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Chinese Prescription Kangen-karyu as Potential Anti-Alzheimer’s Disease Therapeutic: Analyses of BACE1 and GSK-3β Inhibitory Activities

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ARTICLE INFO

Article history
Received: 9 July 2021
Accepted: 16 July 2021
Published Online: 30 August 2021

Keywords:
Alzheimer’s disease
β-Site amyloid precursor protein-cleaving enzyme 1
Glycogen synthase kinase-3β
Kangen-karyu
Salviae Miltiorrhizae Radix
Salvianolic acid C
Salvianolic acid B

ABSTRACT
Inhibition of β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) or glycogen synthase kinase-3β (GSK-3β) is estimated to be the central therapeutic approach for Alzheimer’s disease (AD). In this study, water extract of Kangen-karyu, its crude drug and chemical composition used in oriental medicine were evaluated regarding their BACE1 and GSK-3β inhibitory activities. Fluorescence resonance energy transfer was used to characterize the BACE1 inhibitory effect of Kangen-karyu, its crude drug and chemical composition. GSK-3β activity was determined using the Kinase-Glo Luminescent Kinase Assay Platform. The water extract of Kangen-karyu inhibited BACE1 and GSK-3β in concentration-dependent manners when compared with reference drugs, quercetin and luteolin. Among six components of Kangen-karyu, the water extracts of Salviae Miltiorrhizae Radix or Cyperi Rhizoma exhibited significant inhibitory effects on BACE1 and GSK-3β. Among the constituents of Salviae Miltiorrhizae Radix extract, salvianolic acid C, salvianolic acid A, rosmarinic acid, and magnesium lithospermate B significantly inhibited BACE1. In addition, they inhibited GSK-3β with an IC₅₀ value range of 6.97 to 135.35 μM. From these results, one of the effectiveness and its mechanisms of action of Kangen-karyu against AD may be the inhibition of BACE1 and GSK-3β, and one of the active ingredients of Kangen-karyu is Salviae Miltiorrhizae Radix and its constituents.

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1. Introduction

Alzheimer’s disease (AD) is the most prevalent neurodegenerative dementia with two major pathological features: extra- and intracellular amyloid plaques and intraneuronal neurofibrillary tangles formed of amyloid β-protein (Aβ) and phosphorylated tau protein, respectively \([1]\). Aβ toxicity is believed to play a major role in the pathogenesis of AD \([2]\). For that reason, anti-amyloid strategies have been a major focus of AD drug development.

Aβ is a cleavage product of amyloid precursor protein (APP) by two proteases: β-site APP cleaving enzyme 1 (BACE1) and the γ-secretase complex. APP is firstly cleaved by BACE1, producing secreted APP-β and a C-terminal fragment (CTF) known as β-CTF. β-CTF is subsequently cleaved by γ-secretase to release Aβ \([3]\). BACE1 is a rate-limiting enzyme for Aβ generation and is considered one of the major therapeutic targets for AD \([4]\). However, the increased production of Aβ peptides derived from APP via the sequential proteolytic cleavages catalyzed by BACE1, has a controversial link to the glycogen synthase kinase 3 (GSK-3) enzyme. In AD, GSK-3β is responsible for the phosphorylation of microtubule-related tau protein, which affects microtubule stability and delocalization of the abnormal tau protein to brain cells and dendrites \([5]\). Due to the aggregation of hyperphosphorylated tau proteins, neurofibrillary tangles form, and trigger synaptic dysfunction and neuronal apoptosis, which lead to cognitive impairment \([6]\). Inhibition of BACE1 or GSK-3β is considered as the central therapeutic approach against AD.

Traditional Chinese medicine has been widely used in China for thousands of years. Traditional Chinese medicine has now established its position in the Western world, and has become a prime source of drug discovery. Kangen-karyu, which consists of six medicinal herbs, has attracted considerable attention due to their biological activities and potential health benefit effects: Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma (Table 1). Kangen-karyu is a traditional herbal formula slightly modified the composition of Chinese prescription (Guan-xin No.2) that has been used to treat blood circulation-related various symptoms, neuro-degenerative disorders, diabetes, and diabetic complications. The recent clinical trials have reported the potential clinical applications of using Kangen-karyu extract, such as for cognitive dysfunctions in type 2 diabetes symptoms trough improving central cholinergic dysfunction \([7]\), age-related memory deficit by normalizing neuroplasticity-associated neuronal signaling system, and the VEGF signaling system in the brain \([8]\) against oxidative stress mediated tissue injury \([9]\), type 1 and type 2 diabetes and diabetic complications \([10,11]\), a neuroprotective effect \([12]\). However, there are as yet no reports of BACE1 and GSK-3β, regarded as the central therapeutic approach against AD.

| Common name                  | Botanical name       | Family name  |
|------------------------------|----------------------|--------------|
| Aucklandiae Radix            | Aucklandia lappa     | Compositae   |
| Cyperi Rhizoma               | Cyperus rotundus L.  | Cyperaceae   |

Therefore, we present the performance evaluation for the in vitro BACE1 and GSK-3β inhibition potential of water extract of Kangen-karyu, its crude drug and chemical composition.

2. BACE1 Inhibition

We performed a comparative study on BACE1 inhibition with a boiled water extract of Kangen-karyu and its components. As shown in Table 2, the boiled water extract of Kangen-karyu demonstrated moderate inhibition. Among the components, moderate inhibition was also observed with a boiled water extract of Salviae Miltiorrhizae Radix and Cyperi Rhizoma followed by mild inhibition by Cnidii Rhizoma, Paeoniae Radix, and Carthami Flos. No inhibition was observed with a boiled water extract of Aucklandiae Radix.

| Sample                        | IC₅₀ values \(^{a}\) |
|-------------------------------|---------------------|
| Kangen-karyu                  | 77.40 ± 4.58        |
| Salviae Miltiorrhizae Radix   | 89.84 ± 1.87        |
| Cnidii Rhizoma                | 147.09 ± 0.93       |
| Paeoniae Radix                | 143.57 ± 1.79       |
| Carthami Flos                 | 233.34 ± 0.05       |
| Aucklandiae Radix             | >400                |
| Cyperi Rhizoma                | 91.16 ± 2.21        |
| Quercetin \(^{b}\)            | 10.49 ± 0.28        |

\(^{a}\)The 50% inhibitory concentrations (IC₅₀, μg/mL) are expressed as the mean ± SED. \(^{b}\)Used as positive control.

Values are expressed in μM.

Furthermore, we studied the compositions of Salviae Miltiorrhizae Radix (Figure 1). Among the six compounds tested, salvianolic acid C exhibited significant inhibition...
of BACE1 with an IC$_{50}$ value of 9.18 ± 0.03 μM, while IC$_{50}$ of the positive control quercetin was 10.49 ± 0.54 μM, as shown in Table 3. Likewise, salvianolic acid A exhibited similar maximum inhibition of BACE1 with marked IC$_{50}$ inhibitory activity, but salvianolic acid B and caffeic acid showed weak inhibitory activities against BACE1. Rosmarinic acid and magnesium lithospermate B displayed moderate to mild activity against BACE1.

Table 3. BACE1 inhibitory potentials of compounds identified in Salviae Miltiorrhizae Radix.

| Sample                  | IC$_{50}$ values$^a$ |
|-------------------------|----------------------|
| Salvianolic acid A      | 13.01 ± 0.32         |
| Salvianolic acid B      | 168.90 ± 0.70        |
| Salvianolic acid C      | 9.18 ± 0.03          |
| Caffeic acid            | >200                 |
| Rosmarinic acid         | 29.77 ± 0.70         |
| Magnesium lithospermate B | 30.35 ± 2.67       |

$^a$The 50% inhibitory concentrations (IC$_{50}$, μM) are expressed as the mean ± SEM.

3. GSK-3β Inhibition

As shown in Table 4, the boiled water extract of Kangen-karyu potently suppressed GSK-3β with an IC$_{50}$ value of 17.05 ± 1.14 μg/mL. All components of the boiled water extract of Kangen-karyu’s individual components showed potent suppression against GSK-3β with IC$_{50}$ values ranging from 7.77 to 93.61 μg/mL. The extract of Salviae Miltiorrhizae Radix (IC$_{50}$: 7.77 ± 1.38 μg/mL) was the most potent among them, followed by Cyperi Rhizoma (IC$_{50}$: 20.68 ± 2.50 μg/mL). The extracts of other herbal components showed weak and moderate inhibitory activity in GSK-3β assays.

Table 4. GSK-3β inhibitory potentials of water extract of Kangen-karyu and its constituents.

| Sample                  | IC$_{50}$ values$^a$ |
|-------------------------|----------------------|
| Kangen-karyu            | 17.05 ± 1.14         |
| Salviae Miltiorrhizae Radix | 7.77 ± 1.38       |
| Cnidii Rhizoma          | 66.74 ± 2.05         |
| Paeoniae Radix          | 62.51 ± 1.89         |
| Carthami Flos           | 93.61 ± 3.99         |
| Aucklandiae Radix       | 85.04 ± 6.32         |
| Cyperi Rhizoma          | 20.68 ± 2.50         |
| Luteolin$^b$            | 2.18 ± 0.13$^*$      |

$^a$The 50% inhibitory concentrations (IC$_{50}$, μg/mL) are expressed as the mean ± SED. $^b$Used as positive control.

Next, we evaluated the constituent compounds of Salviae Miltiorrhizae Radix which showed excellent GSK inhibitory effect. As listed in Table 5, magnesium lithospermate B, salvianolic acid A, salvianolic acid B, and salvianolic acid C displayed strong inhibition against GSK-3β. Especially, salvianolic acid B was the most effective, inhibiting the enzyme by 50% at 6.97 ± 0.96 μM. Magnesium lithospermate B, salvianolic acid

Figure 1. Chemical structures of active compounds from S. miltiorrhiza.
A, and salvianolic acid C showed roughly one-fifth the activity of salvianolic acid B with similar IC_{50} values of approximately 30 μM. On the other hand, rosmarinic acid (IC_{50}: 135.35 ± 4.69 μM) and caffeic acid (IC_{50}: 425.01 ± 7.61 μM) showed moderate or mild inhibitory activity in GSK-3β assays.

**Table 5.** GSK-3β inhibitory potentials of compounds identified in Salviae Miltiorrhizae Radix.

| Sample                        | IC_{50} values$^a$ |
|-------------------------------|--------------------|
| Salvianolic acid A            | 30.21 ± 3.14       |
| Salvianolic acid B            | 6.97 ± 0.96        |
| Salvianolic acid C            | 31.82 ± 2.08       |
| Caffeic acid                  | 425.01 ± 7.61      |
| Rosmarinic acid               | 135.35 ± 4.69      |
| Magnesium lithospermate B    | 33.07 ± 3.88       |
| Luteolin$^b$                  | 2.18 ± 0.13        |

$^a$The 50% inhibitory concentrations (IC_{50}, μM) are expressed as the mean ± SEM. $^b$Used as positive control.

4. Discussion

Under pathological patterns, the permitted ongoing treatment approaches for AD are acetylcholinesterase inhibitors (AChEIs: donepezil, galanthamine, and rivastigmine, all enhancing acetylcholine levels) and the non-competitive/low affinity N-methyl-D-aspartate receptor antagonist (NMDA antagonist: memantine, activating glutamate neurotransmission) $^{[13]}$. However, these types of drugs only provide the effect of delaying or relieving symptoms. Since AChEIs (cholinergic) and memantine (glutamatergic) target two different aspects of AD pathology, combination treatment is beneficial effective in patients with moderate to severe AD, by comparison with the monotherapy $^{[14]}$. Furthermore, discovery of different targets that are decisive importance in promoting AD pathogenesis is the current main focus. Currently, it has been hypothesized that BACE1 and GSK-3β contribute distinctly to suppressing the development of AD as a linking bridge between Aβ and tau protein.

A number of recent studies demonstrated a correlation between AD and oxidative stress $^{[15-17]}$. Specifically, AD may be associated with cellular oxidative stress including augmentation of protein oxidation, protein nitration, glycoloxidation, and lipid peroxidation as well as the accumulation of Aβ $^{[15,17,18]}$. In general, oxidative stress involves the production of superoxide (O$_2^-$), and the formation of nitrotyrosine and peroxynitrite (ONOO$^-$) derived from nitric oxide (NO), all of which are destructive free radical oxidants $^{[19]}$. Therefore, drugs that are effective against all destructive free radical oxidants may by important therapies for AD $^{[20-23]}$. Simultaneous inhibition of nitrotyrosine and ONOO$^-$ along with BACE1 and GSK-3β may represent a promising avenue of research for the development of anti-AD agents.

In a previous study, we reported that Kangen-karyu inhibited reactive oxygen species (ROS) production in the presence of high glucose-induced oxidative stress using LLC-PK$_1$ cells, renal tubular cells, which are the most vulnerable renal tissue to oxidative stress. The intracellular ROS (O$_2^-$, NO, and ONOO$^-$) induced by high glucose was concentration-dependently inhibited by Kangen-karyu treatment. Kangen-karyu also reduced the overexpression of inducible nitric oxide synthase, cyclooxygenase-2 proteins induced by high glucose. Furthermore, treatment with Kangen-karyu inhibited the nuclear translocation of nuclear factor-kappa B $^{[24]}$. Moreover, Kangen-karyu had a pleiotropic effect on several oxidative stress-related parameters and exerted a renoprotective effect on the development of diabetic nephropathy in type 2 diabetic db/db mice $^{[11]}$. These findings indicate that Kangen-karyu is a potential therapeutic agent that will reduce the damage caused by hyperglycemia-induced oxidative stress associated with diabetes. In addition, Kangen-karyu might prevent AD by attenuating the increased oxidative biomarkers, including the generation of ROS.

In the present study, we investigated the anti-AD potential of Kangen-karyu and its components using BACE1 and GSK-3β inhibitory assays. Based on the results shown in Tables 2 and 4, the water extract of Kangen-karyu shows inhibitory potential against BACE1, as well as GSK-3β. Among the six components, Salviae Miltiorrhizae Radix and Cyperi Rhizoma showed moderate inhibition of BACE1, while Salviae Miltiorrhizae Radix exhibited stronger inhibitory activity. In contrast, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, and Aucklandiae Radix showed weak inhibitory activity against BACE1 and GSK-3β. Focusing on GSK-3β inhibition, Salviae Miltiorrhizae Radix was about 2.7 times more active than Cyperi Rhizoma and, thus, was selected for further study. Therefore, we estimated the potentials of those chemical composition from the active water extract of Salviae Miltiorrhizae Radix.

Phytochemical constituents of Salviae Miltiorrhizae Radix have been extensively studied. Water-soluble fraction of Salviae Miltiorrhizae Radix contains caffeic acid and its derivatives such as rosmarinic acid (dimer), salvianolic acid A (trimer), salvianolic acid B (tetramer), salvianolic acid C (trimer), and magnesium lithospermate B (tetramer). Chief of all, salvianolic acids are the principal water-soluble ingredients in Salviae Miltiorrhizae Radix, among which salvianolic acid A

DOI: https://doi.org/10.30564/jim.v10i2.3441
(caffeic acid trimer) and salvianolic acid B (caffeic acid tetramer) are the most abundant phytochemicals. A review of the BACE1 and GSK-3β inhibitory activities of our evaluated compounds showed that a monomeric caffeic acid showed weak activity while its dimer, trimer, and tetramer displayed a significant increase in activity. Especially, it exhibited remarkable activity in trimers and tetramers rather than dimers. This pattern means that the activity is enhanced when caffeic acid is condensed. Simultaneous inhibition of both BACE1 and GSK-3β may provide efficient benefits in the treatment of AD.

In the present study, we evaluated the anti-AD activity of Kangen-karyu and its indvidual ingredients via BACE1 and GSK-3β assays. The results demonstrated that Salviae Miltiorrhizae Radix is the main active component of Kangen-karyu against the two enzymes. Furthermore, rosmarinic acid derivatives were found to be marked inhibitors. Among them, salvianolic acid C was a potent mixed inhibitor of BACE1 and showed the lowest IC₅₀ value (9.18 ± 0.03 μM), while salvianolic acid B exhibited the highest inhibitory activity against GSK-3β with an IC₅₀ value of 6.97 ± 0.96 μM. Therefore, one of the mechanisms of action of Kangen-karyu against AD may be the inhibition of BACE1 and GSK-3β, and one of the active components of Kangen-karyu is Salviae Miltiorrhizae Radix and its constituents, salvianolic acids C and B.

5. Conclusions

Alzheimer’s disease drugs generally developed as a single target strategy are not only unsatisfactory for AD treatment, but also have several side effects. Therefore, as a potential effective strategy in the treatment of AD, the choice of the multi-target strategy has been proposed. Therefore, Kangen-karyu and its herbal formula (Salviae Miltiorrhizae Radix) containing caffeic acid derivatives with both BACE1 and GSK-3β inhibitory activities could be a promising herbal medicine for the treatment drug of cognition deficit disorders, such as AD-type dementia.

Funding

No funding was received for this study.

Conflict of Interest Statement

The authors declare no conflict of interest.

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