceptor filter plate was carefully put on the donor plate to form a 'sandwich', which was left undisturbed for 10 hrs at 25 ºC. The concentration of drug in the acceptor, the donor, and the reference wells was determined using the UV plate reader (Tecan Infinite® M1000). P_e can be calculated from the following equation as reported by Faller et al[9, 10] and Sugano et al[11].

$$P_e = -\left(\frac{V_d \cdot V_a}{(V_d + V_a)\, A \cdot t}\right) \cdot \ln\left(1 - \frac{[drug]_{acceptor}}{[drug]_{equilibrium}}\right)$$

V_d = volume of donor well, V_a = volume in acceptor well, A = filter area, t = permeation time, $[drug]_{acceptor}$ = the absorbance of compound found in the acceptor

well, $[drug]_{equilibrium}$ = the theoretical equilibrium absorbance. All compounds were tested in triplicate at pH 7.4 and the results were given as the mean ± standard deviation.

3. Results and discussion

3.1 Inhibition of AChE and BuChE

To evaluate the biological profiles of the synthesized compounds for AD, AChE (Electrophorus electricus) and BChE (equine serum) inhibition was assayed in comparison with tacrine as reference compound. The inhibitory potency against AChE and BChE was evaluated by the method of Ellman et al[26]. The IC_{50} values for AChE and BChE inhibition are summarized in Table 1. All the synthesized compounds demonstrated much higher inhibitory potency against AChE than their precursor compound **3**, with inhibitory activity IC_{50} values in the submicromolar range. This result indicated that introduction of the amino group side chains could increase the inhibitory capacity and selectivity. The structure of terminal groups of side chain has effects on their inhibitory activities. Highest inhibitory potency was found to be associated with diethylamine at the end of side chain (compound **8**) with IC_{50} values in 0.23μM. Dimethylamine derivatives showed less potency. Variation of chain length (n = 2 or 3) had less influence on activity than the functional group structure.

3.2 Inhibiton of self-induced Aβ Aggregation

Besides assessing the ability to inhibit AChE and BChE, which is likely to be relevant on the brain of AD patients, we tested all the compounds (inhibitor:Aβ ratio 1:2) to assess the structural elements responsible for the *in vitro* inhibition of the self-assembly

of Aβ(1-42), which is the most amyloidogenic Aβ fragment found in the AD plaques[32-34]. To determine the amyloid-β(1-42) aggregation inhibition of the new sampangine derivatives (**3**-**12**), thioflavin-T (ThT) assay was performed compared with Curcumin as reference compound[27-29].

Interestingly, all the synthesized compounds presented better inhibitory potency than curcumin on self-induced Aβ(1-42) aggregation. Data in Table 1 showed that compounds **3**-**12** at 10 μM inhibited Aβ(1-42) self-aggregation in a range from 46.5% to 72.3%. The most effective compound is **7**, followed by **8** and **9**, their inhibitory potency was 72.3%, 70.5% and 68.8%, respectively. The result revealed a slight trend of increased efficacy with the reduction of the chain length. As a matter of fact, increasing the methylene chain length reduced the inhibition of Aβ(1-42) self-aggregation.

3.3 Derivatives reduced Aβ42 secretion level in APPsw cells

To test the effects of synthesized derivatives on APPsw SH-SY5Y cell viability and to examine the safety of these compounds, the *in vitro* cytotoxicity on APPsw SH-SY5Y cell of compounds **5**-**12** was evaluated by MTT assay. As shown in Table 1, introducing the side chain in sampangine alkaloid greatly reduced its toxicity. All the derivatives (**5**-**12**) showed at least 3 times less toxic than sampangine **3**. Particularly, compound **8** showed lowest neurotoxicity with stronger AChE and Aβ(1-42) self-aggregation inhibitory potency suggesting the wide therapeutic safety ranges.

It is known that AChE inhibitors enhance the release of non-amyloidogenic soluble derivatives of amyloid precursor protein (APPs) *in vitro* and *in vivo* and possibly slow down the formation of amyloidogenic compounds in brain[35, 36]. Moreover, AChE directly promotes *in vitro* the assembly of Aβ peptide into amyloid fibrils forming stable AChE-Aβ complexes[2]. Thus, the effects of the synthesized compounds on Aβ42 secretion level in APPsw SH-SY5Y cells were tested by human Aβ42 Elisa assay kit. The viability of APPsw cells did not change significantly when concentration of derivatives was less than 10 μM. Thus, two different concentrations (5μM and 10μM) of compounds 5-12 were selected for Aβ42 secretion level testing. Since sampangine **3** showed stronger toxicity than its derivatives and had a great influence on cell viability in 10μM, only 5μM sampangine was selected. As shown in Fig. 2, after treatment with derivatives, APPsw cells showed various degrees of reducing the Aβ42 secretion level. Moreover, the decrease was concentration dependent. The Aβ42 secretion level always decreased with the increase of derivatives concentration. The most effective compound is **8** which treatment significantly reduced the Aβ42 production level by 54% (DMSO, 265.1 pg/ml vs compound **8**, 146.0 pg/ml) in 10μM. The Aβ42 secretion level was even lower than wild type SH-SY5Y cells (control).

3.4 Prediction of blood-brain barrier (BBB) permeability

While protective in nature, the inability of molecules to permeate the BBB is a significant source of attrition in central nervous system (CNS) drug discovery[37, 38]. For this reason, BBB permeability properties of CNS drug candidates should be determined as early as possible in the drug discovery process. To explore whether the

selected compounds would be able to penetrate into the brain, we used a parallel artificial membrane permeation assay for BBB (PAMPA-BBB). The PAMPA-BBB model applied in this study was based on the BBB model described by Di et al[31]. This simple and rapid model was capable of identifying compounds as either BBB permeable (BBB+) or non-permeable (BBB−) with reasonable accuracy by modifying the lipid composition of the artificial membranes[39]. In this paper, a lipid extract of porcine brain was used. Assay validation was made comparing experimental permeabilities of 7 commercial drugs with reported values (Table 2).

A plot of experimental data versus bibliographic values gave a good linear correlation, P_e(exp.) = 0.79P_e(bibl.) + 0.57 (R^2=0.96). From this equation and taking into account the limit established by Di et al[31] for blood-brain barrier permeation, we classified compounds as follows:

a）‘CNS +’ (high BBB permeation predicted); P_e (10^{-6} cm s^{-1}) > 3.7

b) ‘CNS -’ (low BBB permeation predicted); P_e (10^{-6} cm s^{-1}) < 2.1

c) ‘CNS +/-’ (BBB permeation uncertain); P_e (10^{-6} cm s^{-1}) from 3.7 to 2.1

Finally, new synthetical derivatives were tested in the PAMPA-BBB assay, and the results were presented in Table 3. for BBB permeation, we found that molecules with permeability above 3.7×10^{-6} cm s^{-1} would be able to cross the BBB by passive permeation. Compounds **6**-**9** and **11** showed greater permeability values than that limit, pointing out that these molecules would cross the BBB by passive diffusion.

4. Conclusions

Sampangine is a class of alkaloids with a wide range of biological activities. However, their toxicity and poor water solubility and the scarcity from the natural source have limited its development and application. In this article, the alkylamino side chains were first introduced to sampangine on 4-substitution. It not only increased the water solubility of the derivatives, but also reduced their toxicity. Especially for compound **8**, it had the strongest anti-cholinesterase, anti-Aβ aggregating activity and high BBB permeability values but its toxicity was weakest. It makes them promising anti-Alzheimer drug candidates. The results suggested that the novel 4-substitutional sampangine derivatives herein reported were worthwhile to further research.

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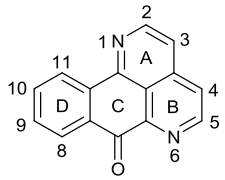


Fig. 1. Structure of sampangine

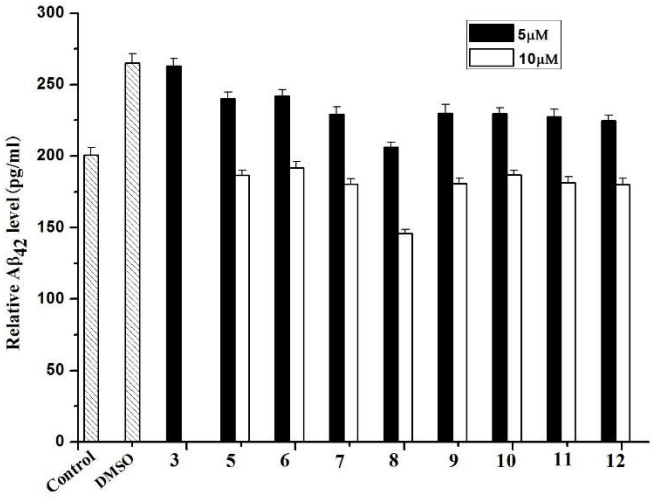
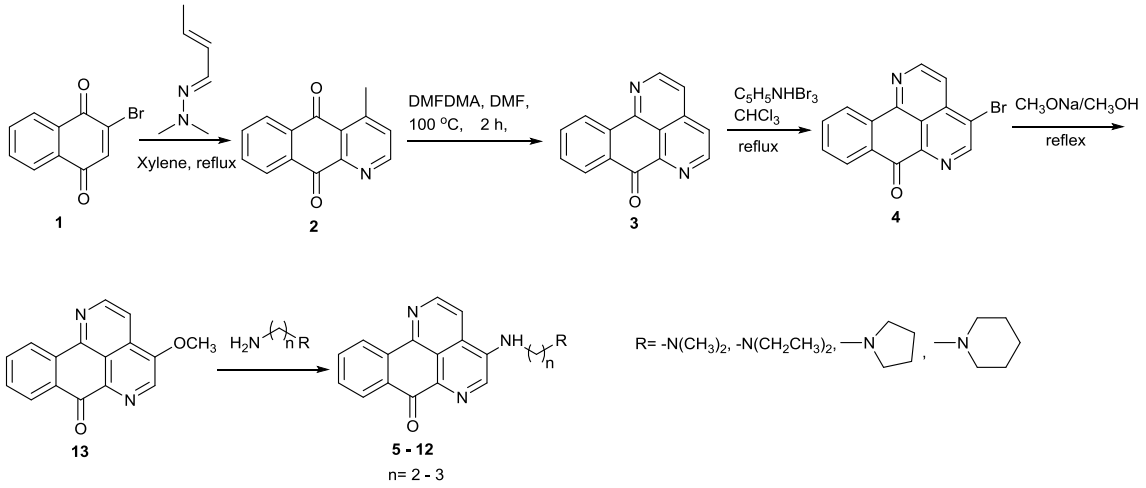
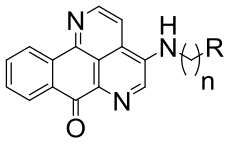


Fig. 2. APPsw cells were incubated for 24 h in a medium containing DMSO or compounds **3-12** (5 μM and 10μM). The Aβ42 levels in the cell medium were determined by ELISA. Control was wild type SH-SY5Y cells.



Scheme 1. Synthesis of 4- Substituted sampangine derivatives.

Table 1. *In vitro* inhibition of AChE, BChE and self-induced Aβ(1-42) aggregation activities of compounds **3, 5-12** and their toxicity in APPsw SH-SY5Y cells



Compound	R	n	IC_{50}(uM) for AChE[a]	IC_{50}(uM) for BChE[a]	Selectivity for AChE/BChE[b]	IC_{50} cytotoxicity (μM) against APPsw SH-SY5Y cells[c]	Inhibition of self-induced Aβ aggregation (%)[d]
3			> 100	> 100		12.4	46.5 ± 2.3
5	$-N(CH_3)_2$	2	12.11 ± 0.66	10.9 ± 0.37	1	41.2	62.1 ± 2.1
6		3	13.60 ± 0.71	> 100	>7	38.8	54.2 ± 1.8
7	$-N(CH_3CH_2)_2$	2	1.82 ± 0.12	38.1 ± 0.64	21	42.1	72.3 ± 2.3
8		3	0.23 ± 0.04	> 100	>435	45.8	70.5 ± 1.9
9		2	4.81 ± 0.32	3.39 ± 0.08	1	39.7	68.8 ± 1.5
10		3	8.86 ± 0.41	12.39 ± 0.36	1	40.7	66.7 ± 2.2
11		2	1.15 ± 0.09	13.14 ± 0.28	11	30.7	59.4 ± 1.9
12		3	1.65 ± 0.15	3.58 ± 0.15	2	35.7	60.9 ± 2.1
Tacrine			0.22 ± 0.02	0.026 ± 0.001	0.1	n.d.[e]	n.d.[e]
Curcumin			n.d.[e]	n.d.[e]		n.d.[e]	44.7 ± 1.9

[a] IC_{50}: 50% inhibitory concentration (means ± SEM of three experiments) of AChE and BChE.

[b] Apparent selectivity for AChE is calculated as IC_{50}(BChE)/IC_{50}(AChE).

[c] Data derived from the mean of three independent assays.

[d] Inhibition of self-induced Aβ(1-42) aggregation (20 μM) produced by the tested compound at 10 μM concentration. Values are expressed as means ± SEM of three experiments.

[e] Not determined.

Table 2. Permeability ($P_e \times 10^{-6}$ cm s^{-1}) in the PAMPA-BBB assay for 7 commercial drugs, used in the experiment validation

Compoud	bibliography [a]	experiment [b]
Imipramine	13	10.2
Chlorpromazine	6.5	6.9
Clonidine	5.3	5.2
Hydrocortisone	1.9	1.3
Enoxacin	0.9	1.2
Corticosterone	5.1	4.6
Dopamine	0.2	0.6

[a] Taken from ref 29.

[b] Data are the mean of three independent experiments.

Table 3. Permeability ($P_e \times 10^{-6}$ cm s^{-1}) in the PAMPA-BBB assay for 4- substituted sampangine derivatives and their predictive penetration in the CNS

Compoud	$P_e \times 10^{-6}$ cm s^{-1} [a]	prediction
3	0.2 ± 0.04	CNS -
5	0.8 ± 0.1	CNS -
6	4.9 ± 0.3	CNS +
7	6.4 ± 0.2	CNS +
8	5.6 ± 0.1	CNS +
9	7.8 ± 0.2	CNS +
10	3.1 ± 0.1	CNS +/-
11	7.0 ± 0.3	CNS +
12	3.5 ± 0.1	CNS +/-

[a] Data are the mean (n=3) ± SD.