

# *In vitro* Anti- $\beta$ -secretase and Dual Anti-cholinesterase Activities of *Camellia sinensis* L. (tea) Relevant to Treatment of Dementia

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The primary target of licensed drugs for the treatment of Alzheimer's disease is the inhibition of the enzyme acetylcholinesterase, although preventing  $\beta$ -amyloidosis is a prime target for drugs in development. The *in vitro* dual anti-cholinesterase and  $\beta$ -secretase activities of *Camellia sinensis* L. extract (tea) is reported. Green and black tea inhibited human acetylcholinesterase (AChE) with IC<sub>50</sub> values of 0.03 mg/mL and 0.06 mg/mL respectively, and human butyrylcholinesterase (BuChE) with IC<sub>50</sub> values 0.05 mg/mL. Green tea at a final assay concentration of 0.03 mg/mL inhibited  $\beta$ -secretase by 38%. These novel findings suggest that tea infusions contain biologically active principles, perhaps acting synergistically, that may be used to retard the progression of the disease assuming that these principles, yet to be identified, reach the brain. Copyright © 2004 John Wiley & Sons, Ltd.

**Keywords:** cholinergic; cholinesterases;  $\beta$ -secretase; dementia; *Camellia sinensis*.

## INTRODUCTION

The functioning of the brain cholinergic system, which is involved in attention and memory (Perry *et al.*, 2000) declines during normal aging and is further affected in Alzheimer's disease (AD) (Perry *et al.*, 1978). In AD and related disorders such as vascular dementia and dementia with Lewy bodies there is a decline in cholinergic activity in the key areas involved in memory and cognition (Grantham and Geerts, 2002). Current drugs for the symptomatic treatment of dementia are aimed at enhancing the associated cholinergic deficit by inhibiting AChE (Rosler *et al.*, 1999), an enzyme which cleaves the neurotransmitter acetylcholine (ACh). Inhibiting the activities of cholinesterases (AChE and also BuChE), increases the level of the neurotransmitter with positive effects on cognitive function (Costa *et al.*, 1999; Nordberg *et al.*, 2001; Giacobini *et al.*, 2002). BuChE increases in the brains of people with AD and may play a role in the progression of the disease by its ability *inter alia* to hydrolyse the neurotransmitter ACh (Giacobini, 2001). It has been suggested that inhibition of both enzymes should be one of the objectives in the treatment of cognitive dysfunctions associated with diseases such as AD (Greig *et al.*, 2001).

An additional therapeutic target in the treatment of AD is the inhibition of the proteolytic enzyme  $\beta$ -secretase. This enzyme is involved in production of  $\beta$ -amyloid peptides (A $\beta$ ) via cleavage of amyloid precursor protein (APP). The deposition and aggregation of A $\beta$  are a key event in the onset, progression and

pathogenesis of AD (Selkoe, 2001). Overexpression of APP in transgenic mice has been shown to lead not only to increased A $\beta$  levels but to the development of the pathological hallmarks of AD, including numerous extracellular A $\beta$  deposits, neuritic plaques and synaptic loss (Games *et al.*, 1995). Further, recent reports (Cai *et al.*, 2001; Luo *et al.*, 2001; Roberds *et al.*, 2001) demonstrated that  $\beta$ -secretase knockout mice lack brain A $\beta$  without observed side-effects. In addition, it has been demonstrated *in vitro* that selective inhibition of  $\beta$ -secretase by a synthetic compound suppresses A $\beta$  secretion in human embryonic kidney cells (Hom *et al.*, 2003).

Here we report that tea infusions *in vitro* have dual anti-cholinesterase and anti- $\beta$ -secretase activities relevant to the treatment of dementia.

## MATERIALS AND METHODS

**Extract preparation.** An extract of green tea (Fempic of Heaven, China Green Tea, special gun powder, Shanghai Tea Import & Export Company) was made using freshly boiled water (1:25 w/v) for 45 min. For black tea (PG Tips, Unilever Bestfoods, UK) a 30 min infusion was prepared in boiled water (1:40 w/v). The coffee (Douwe Egberts 'continental gold' instant, medium roast, Marks & Spencer, UK) was prepared in boiled water (1:40 w/v). The infusions were left to cool to room temperature and centrifuged (12 000 rpm, 15 min, supernatant re-centrifuged). 1 mL aliquots of the supernatant were freeze dried in order to determine their dry weight equivalents. The freeze dried aliquot was reconstituted in water and assayed using serial dilutions.

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**Cholinesterase assays.** Acetylcholinesterase (EC 3.1.1.7) from human erythrocytes, acetylthiocholine iodide (ATChI), butyrylcholinesterase (EC 3.1.1.8) from human serum, butyrylthiocholine iodide (BTChI), 5:5-dithiobis-2-nitrobenzoic acid (DTNB) and sodium bicarbonate were purchased from Sigma Co., UK.

An assessment of cholinesterase inhibition was carried out on 96-well microtitre plates using a colorimetric method of Ellman *et al.* (1961) A typical run consisted of 5  $\mu$ L of human AChE or BuChE solution, at final assay concentrations of 0.03 U/mL and 0.01 U/mL respectively; 200  $\mu$ L of 0.1 M phosphate buffer pH 8; 5  $\mu$ L of DTNB, at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer pH 7 with 0.12 M of sodium bicarbonate; and 5  $\mu$ L of the test solution. The reactants were mixed in a 96-well microtitre plate and the mixture preincubated for 30 min at 30 °C. The reaction was initiated by adding 5  $\mu$ L of ATChI or BTChI at final concentrations of 0.5 mM. As a control the inhibitor solution was replaced with buffer. The control was assayed in triplicate. To monitor any non-enzymatic hydrolysis in the reaction mixture two blanks for each run were prepared in triplicate. One blank consisted of buffer replacing enzyme and a second blank had buffer replacing substrate. Change in absorbance at 405 nm was measured on a Titertek Multiscan MCC/340 96-well plate reader for a period of 6 min at 30 °C.

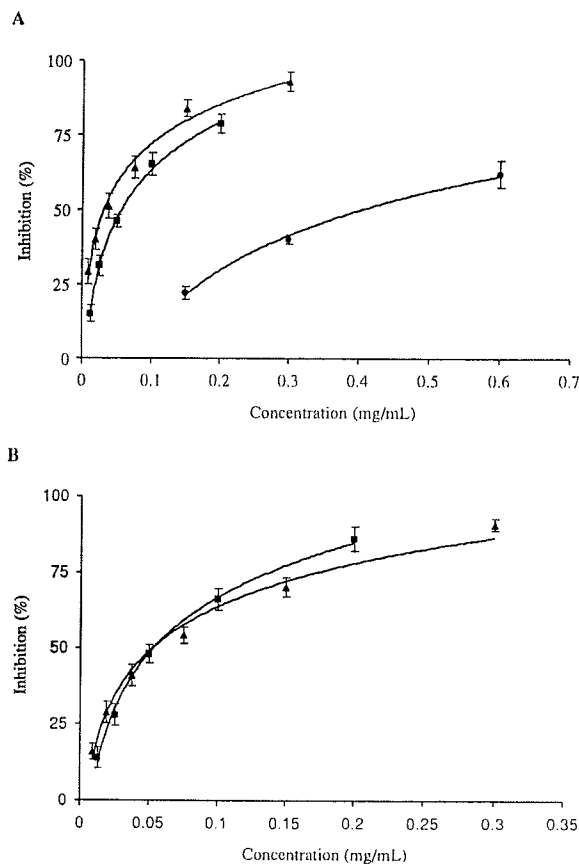
**$\beta$ -secretase assay.** Secretase activity kit and BACE-1 ( $\beta$ -secretase) were purchased from R&D systems Europe, Ltd, UK. The EDANS/DABCYL substrate corresponds to the amino acid sequence associated with  $\beta$ -secretase cleavage of amyloid precursor protein (aa 668–675) and includes substitution of Asn and Leu for amino acids Lys<sub>670</sub> and Met<sub>671</sub> known as the Swedish mutation. Recombinant human BACE-1: A cDNA sequence encoding the ectodomain (amino acid residues 1–460) of the recombinant human  $\beta$ -secretase was expressed as secreted protein with a COOH-terminal His tag in a murine myeloma cell line, NS0. Purity > 90%. Cleavage of the substrate, conjugated to the reporter molecules EDANS and DABCYL, by BACE-1 separates the EDANS and DABCYL allowing the release of a fluorescent signal. The level of secretase enzymatic activity is proportional to the fluorometric reaction. A measurement of fluorescence was performed on a Tecan SPECTRAFluoro Plus fluorometer with Magellan software.

**Assay procedure.** BACE-1 was reconstituted and further diluted with deionized water. 40  $\mu$ L BACE-1 (4.8  $\mu$ g/mL final assay concentration) was mixed with 50  $\mu$ L reaction buffer (supplied) and 10  $\mu$ L green tea extract solution. The reaction mixture was incubated over 5, 30, 60, 90 and 120 min in the dark at 37 °C. 5  $\mu$ L substrate was added after the periods of incubation time. A 96-well flat bottom microtitre plate was read on a fluorescence micro-plate reader in endpoint mode at an excitation wavelength of 360 nm and emission at 535 nm. The negative control was as above with the exception that a 10  $\mu$ L test solution was replaced with 10  $\mu$ L deionized water. Two blanks were prepared. Blank 1 was made as above with the exception that 40  $\mu$ L BACE was replaced with 40  $\mu$ L deionized water and 10  $\mu$ L of deionized water was used instead of inhibitor, whereas in blank 2, 5  $\mu$ L substrate was

replaced with 5  $\mu$ L deionized water and 10  $\mu$ L deionized water was added instead of inhibitor. All controls, blanks and samples were run in triplicate.

## RESULTS

Green and black tea inhibited the activity of AChE in a concentration dependent manner (Fig. 1a), with green tea as prepared, being the most potent. Coffee was a less potent inhibitor of AChE. IC<sub>50</sub> values were 0.03  $\pm$  0.004 mg/mL, 0.06  $\pm$  0.005 mg/mL and 0.41  $\pm$  0.004 mg/mL for green tea, black tea and coffee, respectively. Both teas exhibited concentration dependent inhibition of BuChE activity with IC<sub>50</sub> values of 0.05  $\pm$  0.005 mg/mL and 0.05  $\pm$  0.007 mg/mL, respectively (Fig. 1b), whereas coffee was inactive on this enzyme. Green tea at a final assay concentration of 0.03 mg/mL inhibited  $\beta$ -secretase by 27% after 5 min incubation, whereas after 60 min inhibition reached 38%. There was no further increase in the activity after 60 min incubation (Fig. 2). Statistical analysis (ANOVA) showed significant differences ( $p < 0.05$ ) in activity between the 5 min incubation and all the other incubation periods. No significant differences were shown among 30–120 min incubation times.



**Figure 1.** Inhibition of human AChE and BuChE by green tea, black tea and coffee extracts. (A) Dose response curves for AChE inhibition: green tea (▲), black tea (■), coffee (●). (B) Dose response curves for BuChE inhibition: green tea (▲), black tea (■). Concentrations are dry weight equivalent of the extracts in the assay ( $n = 6$ , mean  $\pm$  SD).

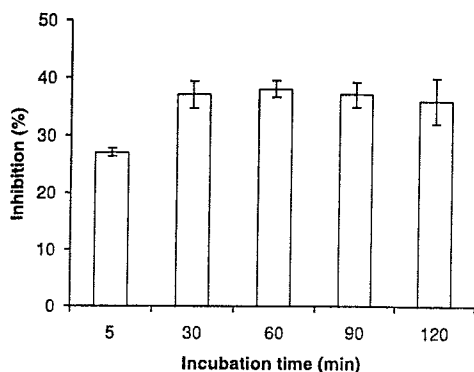


Figure 2. Inhibition of  $\beta$ -secretase activity of green tea extract ( $n = 3$ , mean  $\pm$  SEM).

## DISCUSSION

Previous studies have shown that both green and black tea possess pharmacologically protective, properties such as antioxidative (Leung *et al.*, 2001), anti-carcinogenic (Yang and Wang, 1993), neuroprotective (Lee *et al.*, 2000) and hypocholesterolaemic effects (Imai and Nakachi, 1985). Our study indicates that *C. sinensis* has the potential to enhance cholinergic function and therefore may have a role in ameliorating the cholinergic deficit in AD and other age related memory impairments. Relating cholinomimetic effects to the individual constituents of tea is challenging since the chemistry of *C. sinensis* is complex (Harbowy and Balentine, 1997).

One of the major constituents of tea, epigallocatechin gallate, has been reported to facilitate cholinergic transmission in guinea-pig myenteric plexus (Katayama *et al.*, 2002). Caffeine, theophylline and theobromine, showed no anti-cholinesterase activity at concentrations present in the infusions (data not shown).

We have also shown that green tea, as prepared, possesses anti- $\beta$ -secretase activity. *In vivo* 50% inhibition of the enzyme was effective in reducing  $A\beta$  in mice (Dewachter and Van Leuven, 2002), hence the activity of the tea extract could also be physiologically relevant. To date, only a few inhibitors of  $A\beta$  have been reported (Sinha *et al.*, 1999; Gosh *et al.*, 2001a; Gosh *et al.*, 2001b), all of them synthetic chemicals and of limited therapeutic potential because of high molecular weights likely to restrict transfer across the blood – brain barrier. There are no reports (to our knowledge) of natural plant product inhibitors of  $\beta$ -secretase.

The effects of tea infusions on the cerebral cholinergic system and  $\beta$ -secretase *in vivo* will depend on the levels of the enzymes in the brain (i.e. severity of the disease), the type and chemistry of the tea, infusion concentration (strength), dose (number of cups per day) and duration of consumption. It is also possible that regular consumption of tea by patients with dementia prescribed cholinesterase inhibitors may alter the effects of such drugs. Clinical and scientific investigation of the chemistry and activities of cholinomimetic and anti- $\beta$ -secretase compounds in *C. sinensis*, and cognitive effects of tea consumption is warranted in order to establish the relevance of our novel findings to the maintenance of cognitive function in old age and in diseases such as AD.

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