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Silibinin: A novel inhibitor of Aβ aggregation

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ABSTRACT

Alzheimer's disease (AD) is characterized by the abnormal aggregation of amyloid β peptide (Aβ) into extracellular fibrillar deposits known as amyloid plaque. Inhibition of Aβ aggregation is therefore viewed as a potential method to halt or slow the progression of AD. It is reported that silibinin (silybin), a flavonoid derived from the herb milk thistle (Silybum marianum), attenuates cognitive deficits induced by Aβ25-35 peptide and methamphetamine. However, it remains unclear whether silibinin interacts with Aβ peptide directly and decreases Aβ peptide-induced neurotoxicity. In the present study, we identified, through employing a ThT assay and electron microscopic imaging that silibinin also appears to act as a novel inhibitor of Aβ aggregation and this effect showed dose-dependency. We also show that silibinin prevented SH-SY5Y cells from injuries caused by $A\beta_{1-42}$-induced oxidative stress by decreasing H_2O_2 production in $A\beta_{1-42}$-stressed neurons. Taken together, these results indicate that silibinin may be a novel therapeutic agent for the treatment of AD.

1. Introduction

Alzheimer's disease (AD) is a common form of progressive neurodegeneration that manifests itself as a learning and memory disorder which leads to a marked impairment of cognition and progressive loss of higher order functions ultimately leading to a significant shortening of lifespan (Selkoe, 2002). Extracellular aggregation of amyloid β (Aβ) peptides, which are the main proteinaceous components of senile plaques found in the brain of AD patients, is the major post mortem pathological feature of AD (Coria et al., 1994; Crouch et al., 2008). A large body of *in vitro* evidence shows that Aβ in either the oligomeric or fibril form has a stronger neurotoxicity than its monomeric form (Dahlgren et al., 2002). Inhibition of Aβ aggregation is, therefore, viewed as a potential therapeutic approach to slow or mitigate the progression of AD (Estrada and Soto, 2007; Findeis, 2002).

Accordingly, intense drug discovery and screening efforts have been undertaken in recent years to identify potential inhibitors of Aβ aggregation. A variety of analytical methods have been developed to monitor the aggregation of amyloid peptides, with some being found to simulate the mechanism of Aβ-induced neurotoxicity observed *in vitro*. These model systems have provided useful means to study the mechanism of Aβ-mediated cell cytotoxicity and can also be used in the development of novel therapeutics aimed at inhibiting Aβ aggregation or compounds that can reduce or solubilize existing Aβ deposits (LeVine, 1993; Walker et al., 2005). Thioflavin T (ThT) is the most commonly used dye for the detection of Aβ aggregation as it undergoes significant increases in fluorescence (around 482 nm, when excited at 450 nm) upon binding to amyloid fibrils (Bourhim et al., 2007; Sabate and Saupe, 2007). In the present study, we have used the ThT assay to determine the effects of silibinin on Aβ aggregation.

Silibinin (Sylibin), a flavonoid derived from the herb milk thistle (*Silybum marianum*), has been shown to have anti-oxidative and anti-inflammatory properties (Shanmugam et al., 2008). Further, an *in vitro* study conducted on some food constituents including silymarin (refined flavonoids extract from *S. marianum* containing silibinin) demonstrated that silibinin inhibits the activity of monoamine oxidase that catalyzes the oxidative deamination of monoamines (Mazzio et al., 1998). These studies suggest that silibinin may have neuroprotective effect on cognitive deficits. More recently, silibinin was shown to attenuate Aβ-induced memory impairment in mice through amelioration of oxidative stress and inflammatory response (Lu et al., 2009a,b). Silymarin was also shown to inhibit Aβ fibril formation in PC12 cells, and also improve behavioral abnormalities in mouse model of Alzheimer's disease (Murata et al., 2010). This present study reports our finding that silibinin is a novel inhibitor of Aβ aggregation, and also antagonizes the oxidative damage induced by $A\beta_{1-42}$ through decreasing H_2O_2 levels in SH-SY5Y cells.

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2. Materials and methods

2.1. Materials and reagents

SH-SY5Y human neuroblastoma cells were purchased from American Type Tissue Culture (Manassas, VA, USA). Silibinin was obtained from Sigma, whose purity is over 98% (S0417, St. Louis, MO, USA). Dulbecco's modified Eagle's medium, fetal bovine serum Glycine, Thioflavin T (ThT), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT). $A\beta_{1-42}$ was purchased from Anaspec (San Jose, CA, USA). Hydrogen peroxide assay kit was purchased from Biovision (Mountain View, CA, USA).

2.2. Preparation of $A\beta_{1-42}$ aggregates

For aggregation experiments, $A\beta_{1-42}$ was dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 mg/mL, sonicated in a water bath for 10 min, and aliquoted into microcentrifuge tubes to obtain 0.1 mg stocks. The stocks were stored at room temperature and protected from light for 5–24 h before removing HFIP by evaporation under N_2, leaving a thin transparent film of peptides at the internal surface of the tube, and then stored at −80 °C. Prior to use, the HFIP-treated $A\beta_{1-42}$ was dissolved in dimethylsulfoxide (DMSO) at 1 mg/mL and diluted to 20 μM in PBS, pH 7.4. A 25 mg/mL silibinin stock solution (in DMSO) was prepared. $A\beta_{1-42}$, with a final concentration of 10 μM, was mixed with silibinin or other tested chemicals at indicated concentrations.

2.3. ThT binding assay

The aggregation potential of Aβ was assessed using the ThT-binding assay. The extent of Aβ aggregation in the samples prepared as stated above was measured by taking 10 μL of $A\beta_{1-42}$ from the incubated samples and adding that to 200 μL of 10 μM ThT in a 0.1 M glycine buffer at pH 8.9. For each sample, the mixture of peptide and ThT was pipetted into a single well on a standard 96-well plate. The plates were loaded into Varioskan microplate reader (Thermo, USA) and the fluorescence measured with excitation at 450 nm and emission at 482 nm. Each value reported is the average of three readings for every sample after subtraction of ThT fluorescence background. All ThT fluorescence experiments were performed in triplicate.

2.4. Transmission electron microscopy (TEM) imaging

To prepare specimens for TEM imaging, 100 μM $A\beta_{1-42}$ was incubated in the presence or absence of 50 μM silibinin for 3 days at room temperature. An aliquot (5 μL) of each sample was spotted onto a glow-discharged, carbon-coated Formvar grid, which was then stained with 5 μL uranyl acetate for 1 min. Samples were examined with a Hitachi H7500 transmission electron microscopy (Hitachi, Japan). All images were captured at voltage of 80 kV.

2.5. H_2O_2 assay

SH-SY5Y cells were seeded onto 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were washed once with PBS and phenol red-free and serum-free DMEM was added. After the cells were preincubated with silibinin at various concentrations for 2 h, $A\beta_{1-42}$ was added to achieve a final concentration of 2.5 μM and subjected to an incubation time of 24 h. The media was analyzed for the H_2O_2 levels in accordance to the protocol provided by the supplier (BioVison, San Francisco, USA).

2.6. MTT assay

The cytotoxicity of $A\beta_{1-42}$ was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. SH-SY5Y cells were seeded onto 12-well plates and cultured for 24 h before beginning the experiment. After one wash with PBS, the media was changed to a serum-free and phenol red-free DMEM. At this time, the cells were pretreated with silibinin for 2 h. After that, 2.5 μM $A\beta_{1-42}$ was added and incubated for an additional 24 h. The MTT assay was performed by adding 0.5 mg/mL MMT and incubating 2 h at 37 °C. The formazan salt generated by viable cells (as a result of conversion of MTT) was dissolved in DMSO and absorbance measured at 570 nm and 630 nm, respectively.

3. Results

3.1. Screening of inhibitors for Aβ aggregation

Many studies have reported that ThT binding with Aβ fibrils produces a hypochromic shift to increase the fluorescence intensity, and the reaction starts immediately upon mixing Aβ with ThT is complete within 1 min. We used the ThT assay to assess the ability of a compound to inhibit the aggregation of Aβ and the

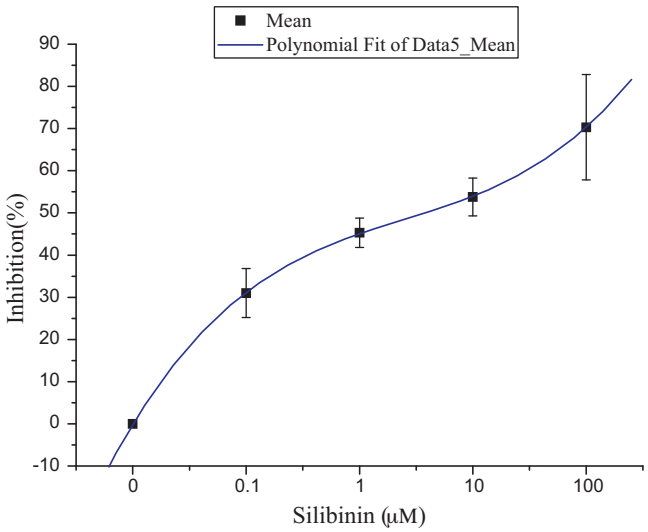

Fig. 1. ThT assay on the aggregation of $A\beta_{1-42}$ peptide in the absence or presence of silibinin at indicated concentrations. After incubation of $A\beta_{1-42}$ at 20 μM with indicated dose of silibinin for 72 h, 10 μL peptide was mixed with 200 μL ThT (10 μM). The fluorescence was determined with Varioskan plate reader at Ex/Em 450/482 nm. Data are mean from 6 wells of two independent experiments ($n = 6$, $R^2 = 0.99834$).

Z-factor, calculated using the equation proposed by Zhang et al. (1999), to evaluate the reliability of ThT assay. It is suggested that the assay has high statistical reliability if the *Z*-factor is over 0.5. The *Z*-factor in our study was 0.9811 when the concentration of $A\beta_{1-42}$ was 10 μM and ThT was 5 μM, indicating that our assay is a reliable and a valid screening method as previously described (Zhang et al., 1999).

We carried out a primary screening using the ThT assay on 215 samples (plant extracts, fractions, or pure compounds) derived from Atlantic Canada plants or acquired from commercial sources for their ability to inhibit Aβ agrregation. The assay detected 9 potential hits as we found 7 extracts, 1 pure compound, and 2 fractions inhibited the aggregation of $A\beta_{1-42}$ by more than 80%. One compound of interest was silibinin which showed an IC_{50} of 2.36 μM (Fig. 1). To confirm the effect of silibinin on $A\beta_{1-42}$ aggregation, we examined the microscopic structure of $A\beta_{1-42}$ after incubation with silibinin. As shown in Fig. 2, after a 3 day incubation of $A\beta_{1-42}$ at 50 μM with 50 μM of silibinin which showed marked inhibition of $A\beta_{1-42}$ aggregation.

3.2. Silibinin decreases the cytotoxicity of $A\beta_{1-42}$ in SH-SY5Y cells

To test the influence of silibinin on the cytotoxicity of $A\beta_{1-42}$ aggregation, a MTT assay was employed; $A\beta_{1-42}$ at 100 μM was incubated with silibinin 10 μM for 3 days. The mixture/preparation was then added to SH-SY5Y cells at a final concentration of 2.5 μM and incubated for 48 h. Before the treatment with Aβ, the media of SH-SY5Y cells was replaced with phenol red-free and serum-free DMEM. The cell cytotoxicity was assessed with the MTT assay. The results showed a strong cytotoxicity of Aβ, which was significantly decreased following the incubation with silibinin (Fig. 3). To correct the influence of silibinin itself on the cell viability, equal amount of silibinin was added to cell medium and the cells were treated with freshly prepared $A\beta_{1-42}$ aggregates.

3.3. Silibinin prevents $A\beta_{1-42}$ induced cell injury in SH-SY5Y cells

To evaluate the effect of silibinin on $A\beta_{1-42}$-induced cell damage in SH-SY5Y cells, we preincubated cells with silibinin prior to adding $A\beta_{1-42}$. After treatment with 2.5 μM $A\beta_{1-42}$ for 24 h, cell

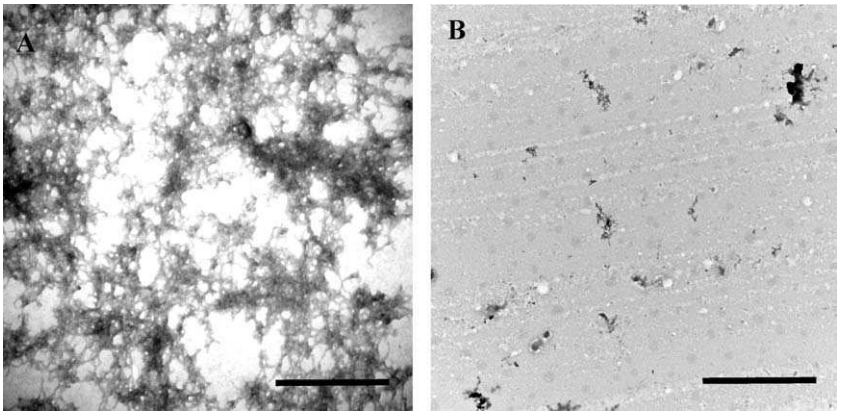

Fig. 2. Electron microscopy imaging of $A\beta_{1-42}$ aggregates in the presence or absence of Silibinin. Electron microscopy analysis of $A\beta_{1-42}$ aggregates was carried out after 3 days of incubation without (A) or with (B) silibinin at 50 μM. Scale bar = 500 nm. Images were acquired using Hitachi 7500 transmission electron microscopy at 80 kV with 100,000× magnification.

viability was determined using the MTT assay. The results showed that silibinin prevented cell damage induced by the addition of $A\beta_{1-42}$. The effect was dose dependent and we found that 10 μM silibinin was capable of increasing SH-SY5Y cell viability by 21% as compared to the effects of $A\beta_{1-42}$ under control conditions (Fig. 4).

3.4. Silibinin attenuates the production of H_2O_2 in $A\beta_{1-42}$-treated SH-SY5Y cells

To further explore the protective mechanism of silibinin on $A\beta_{1-42}$ induced cell damage in SH-SY5Y cells, we measured the level of H_2O_2 in $A\beta_{1-42}$ treated cells. The results demonstrated that incubation with 2.5 μM $A\beta_{1-42}$ peptide increased H_2O_2 levels compared to control, and by contrast, silibinin dose-dependently decreased the level of H_2O_2 in $A\beta_{1-42}$ peptide treated SH-SY5Y cells (see Fig. 5).

4. Discussion

There is an accumulating body of evidence linking oxidative damage to the brain and a wide range of neurodegenerative diseases, including Alzheimer's disease (AD) which is characterized by extracellular amyloid plaques and intracellular neurofibrillary tangles (Goedert and Spillantini, 2006; Selkoe, 2002). Furthermore, increasing data showed that Aβ is responsible for senile plaque formation and cell damage in AD (Cras et al., 1991; Yamaguchi et al., 1994). Several different hypotheses have been put forward to explain this toxic effect, including the formation of ion channels in cell membrane, the spontaneous fragmentation of Aβ to generate peptidyl radicals, and the direct formation of H_2O_2 by the peptides. According to the latter hypothesis, Aβ generates hydrogen peroxide from molecular oxygen through electron transfer interactions involving bound redox-active metal ion (Curtain et al., 2001; Huang et al., 1999). Hydrogen peroxide is readily converted to a highly reactive hydroxyl radical by Fenton reaction and both forms may be responsible for some of the oxidative damage observed during post-mortem examination of AD patient's brain tissue (Hong and Schoneich, 2001; Rival et al., 2009).

Silibinin, a flavonoid isolated from milk thistle (*Silybum marianum*) and has been used clinically for thousands of years in China and Europe as an anti-hepatotoxic agent to treat liver diseases, especially alcoholic liver disease (Brandon-Warner et al.,

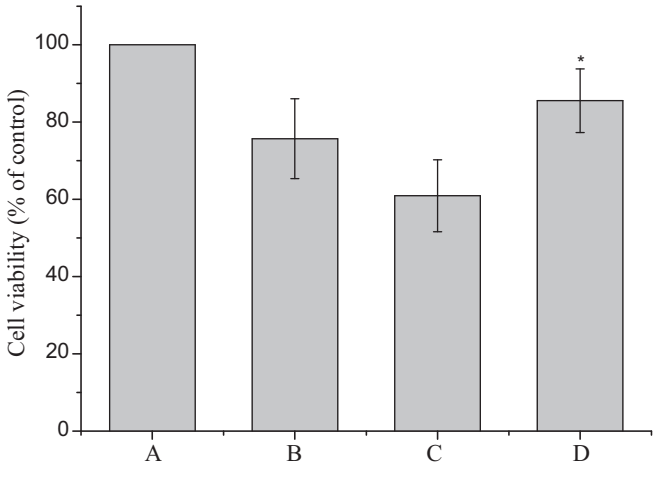

Fig. 3. Silibinin (Sil) decreases $A\beta_{1-42}$ cytotoxicity by inhibiting its aggregation. After incubation of 100 μM $A\beta_{1-42}$ for 72 h in the presence (D) or absence (C) of 10 μM silibinin, these preincubated $A\beta_{1-42}$ along with the freshly prepared $A\beta_{1-42}$ (B) were used at indicated concentrations (2.5 μM) to treat SH-SY5Y cells. After 24 h of incubation, cell viability was determined using the MTT assay. A, control; B, freshly prepared $A\beta_{1-42}$; C, preincubated $A\beta_{1-42}$ alone; D, $A\beta_{1-42}$ preincubated with silibinin for 3 days. Data are mean ± SD from three independent experiments. *, $P < 0.05$ vs. group C.

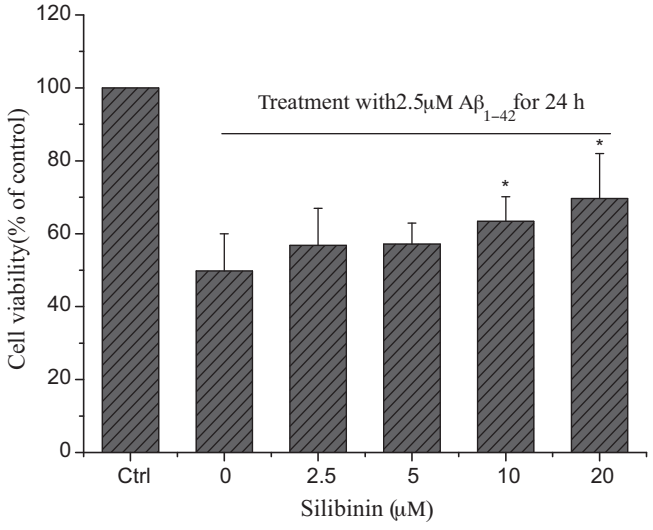

Fig. 4. Effect of silibinin on the cell viability of SH-SY5Y in the presence or absence of $A\beta_{1-42}$ aggregates. After the cells were treated with the indicated concentration of silibinin for 2 h in the phenol red-free and serum-free media, the cells were incubated for 48 h in the presence or absence of 2.5 μM $A\beta_{1-42}$. The cell viability was determined using the MTT assay. Data are means ± SD of three independent experiments. *, $P < 0.05$ vs. the Aβ group alone.

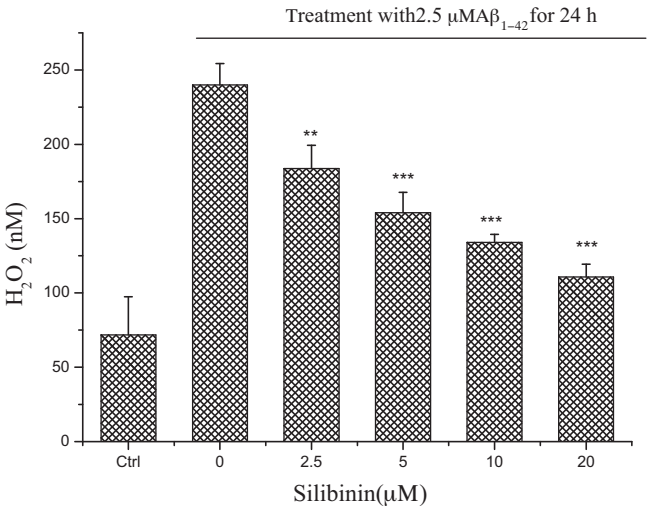

Fig. 5. Silibinin decreases the concentration of H_2O_2 in Aβ-treated SH-SY5Y cells. After SH-SY5Y cells were preincubated with silibinin for 2 h, $Aβ_{1-42}$ was added with the final concentration of 2.5 μM, and incubated 24 h. The concentration of H_2O_2 in the medium was determined with a peroxide hydrogen assay kit as per directions. Data are means ± SD from 4 wells of two independent experiments. **, $P < 0.01$ vs. control, and #, $P < 0.05$, ##, $P < 0.01$ vs. the group of Aβ alone.

2010; Guigas et al., 2007). Recent studies have shown that silibinin has a strong antioxidant activity, increases cellular glutathione content, induces superoxide dismutase (SOD), antagonizes manganese-induced neurotoxicity in adult mice, and inhibits lipid peroxidation (Schumann et al., 2003). In addition, silibinin also possess anti-inflammatory (Min et al., 2007), growth-modulating and anti-carcinogenic effects (Chu et al., 2004). In the current study, we discovered, using a ThT assay and electron microscopic imaging analysis, that silibinin appears to be a strong inhibitor of Aβ aggregation. Although similar result was just reported using a flavonoid mixture including silibinin (Murata et al., 2010), our finding on the actions of this pure compound silibinin further confirmed the inhibitory activity on that this natural falvanoid has on Aβ aggregation.

In addition to the inhibition of $Aβ_{1-42}$ aggregation, treatment of SH-SY5Y cells with silibinin markedly and dose-dependently increased cell viability in the presence of $Aβ_{1-42}$ aggregates. Further experiments showed silibinin also decreased the level of H_2O_2 induced by $Aβ_{1-42}$ in human neuroblastoma SH-SY5Y cell line based assay. The increased production of H_2O_2 due to the presence of Aβ aggregates is consistent with the findings reported by others (Tabner et al., 2005).

In conclusion, we report that silibinin appears to act as an inhibitor of Aβ aggregation and protects SH-SY5Y cells from Aβ-induced neuronal damage and neurotoxicity. The neuroprotective effect of silibinin seems to be the result of an attenuation of H_2O_2 production induced by Aβ aggregates. Silibinin has an established safety profile and has a long history of use as a clinical treatment of liver diseases (Feher et al., 1987). The present results, along with other studies might broaden the application of silibinin in the treatment, mitigation or slowing of neurodegenerative diseases and disorders involving aggregation and/or oxidative stress. Our findings suggest that silibinin or derivatives thereof may have potential therapeutic benefits for neurodegenerative disease, including AD.

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