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Effect of Mycelial Extract of *Clavicorona pyxidata* on Acetylcholinesterase and β-Secretase Activity *in vitro*

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In a previous study, an extract of *Clavicorona pyxidata* DGUM 29005 mycelia demonstrated an inhibitory effect against enzyme-associated perceptual disorders. We have attempted to determine whether this mycelial extract is also capable of inhibiting the activities of acetylcholinesterase (AChE) and β-secretase (BACE) activity. Butanol, ethanol, and water extracts of *C. pyxidata* DGUM 29005 mycelia were shown to inhibit AChE activity by 99.3%, 93.7%, and 91.7%, respectively. The inhibitory value of the butanol extract was more profound than that of tacrine (95.4%). The ethanol extract also exerted an inhibitory effect against BACE activity; this fraction may harbor the potential for development into a pharmocotherapeutic modality for the treatment of Alzheimer's disease (AD) patients. Rat pheochromocytoma PC12 cells in culture were not determined to be susceptible to the cytotoxic activity evidenced by the mycelial extract. The ethanol extract inhibited endogenous AChE activity in PC12 cellular homogenates, with an IC_{50} of 67.5 μg/ml, after incubation with intact cells, and also inhibited BACE activity in a dose-dependent fashion. These results suggest that the *C. pyxidata* mycelial extract has the potential to enhance cholinergic function and, therefore, may perform a function in the amelioration of the cholinergic deficit observed in cases of AD, as well as other types of age-associated memory impairment.

Keywords: *Clavicorona pyxidata*, Acetylcholinesterase, β-secretase, Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder which develops gradually and induces memory loss, unusual behavior, personality changes, and a general decline in thinking abilities (Parihar and Hemani, 2004). More than 12 million individuals worldwide have this disease, and as average life expectancy increases, the number of individuals with AD will necessarily increase, due to the increasing susceptibility to AD inherent to advanced age (Citron, 2004). AD is debilitating in patients who have had it for several years, and the cost of treatment can be substantial. AD care places a heavy social and financial burden both on society and on the families of patients. Therefore, the development of effective drugs for the prevention and treatment of AD is considered to be a matter of great importance. Acetylcholinesterase (AChE) inhibitors have been approved for AD therapy. These drugs have indicated obvious symptom-reducing effects, but are insufficient to effect a cure for the disease (Enz *et al.*, 1993). Extensive research has been conducted in an attempt to discover effective anti-AD drugs, including studies of AD pathogenesis and the identification of novel therapeutic targets (Wolfe, 2001). Recently, a novel aspartyl protease, β-secretase (BACE), as a promising new target for AD, has received considerable attention as a promising new target for AD (Ghosh *et al.*, 2002). BACE has been identified as a member of the pepsin family of aspartyl proteases. Several studies have shown increased β-secretase activity in patients with sporadic AD. Additionally, BACE knockout mice that were otherwise healthy, fertile, and phenotypically normal throughout adulthood are deficient with regard to amyloid-β (Aβ) production (Cai *et al.*, 2001; Luo *et al.*, 2001; Li *et al.*, 2004). As BACE was first discovered in 1999 (Sinha *et al.*, 1999), a large number of BACE inhibitors have been developed. Unfortunately, the majority of these BACE inhibitors

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(Ghosh *et al.*, 2000), which, if used as drugs, will face formidable obstacles to efficacy, as their peptide structures render them vulnerable to degradative enzymes, rapid biliary clearance, and poor oral absorption (Plattner and Norbeck, 1990). The development of more therapeutically promising low molecular-weight, potent, nonpeptide BACE inhibitors is clearly a matter of necessity.

Clavicorona pyxidata is a common basidiomycete, which is distributed worldwide distribution (Corner, 1970). *C. pyxidata* is an edible fungus with a generally favorable reputation among mycophiles (Miller, 1977). The mycelial and culture broth extracts of *C. pyxidata* have demonstrated inhibitory effects against AChE and proryl endopeptidase activity (Lee *et al.*, 1999; Lee, 2004). In this study, we have attempted to determine whether the *C. pyxidata* mycelial extract exerts an inhibitory effect on the activities of AChE and BACE, including endogenous AChE and BACE activity in rat pheochromocytoma PC12 cells.

Materials and Methods

Materials

All chemicals used in this study were of the highest purity. Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, heat-inactivated horse serum, and fetal bovine serum (FBS) were acquired from Gibco (USA). Acetylthiocholine, acetylcholinesterase (AChE), dimethyl sulfoxide (DMSO), 5, 5'-dithiobis-(2-nitro)-benzoic acid (DTNB), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma Co. (USA). β-Secretase (BACE1) was obtained from the Invitrogen Co. (USA). The β-secretase assay kit was purchased from ANASPEC (USA).

Clavicorona pyxidata and preparation of extract

C. pyxidata used in this study was isolated from the fruiting body, identified, and designated as *C. pyxidata* DGUM 29005. The RNA sequences of ITS1, 5.8S and ITS2 of *C. pyxidata* DGUM 29005 (Lee *et al.*, 2006) were deposited in the Genbank database (accession no. AY 588248). The mycelia of *C. pyxidata* were cultivated for 20 days in YMG broth (0.4% yeast extract, 1.0% malt extract, and 0.4% glucose) at 24°C with shaking at 120 rpm. The mycelia were harvested via vacuum filtration and washed 3 times in distilled water. The 10 g of mycelia were homogenized by Ultra-turrax T-50 (Janke Kunkel IKA-Labortechniker, Germany) for 10 min at 7,000 rpm. The homogenized mycelia were extracted for 24 h with 100 ml ethanol at 4°C. The extracts were then evaporated and freeze-dried. The ethanol extract was dissolved in distilled and demineralized water and added to an equal volume of *n*-hexane. The aqueous extract was then fractionated via successive extractions with ethyl acetate and butanol. These extract fractions were designated as *n*-hexane, ethyl acetate, butanol, and water, respectively. After each of the extract fractions was evaporated and freeze-dried, the water extract fraction was dissolved in water and the other fractions were dissolved in DMSO.

Cell culture

Rat pheochromocytoma PC12 cells were obtained from the American Type Culture Collection (ATCC, USA). They were maintained in medium consisting of a 1:1 mixture of DMEM/F-12 supplemented with 5% heat-inactivated FBS and 10% heat-inactivated horse serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. When the PC12 cells growing on the 100-dishes had achieved 90% confluence, they were added to various concentrations of mycelial extract then incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. After incubation, the PC12 cells were lysed in order to release pure AChE and BACE, which were used as endogenous enzyme sources.

Cytotoxicity assay

In vitro cytotoxicity was determined using a rapid colorimetric MTT assay, in accordance with the method previously described by Plumb *et al.* (1989) and Page *et al.* (1988), with some modifications. To carry out this assay, we added 50 μl of MTT solution (2 mg/ml) to each well in a 96-well plate and incubated it 4 h. The supernatant was then removed, and the formazan crystals produced were dissolved in 150 μl of DMSO and via measurements of the optical density of the solution at 540 nm using an ELISA reader (Molecular Devices, USA).

Assay of AChE inhibition activity

An AChE inhibition assay was conducted in accordance with the method previously described by Ellman *et al.* (1961), with some minor modifications. To obtain an endogenous enzyme source, the PC12 cells were homogenized with a Glass-Col homogenizer in 5 volumes of buffer (10 mM Tris-HCl: pH 7.2, with 1 M NaCl, 50 mM $MgCl_2$, and 1% Triton X-100) containing 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 100 μg/ml Na_3VO_4. The lysed cells were then transferred to tubes on ice and then clarified by centrifugation at 15,000 × g for 10 min at 4°C to remove the cellular debris. The supernatant was used as an endogenous source of AChE. A typical run consisted of 20 μl of the AChE solution (1 unit/ml), 860 μl of 50 mM sodium phosphate buffer (pH 8.0), and 20 μl of the test solution. The reactants were mixed and incubated

at 37°C for 15 min. The absorbance was read at 412 nm immediately after the addition of 100 μl of Ellman reaction mixture (0.5 mM acetylthiocholine and 1 mM DTNB). The reading was repeated for 10 min in 2 min intervals to verify that the reaction had occurred in a linear fashion. A blank reaction was measured via the substitution of the suspended solvent for the enzyme solution. The degree of enzyme inhibition was calculated in accordance with the following equation: Inhibition (%) = 100 × (1-inhibited reaction/ uninhibited reaction). All controls, blanks, and samples were run in triplicate.

Assay of BACE inhibition activity

The BACE inhibition assay was conducted using a β-secretase assay kit from ANASPEC (San Jose, USA) in accordance with the manufacturer's recommended protocol. To obtain an endogenous enzyme source, PC12 cells were homogenized with a Glass-Col homogenizer in 150 μl of β-secretase buffer (BioVison, USA). These lysed cells were then incubated for 10 min on ice, and then clarified by centrifugation at 15,000 × g for 10 min at 4°C to remove the cellular debris. The resultant supernatants were used as endogenous sources of BACE. This method is premised on the secretase-dependent cleavage of a secretase-specific peptide conjugated to the fluorescent reporter molecules HiLyte Flour™ 488 and QXL™ 520, which results in the release of a fluorescent signal that can be detected on a fluorescent microplate reader (excitation wavelength: 488 nm; emission wavelength: 520 nm).

Statistical analysis

The data were expressed as the average ± SD (standard deviation). The mean values were compared using Ducan's Multiple Range Test using SPSS software (SPSS Inc., USA); P values of < 0.05 were considered to be significant.

Results

Inhibitory effect of AChE activity

We evaluated the solvent extracts of *C. pyxidata* mycelia to determine the presence of an inhibitory effect on AChE (Table 1). Among the tested extracts, the butanol extract exhibited the strongest inhibitory activity (99.3%), followed by the ethanol extract (93.7%), water extract (91.7%), ethyl acetate extract (75.3%), and hexane extract (52.9%). Lee *et al.* (1999) reported that a methanol extract of *C. pyxidata* demonstrated 27% to 31% AChE inhibitory activity. The value of the butanol extract was higher than that determined for the positive control (95.4%). These results show that *C. pyxidata* mycelia extracts exert a

Table 1. Inhibitory effect of extract fraction of *C. pyxidata* mycelia on AChE activities

Fraction	Inhibition of AChE activity (%)
Ethanol	93.7 ± 2.3
Hexane	52.9 ± 1.6
Ethyl acetate	75.3 ± 0.2
Butanol	99.3 ± 2.1
Water	91.7 ± 1.6
Tacrine[a]	95.4 ± 2.3

The final concentration of sample was 100 μg/ml. Inhibition (%) = 100 × (1-inhibited reaction/uninhibited reaction). Values represent the means ± SD of three separate experiments that were each performed in triplicate.
[a] Positive control

Table 2. Inhibitory effect of extract fraction of *C. pyxidata* mycelia on BACE activities

Fraction	Inhibition of BACE activity (%)
Ethanol	28.7 ± 2.2
Hexane	20.8 ± 1.2
Ethyl acetate	0
Butanol	20.2 ± 2.4
Water	0

The final concentration of sample was 100 μg/ml. Inhibition (%) = 100 × (1-inhibited reaction/ uninhibited reaction). Values represent the means ± SD of three separate experiments that were each performed in triplicate.

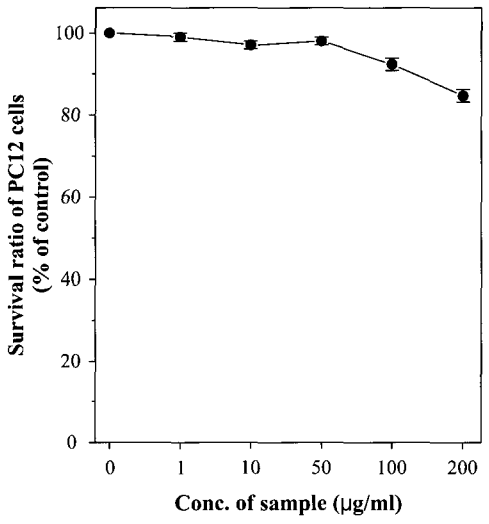

Fig. 1. Effect of the ethanol extract of *C. pyxidata* mycelia on the growth of PC12 cells. The PC12 cells were incubated with various concentrations of mycelial extract for 48 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. The survival ratio, which was expressed as a percentage of the survival of the controls, was determined using the MTT assay. The values represent the means ± SD of three separate experiments, each of which was conducted in triplicate.

beneficial AChE-inhibitory effect.

Inhibitory effect of BACE activity

We evaluated the inhibitory effects of *C. pyxidata* mycelia solvent extracts on BACE activity (Table 2). Among the tested extracts, the ethanol extract, hexane extract, and butanol extract of *C. pyxidata* demonstrated 28.7%, 20.8%, and 20.2% inhibitory effect against BACE, respectively. Other extract fractions exerted no effects against BACE activity.

Effect of the ethanol extract of C. pyxidata mycelia on AChE and BACE activity in PC12 cells

We also attempted to determine whether a *C. pyxidata*

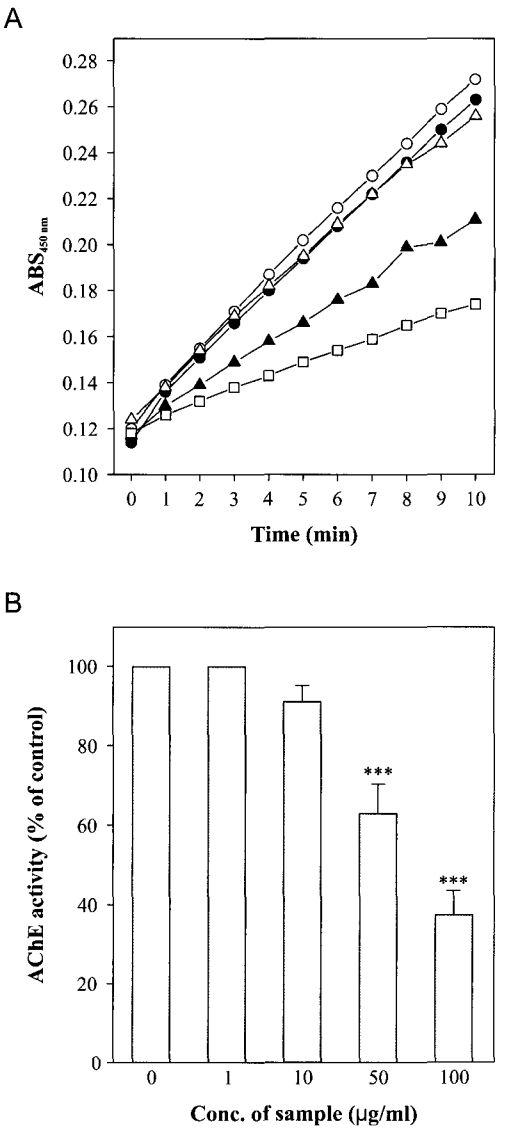

Fig. 2. Inhibitory effects of the ethanol extract of *C. pyxidata* mycelia on endogenous AChE activity. (A) Kinetics of AChE activity in PC12 cells incubated with various concentrations of mycelial extract-0 μg/ml (○), 1 μg/ml (●), 10 μg/ml (△), 50 μg/ml (▲), 100 μg/ml (□) - for 48 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. (B) A histogram was constructed in order to compare the inhibitory activity of AChE in various concentrations of mycelial extract. The values represent the means ± SD of three separate experiments, each of which were conducted in triplicate. *** $P < 0.001$ compared with control group (0 μg/ml).

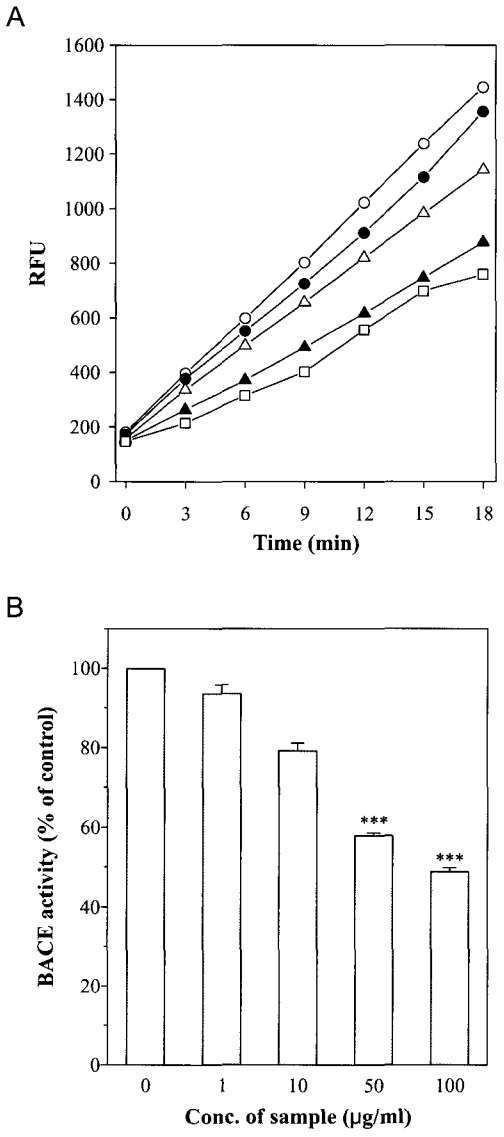

Fig. 3. Inhibitory effects of the ethanol extract of *C. pyxidata* mycelia on endogenous BACE activity. (A) Kinetics of BACE activity in PC12 cells incubated with various concentrations of mycelial extract-0 μg/ml (○), 1 μg/ml (●), 10 μg/ml (△), 50 μg/ml (▲), 100 μg/ml (□) - for 48 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. The increase in fluorescence intensity was monitored at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. (B) A histogram was constructed in order to compare the inhibitory activity of BACE in various concentrations of mycelial extract. The values represent the means ± SD of three separate experiments, each of which were conducted in triplicate. *** $P < 0.001$ compared with control group (0 μg/ml).

mycelial extract inhibited intracellular AChE and BACE activity following incubation with intact PC12 cells. These cells were determined to be insensitive to *C. pyxidata* mycelial extract, as shown in Fig. 1. An AChE inhibitory effect was detected at various concentrations of the ethanol extract of mycelia (Fig. 2). A 50 μg/ml and 100 μg/ml sample of the ethanol extract of mycelia resulted in 37% ($P < 0.001$) and 63% ($P < 0.001$) inhibitions of AChE activity, respectively. The ethanol extract of mycelia manifested a dose-dependent inhibitory effect against AChE activity, evidencing an IC_{50} value of 67.5 μg/ml.

As is shown in Fig. 3, 100 μg/ml of the ethanol extract effected a in 53% inhibition of BACE activity ($P < 0.001$). The mycelial ethanol extract also demonstrated a dose-dependent inhibitory effect on BACE activity, with an IC_{50} value of 92.5 μg/ml.

Discussion

Acetylcholinesterase (AChE) inhibition plays an important role in Alzheimer's disease (AD), which is caused principally by cholinergic dysfunction. The degree of cognitive impairments in individuals suffering from AD has been reported to be strongly correlated with central cholinergic deficits (Coyle *et al.*, 1983). This provides a rationale for current approaches to AD drug therapy. It has been suggested that the elevation of the acetylcholine level might help to improve the symptoms of cognitive deficits in AD (Chemnitius *et al.*, 1996). Several groups have attempted to enhance the synaptic acetylcholine levels via the adminstration of acetylcholine precursors, cholinergic agents, or AChE inhibitors (McGeer and McGeer, 1995) including tacrine and galantamine, which prevent acetylcholine hydrolysis. Thus, the identification of an AChE inhibitor is a prerequisite for the effective treatment of patients with AD.

BACE is an excellent target for anti-amyloid therapy. Potent peptidic inhibitors of this enzyme have been previously studied (Shinha *et al.*, 1999; Ghosh *et al.*, 2000), but such peptides are unlikely to exhibit the required metabolic properties of a drug, as they will not penetrate the blood-brain barrier to a sufficient extent. Instead, it is necessary to generate small organic molecules, which specifically inhibit BACE specifically. This is a significant challenge, but a rational design approach may result in the identification of such molecules, as has been shown with protease targets (Leung *et al.*, 2000).

The primary objective of the present study was to determine whether the *C. pyxidata* mycelial extract exerted an inhibitory effect against AChE and BACE activity. Based on our findings, we are able to conclude that this extract exerts a dose-dependent inhibitory effect against AChE and BACE activity. Additionally, the cultured cells were found not to be susceptible to the cytotoxic effects of this extract. These results indicate the *C. pyxidata* mycelial extract has the potential to enhance cholinergic functions and, therefore, may perform a significant function in ameliorating the cholinergic deficits associated with AD, as well as with other types of age-related memory impairment. Further studies will be required in order to determine whether or not the *C. pyxidata* mycelial extract inhibits BACE activity *in vivo*.

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