CHAPTER V

DISCUSSION

BM extract could enhance the MTT viability of NGF-deprived PC12 cells brought up in normal-serum RPMI1640. This result was still in congruent with the recent study of Limpeanchob N., et al. (2008) that the primary cortical cultural neurons induced by Aβ25-35 undergo to more cell death when treated with high dose of BM extract. At the dose of 100 μg/ mL, BM extract has not altered their cellular viability induced by Aβ25-35 for 24 hr but when prolong the treatment to 48 and 72 hr, this concentrations of BM extract can significantly enhance their viability 37% and 20% at 48 hr and 72 hr, respectively (Limpeanchob, et al., 2008). In that study, the 50% inhibitory concentration (IC_{50}) is 242.8+1.5 μg/ mL and the concentration of BM extract at 100 μg/ mL does not affect cell viability. Some different from the study by Limpeanchop, N. *et al.* might be attributed to the utilization of different cell culture system. PC12 or pheochromocytoma cell line is derived from rat adrenal medullar which has the same origin from neural crest as the other peripheral neurons. PC12 is NGF-dependent cells that when expose to NGF, they can induce to proliferate and differentiate to be sympathetic neurons (LUO Ben-yan, 2004). There are many findings report that NGF-dependent neurons may impair selectively during aging or AD in the basal forebrain (Gibbs, 1998b, a; Hefti and Schneider, 1989; Perez-Polo, et al., 1990; Salehi, et al., 2000). This might contribute to non-significant enhancement of the cellular viability in the cells treated with 100 μg/ mL concentration of BM extract for 48 hours (2 days). However, BM extract at 150 and 200 μg/ mL concentrations could significantly accentuate the cellular viability in the middle phase (4^{th} day) of the experiment (Figure. 9).

BM extract might accentuate the viability of apoptotic-induced neuronal cells. This study on the viability measurement of NGF-deprived PC12 cells, apoptotic-induced by serum-free medium (Table 5) was in congruent with the study by Shelton, SB and Johnson, GVW. in 2001, which showed that the removal of NGF resulted in a progressive loss of the cellular viability of PC12 cells maintained in serum-free medium (Shelton and Johnson, 2001). In this study on the viability measurement of

the differentiated SH-SY5Y cells, apoptotic-induced by camptothecin (Table 6.), was also relevant to the study by Mookherjee, P. and Johnson, GVW. in 2001 (Mookherjee and Johnson, 2001), which revealed the time-dependent cell death of the differentiated SH-SY5Y cells, apoptotic-induced by 5 μM camptothecin. Some little discrepancy might be attributed to the personal error or the different measuring technique. In this study, the MTT assay was run on the 96-well microplates which could carry only minute volume of solution. If only is little mistake in pipetting occurred, the great fluctuating result is prone to be obtained. Additionally, in those two studies, they measured their cell death from the percentage of LDH (lactase dehydrogenase) released from the death cells. On the other hand, the cellular viability in this study was measured from the reduction of MTT by reductase enzymes in the viable cells.

The capability of BM extract, especially at low concentration, to improve the cellular viability from the two models of apoptotic-induced neuronal cells in this study was again in accordant to the study by Limpeanchop, N., *et al.* (Limpeanchob, et al., 2008) with some exception. The primary cortical neuronal culture is induced with Aβ peptide and neuronal PC12 cell line is neurotrophic deprived are both their contributors to their cellular apoptosis (Ban, et al., 2006; Harada and Sugimoto, 1999; Ju Yeon and Yeon Hee, 2005; Saille, et al., 1999; Yagami, et al., 2003; Yagami, et al., 2002). Since NGF can rescue PC12 cells, brought up in neurotrophic-removed medium, from apoptosis (Macdonald, et al., 2003; Pappas, et al., 2003), PC12 grown in NGF-deprived serum-free medium may greater undergo apoptosis than those in normal-serum medium. For the differentiated SH-SY5Y cells, when they were treated by BM extract, they were also exposed to the DMSO (solvent of BM extract solution) and the camptothecin (apoptotic inducer) at the same time over the experiment. That meant the cells were more experienced to apoptosis than they were treated by DMSO or camptothecin alone. With these reasons, the BM extract could not able to significantly enhance up the cellular viability in these two apoptotic neuronal cells.

BM extract could dose-dependently attenuate Tau phosphoryltion (at Tau-1 site) in NGF-deprived PC12 cells, grown up in normal-serum RPMI1640 medium. This was attributed to the ability of the BM extract at 100 μg/ mL concentration to reduce down the amount of total Tau (Tau 5) expression but to increase up the amount of dephosphorylated Tau (at Tau 1 site) expression in NGF-

deprived PC12 cells, grown up in normal-serum RPMI1640 medium for 48 hours with more extent than the BM extract at 50 μg/ mL concentration, though it could be able to increase the cellular viability less than the BM extract at 50 μg/ mL concentration.

BM extract at 100 μg/ mL concentration could diminish Tau phosphoryltion (at Tau-1 site) in NGF-deprived PC12 cells, grown up in serum-free RPMI1640 medium. Because BM extract at 100 μg/ ml concentration could display more cell death than the BM extract at 50 μg/ ml concentration in NGF-deprived PC12 cells, grown up in serum-free RPMI1640 medium for 48 hours, it exhibited more total Tau than the BM extract at 50 μg/ ml concentration did. However, this tau was highly in the dephosphorylated form (at Tau-1 site).

In this study, BM extract could weaken phosphorylated Tau (at Tau-1 site) in the cells raised up in normal-serum medium more distinctively than the cells brought up in serum-free medium (Figure. 14). This was concordant with the study by Shelton, SB. And Johnson, GVW. that revealed a decrement in Tau-1 immunoreactivity and thus an increment in the phosphorylation of HMW Tau and Tau in PC12 cells deprived of serum and NGF (Shelton and Johnson, 2001).

BM extract tended to dose-dependently abate phosphorylated Tau (at Tau-1 site) in differentiated SH-SY5Y cells, apoptotic-induced by camptothecin. In parallel to the cellular viability that only 5 μM camptothecin caused more cell death than only 0.025% DMSO did, absolute 5 μM camptothecin has propensity to produce more amount of total Tau expression than the only 0.025% DMSO did in differentiated SH-SY5Y cells, apoptotic-induced by camptothecin . This was relevant to the study by Mookherjee, P. and Johnson, GVW that 5 μM camptothecin provided the differentiated SH-SY5Y to experience apoptotic death (Mookherjee and Johnson, 2001). Additionally, 0.025% DMSO impacted on the enhancement of the amount of total Tau expression in these cells. The cells raised up with BM extract were also exposed to both DMSO (BM extract solvent) and camptothecin (apoptotic-inducer) at the same time over the experiment. These two substances synergistically boosted up the amount of total Tau expression in these cells. However, BM extract might facilitate to dose-dependently dephosphorylated Tau (at Tau-1 site) (Figure. 16, 18, and 20) in these cells, especially at the beginning 12 hr of the experiment when most cells were still intact.

Nuydens, et al. (1997) reported that differentiated PC12 undergo programmed cell death after NGF-deprivation with the prominence of neurite retraction and the vivid signal aberrant tau phosphorylation at AT8 site (Ser202/Thr205) (Nuydens, et al., 1997). Immunoblot analysis presents an increasing in tau phosphorylation at tau-1 site (Davis and Johnson, 1999a). Alteration of tau phosphorylation state during PC12 apoptosis can abate tau microtubule-binding ability and tau phosphorylaton at tau-1 site occurs throughout cell body except in nucleus and at perinuclear regions (Davis and Johnson, 1999b). Hyperphosphorylation at several specific sites during PC12 apoptosis is due partly to dysregulation of certain protein kinases including cdk5 and cdc2 (Zhang and Johnson, 2000). Aggravated during neuronal PC12 apoptosis, tau phosphorylation is enhanced in the neuritis of apoptotic cells (Shelton and Johnson, 2001). Those hyperphosphorylated tau contributes to neurofibrillary tangle (NFT) formation (Bancher, et al., 1989; Grundke-Iqbal, et al., 1986; Kidd, M., 1963; Kurt, et al., 2003; Lee, V. M., et al., 1991; Noble, et al., 2003; Rankin, et al., 2008; Vincent, et al., 1998). There are studies both in vivo (Broe, et al., 2001; Ferrer, et al., 2001; Lassmann, et al., 1995; Lucassen, et al., 1997; Sheng, et al., 1998; Smale, et al., 1995; Stadelmann, et al., 1998; Su, et al., 1994; Sugaya, et al., 1997) and in vitro (Behl, 2000; Broe, et al., 2001; Shelton and Johnson, 2001), having addressed the relationship between the NFT formation and the neuronal apoptosis. However, it is uncertain that which one is the forerunner or the follower. A study by Kobayashi, et al. (2003) reveals that in CA and parietal cortex, correlation of TUNEL-stained neuron nuclei with tau positive neurons varies depending on cerebral regions. Density of TUNEL-stained neuron nuclei shows reciprocal relationship with that of AT8-positive and Gallyas-stained NFTs in CA and presents parallel association with AT8- and HT7-positive neurons in the frontal cortex. They conclude that phosphorylation sites of tau at amino acid 159-163 (HT7) and 202-205 (AT8), are probably associated with neuronal apoptosis and that apoptotic alteration follows abnormal phosphorylation of tau (Kobayashi, et al., 2003). Even though tau hyperphosphorylation is either the harbinger or the adherent of apoptosis, they are putatively associated with each other with positive correlation.

As mention earlier, it is indicated that not only could BM extract attenuate total tau proteins but also abate tau protein phosphorylation at tau-1 site in NGF-

deprived PC12 cells brought up in both media. The ability of BM to reduce both total tau proteins and phosphorylated tau protein may be attributed to the same antioxidant capability as the antioxidant property of curcumin which can decrease both total tau (tau 5) and phosphorylated tau (at tau-1 site and at Ser202 AT8 site) in cytotoxic Aβ-induced PC12 cells (Park, et al., 2008). There are several studies consecutively reported on the antioxidant property of BM not only in vivo system (Anbarasi, et al., 2006a; Anbarasi, et al., 2006b; Bhattacharya, S. K., et al., 2000; Jyoti, et al., 2007; Jyoti and Sharma, 2006; Saraf, et al., 2010; Shinomol and Muralidhara, 2011; Uabundit, et al., 2010) but also in vitro system (Limpeanchob, et al., 2008; Russo, et al., 2003a; Russo, et al., 2003b). The capability of BM to attenuate tau phosphorylation (tau-1) may be due to the other properties of BM similar to Icariin, a flavanoid compound from the herb *Epimedium brevicornum Maxim*, that can significantly reduce Aβ25-35-induced cytotoxicity and apoptosis rate by inhibiting tau protein phosphorylation at Ser396, Ser404, and Thr205 sites, respectively through activating PI3K/Akt signaling pathway which can further be inhibiting effect on glycogen synthase kinase (GSK-3β), a major protein kinase responses for tau protein hyperphosphorylation. There are the recent findings demonstrate that BM extract can diminish Aβ 1-40 and 1-42 level in cortex by much as 60% and reverse Y-maze performance and open field hyperlocomotion behavioral change present in PSAPP mice (Holcomb, et al., 2006), and BM extract can preclude neurons from Aβ -induced cell death (Limpeanchob, et al., 2008). Accordingly, some study reveals that Aβ peptide can activate GSK3β (Terwel, et al., 2008). Since BM extract is comprised of several ingredient compounds containing a putative saponin Bacoside A as an active ingredient (Anbarasi, et al., 2006a; Anbarasi, et al., 2006b; Chatterji, 1965; Mathew, et al., 2010a; Mathew, et al., 2010b; Sumathi and Nongbri, 2008) further researches remained to be investigated whether total tau protein and phosphorylated tau protein attenuation effect of BM extract is participated by Bacoside A, and what is the precise mechanism in the PC12 system or other neuronal culture systems.

Tau is hyperphosphorylated during fetal brain development (Mawal-Dewan, et al., 1994), in adult brain in response to wide range of insults (Yen, et al., 1995) and in pathological conditions such as AD (Lee, V. M., et al., 1991). Tau, in its hyperphosphorylated form, is the major component of PHF observed in AD

(Greenberg, et al., 1992; Sergeant ,et al., 1997). During development of fetal brain, tau phosphorylation plays an important role in neurogenesis where large levels of tau proteins are required. Tau hyperphosphorylation may protect tau from proteolysis thereby enhancing available tau levels needed for neurogenesis. Tau hyperphosphorylation decreases its capacity to interact with microtubule. It is possible that, during brain development, tau must be phosphorylated at least at epitopes involved in this interaction to be able to bind to microtubules (Smith, et al., 2002). Levels of dephosphorylated tau should correspond to those of available microtubules. Excess dephosphorylated tau accumulates in neurons. Dephosphorylated tau itself is not deleterious for neurons. However, in the presence of apoptosis trigger, tau dephosphorylation facilitates its cleavage and degradation and accelerates apoptosis (Rametti, et al., 2004). In AD brain, tau proteins are non-reversible hyperphosphorylated (Grundke-Iqbal, et al., 1986; Guillozet, et al., 2003; Novak, et al., 1993; Polydoro, et al., 2009). These hyperphosphorylated tau proteins, which are resistant to cleavage and degrade contribute to the PHF formation (Smith, et al., 2002). Dephosphorylation plays a crucial role not only in facilitating tau binding to microtubule during brain development and neuronal plasticity, but also in clearing highly phosphorylated tau observed in degenerating neurons to prevent the formation of PHF (Rametti, et al., 2004). Recently, the protective effect of tau phosphorylation has been proposed (Lee, H. G., et al., 2005). It seems that hyperphosphorylation of tau may prevent the brain from rapid loss of a majority of neuron by leading the neurons to escape apoptosis, but the neurons with hyperphosphorylation tau are "sick" and no longer competent for normal physiological functions, such as promoting microtubule assembly and maintaining normal axonal transport. Additionally, extended survival time of these sick neurons make them less resistant to environmental/metabolic insults and allows NFTs to evolve from the hyperphosphorylated tau. These incompetent, sick neurons may be the origin of neurodegeneration. Thus, the formation of NFTs in AD brains may result from the ability of neurons to abort acute apoptotic cell death and enter pathways culminating chronic neurodegeneration (Li, et al., 2007).

BM extract at both 50 and 100 µg/ mL concentration could be able to reduce the 3R tau mRNA to express in differentiated SH-SY5Y cells, apoptotic-induced by camptothecin. Tau mRNA content could be able to determined in the

SH-SY5Y cultured cells brought up with the low-serum RPMI 1640 medium only (the control cells), 0.025% DMSO, 5 μM camptothecin, and BM extract at the 50 and 100 μg/ mL concentrations for at least 48 hr (upper panel in Fig. 22 B). Contrary to their protein products which could be revealed since these cells were cultured for 12 hours (Figure. 21 A-C). This may be attributed to the high efficiency in translation of tau mRNA or the high rate of tau mRNA turnover. The tau mRNA might be expressed since these cells were cultured for 12 hours but in a too little content to be displayed from the present PCR or from agarose gel detection. With the very little content, the tau mRNAs might have high efficiency to be translated to their protein products, so the immunoblot could detect. Otherwise, the tau mRNAs might have high rate of tau mRNA turnover, thus their little mRNAs content disappeared in the beginning of the experiment (12 and 24 hr). With time, the more death cells at 48 and 72 hr expressed the more content of tau mRNAs, so they could be revealed by PCR and agarose gel detection though their mRNA had high turnover rate. Most PCR band products from these cultured cells were the 200 bp fragment of 3R isofrom. This is relevant to the study of Uberti D, et al. (Uberti, et al., 1997) which reported that differentiated SH-SY5Y cells not only expressed the fetal tau (0N/3R isofrom) but this isoform was indeed most abundant. PCR band products from SH-SY5Y cells brought up with the low-serum RPMI 1640 medium only (the control cells) or 0.025% DMSO for 48 hours were higher than PCR band products from these SH-SY5Y cells cultured for 72 hours because of more death cells in 72 hours culturing (late phase). In addition, these PCR band products were translated from 3R isoform of tau mRNA. On the other hand, PCR band products from SH-SY5Y cells brought up with BM extract at the 50 μg/ mL concentrations for 48 hours were lower than PCR band products from these SH-SY5Y cells cultured for 72 hours. Additionally, PCR band product from SH-SY5Y cells brought up with BM extract at the 50 μg/ mL concentrations for 48 hours was descended from 4R isoforms of tau mRNA while PCR band product from these cells cultured for 72 hours was passed by the 3R isofroms of tau mRNA. This might be attributed to the impact of BM extract which could induce both 3R and 4R tau mRNA to expression with 4R isoforms prominent at 48 hours of the experiment. 3R tau mRNA might be too little content to be detected in this study. Previous studies have shown that the inclusion of the Exon 10 (4R isoforms) increased the ability of tau

proteins to promote *in vitro* microtubule assemble and to stabilized microtubules in the cells (Lee, G. and Rook, 1992). The expression of 4R tau protein could facilitate in tau-induced process formation (Yu, et al., 2002). Additionally, 3R isoforms of tau protein are more susceptible to oxidative stress than 4R isoforms of tau protein (Utton, et al., 2001). At 72 hours of the experiment, all detected PCR products were 3R tau mRNA progenies. The cultured SH-SY5Y cells exposing to BM extract at both 50 and 100 μg/ mL concentrations for 72 hours expressed less tau mRNA contents than the cells brought up with the low-serum RPMI 1640 medium only (the control cells) or 0.025% DMSO for the same period of times. This might likely suggest that BM extract could reduce expression of tau 3R mRNAs.

All tau PCR band products displayed in this study were formed either 4R (254 bp band) or 3R (200 bp band) isoforms of tau mRNA (upper panels in Fig. 22 a and b) while their corresponding protein products were ranging from 42 to 62 kDa (Figure. 19 and 20). This is relevant to the study of Uberti D, et al. (Uberti, et al., 1997) thatWestern blot analysis of proteins from differentiated SH-SY5Y revealed several bands characteristic by a smear between 45-65 kDa. Different isoforms of tau mRNAs are originated by alternative splicing of only one human gene on chromosome 17q21. Their tau protein products are composed of 352-441 amino acid residues with molecular weight about 37 to 46 kDa. The complexity of the tau isofroms is further increased by various posttranslational modifications including phosphorylation (Figure. 23) (Wang and Liu, 2008).

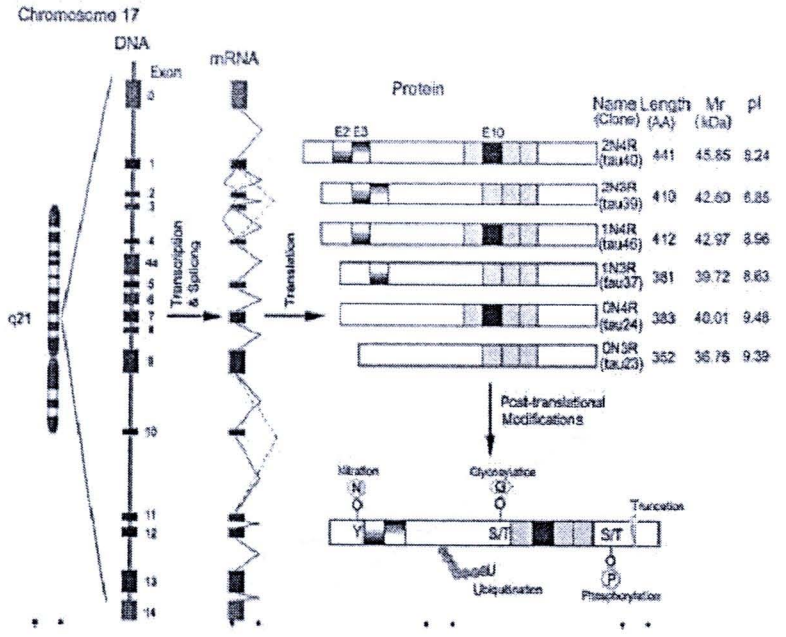

Figure 23 Tau mRNA alternative splicing and Tau protein products

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