Thesis for the Degree of Science

Effect of phlorofucofuroeckol-A on the hepatoprotection in tacrine -induced HepG2 cells

by

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Pukyong National University

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Tacrine으로 처리된 HepG2 세포의 phlorofucofuroeckol-A에 의한 간보호 효과

Advisor: Prof. Hyeung Rak Kim

By
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Contents

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Abstract

Brown sea plants are very popular seafood and many people ingest them as a healthy food in Korea, Japan, and China. The plants are known to contain several phlorotannins, which have been reported to have several biological activities, such as antioxidation, antimutaion, antiskinaging, and hepatoprotective activities. In previous study, we isolated several phlorotannins from the brown algae *Ecklonia stolonifera* through solvent fractionation and HPLC chromatographies. Among isolated phlorotannins from *E. stolonifera*, phlorofucofuroeckol-A (PFF-A) showed the highest antioxidant activity and the hepatoprotective activity against tacrine-induced in HepG2 cells. Our objective was to investigate the hepatoprotective effects of PFF-A on the tacrine-induced HepG2 cells and to assess the possible mechanism of the hepatoprotection by measuring the change of proteins responsible to the cell protection. HepG2 cells were cultured in minimal essential medium containing 10% fetal bovine serum. Cell damage was induced by treatment of 0.3 mM tacrine for 24 hr and cell protective effect was determined by the addition of different concentration of

PFF-A. The expression level of proteins was determined with Western blot. Our result showed that the cytotoxic effects of tacrine to HepG2 cells were caused by ROS generation and induction of Fas, pJNK, cleaved caspase-3, and PARP, which are important proteins on apoptosis, with dose dependent pattern. Treatment of PFF-A showed the suppression of ROS generation and the expression of Fas/FasL-mediated apoptotic proteins with dose dependent manner. Furthermore, the Fas was regulated through inhibition of pJNK by SP600125, pJNK inhibitor. The hepatoprotective effects of PFF-A on tacrine-induced HepG2 cells appeared through reducing ROS generation and down-expression of Fas, Fas-L, pJNK, cleaved caspase-3 and PARP. These findings provide a biochemical basis for the use of PFF-A as a functional food component. Also marine brown plant such as *E. stolonifera* may have a potential for the utilization of functional food source for preventing hepatotoxicity.

1. Introduction

Liver plays a pivotal role in metabolism of the intestinal organs and has a numerous function in the body, including glycogen storage, decomposition of erythrocytes, plasma protein synthesis, and detoxification. Because the liver is the first line protection against damage by ingested agent including xenobiotics and drugs, chronic liver disease is caused by a variety of insults such as viral hepatitis, toxic liver damage by poisons or drugs, and ischemia [1]. Drug-induced liver toxicity is a common cause of liver injury. It accounts for approximately one-half of the cases of acute liver failure and mimics all forms of acute and chronic liver disease. An estimated 1000 drugs have been implicated in causing liver disease [2].

A centrally active noncompetitive cholinesterase inhibitor, tacrine or 9-amino-1,2,3,4-tetrahydroaminoacridine (THA, also known as Cognex®), was the first drug approved for treatment of Alzheimer's disease. Unfortunately, longterm-dosage of tacrine may give rise to severe side effects such as nausea, vomiting, salivation, sweating, bradycardia, hypotension, collapse, and convulsions. Tacrine used for clinical purpose also has been demonstrated to induce hepatotoxicity

[3,4]. Although the mechanism of tacrine-induced hepatotoxicity is not clarified, reaction between tacrine and cytochrome P-450, which is the promoter of xenobiotics metabolism in liver, produces many toxic metabolites such as free radicals, and then radicals induce lipid peroxidation and mitochondrial dysfunction. These damages promote cell death through apoptosis or necrosis, two distinct mechanisms of cell death with different biochemical, morphological, and functional characteristics in hepatocytes [5-8].

Reactive oxygen species (ROS) including free radicals, hydrogen peroxide, and singlet oxygen have been implicated in the apoptosis induced by a variety of stimuli [9]. Intracellular ROS levels caused to cell death induced by the increased expression of TNF, Fas, or growth factor deprivation [10-12]. The liver is very sensitive to Fas-mediated apoptosis because Fas antigen is constitutively expressed on hepatocytes [13]. The Fas/FasL system seems to be involved in many pathological situations, such as graft-versus-host disease, liver transplant, hepatitis B and C, acute alcoholic hepatitis, and some biliary diseases [14-16].

The mitogen-activated protein kinases (MAPKs) are well-known signal transduction pathways on cell regulation such as proliferation, differentiation, inflammation, cell cycle arrest, and apoptosis. Among the MAPKs, c-Jun NH2-terminal kinase (JNK), also known as the stress-

activated protein kinase (SAPK), is activated by various cellular stresses such as UV, protein synthesis inhibitor, proinflammatory cytokines, G protein-coupled receptors, and growth factor receptors [17]. In rat PC-12 phenochromocytoma cells, withdrawal of nerve growth factor (NGF) leads to sustained activation of the JNKs and induces apoptosis [18]. In sphingomyelin pathway, the second messenger ceramide initiates apoptosis through the JNKs cascade on U937 human monoblastic leukaemia cells [19].

Ecklonia stolonifera OKAMURA, the representative brown algae of *Ecklonia sp.*, is a member of the family of Laminariaceae, belonging to the order Laminariales as a perennial brown algae [20]. The secondary metabolites of *E. stolonifera* are known to have several biological activities such as antioxidation [21,22], feeding-deterrent effects [23], antimutation [24], and antiskinaging [25], inhibition of tyrosinase, glycosidase, and hyaluronidase [26-28]. Among the phlorotannins isolated from *E. stolonifera*, phlorofucofuroeckol-A (PFF-A, Fig. 1) showed various biological activities such as antioxidant activity, antiplasmin activity, inhibition of acetylcholinesterase, and the hepatoprotective activity against tacrine-induced HepG2 cells [20-24, 29]. Because the liver has many essential functions in our body, prevention of liver injury is very important. The present study was

designed to investigate the mechanism of hepatotoxicity in tacrine-induced HepG2 cells and the protective effect of PFF-A isolated from *E. stolonifera* OKAMURA on the tacrine-induced HepG2 cells.

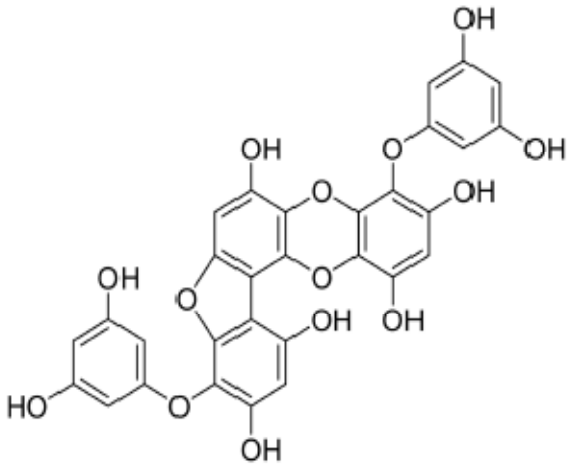

Fig. 1. Structure of PFF-A.

2. Materials and Methods

2.1. Materials

MEM (Minimum essential medium), penicillin-streptomycin mixture, 0.25% tripsin-EDTA, fetal bovine serum (FBS) were purchased from HyClone Laboratory Inc. (UT, USA), sodium pyruvate and non-essential amino acid were purchased from Gibco BRL (MD, USA). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) was purchased from Promega (WI, USA). Tacrine, DMSO (dimethyl sulfoxide), DCFH-DA (dichlorofluorescin diacetate), and HBSS (Hanks' balanced salt buffer) were purchased from Sigma Chemical (MO, USA). Enhanced chemiluminescence (ECL) detection kit was purchased from Perkin Elmer Life science (MA, USA). Polyclonal antibodies against PARP, caspase-3, -8, Bid, pJNK, Fas, FasL, cytochrome c, p53, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Cleaved caspase-3 was purchased from Cell Signaling Technology Inc. (MA, USA). SP600125 was purchased from A.G. Scientific Inc. (CA, USA). PFF-A was isolated from *E. stolonifera* described in Kim et al. (2005).

2.2. Cell culture and treatment

HepG2 cells (ATCC, MD, US) were maintained in MEM containing 2.0 mM L-glutamine, Earle's BSS, 0,1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% heat-inactivated FBS at 37℃ in 5% CO_2. The medium were changed at every 48 hrs. For experiments, cells starved of FBS for 24 hr were cultured in the absence (DMSO alone) or in the presence of tacrine (dissolved in DMSO) without or with PFF-A (dissolved in DMSO) at indicated concentrations and of time. The final concentration of DMSO did not exceed 0.5%.

2.3. Cytotoxicity and Hepatoprotection assay

Cell viability was determined by MTS assay using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega, WI, USA) according to the manufacturer′s manual. Preconfluent HepG2 cells were cultured in 96-well plates at a density of 2.5 x 10^4 cell/well in MEM with 10% FBS. All cells were incubated in serum-free MEM for 24 hr at 37℃ under 5% CO_2 before sample treatment. Tacrine (0-0.75 mM) was added on the serum-starved cells with or without PFF-A (0-70 μM) in dose-

dependent manner and cells were cultured for 24 hr. The culture medium was removed and replaced by 95 μl of fresh culture medium and 5 μl of MTS solution. After 30 min, the abrosbance was measured using microplate reader (Ultraspec® 2100 *pro*, Amersham Biosciences, Piscataway, NJ) at 490 nm.

2.4. Measurement of intracellular ROS

The intracellular ROS scavenging activity of the sample was measured using the oxidant-sensitive fluorescent probe DCFH-DA. DCFH converted from DCFH-DA by deacetylase within the cells is oxidized by a variety of intracellular ROS to DCF, a highly fluorescent compound. The cells were incubated with tacrine with or without PFF-A for 30 min. Cells were harvested by trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA in PBS) and washed with PBS twice. The cells were treated with 20 μM DCFH-DA for 30 min at 37℃. The fluorescence intensity was measured at excitation wavelength of 485 nm and emission wavelength of 528 nm using fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, CA, USA).

2.5. Western immunoblot analysis

HepG2 cells cultured in 6-well plates were washed twice with ice-cold PBS contained protease and phosphatase inhibitors. Lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 200 nM aprotinin, 20 μM leupetin, 50 μM phenanthroline, 280 μM benzamidine-HCl) was added on each well and stood on ice for 30 min for lysis. After centrifuge at 12,000 rpm for 20 min at 4℃, protein content of supernatant was measured, and aliquots (40 μg) of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim-milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 hr and incubated for 16 hr with primary antibody in TBST. The blots were treated with secondary antibody in TBST for 2 hr, and immune complex was detected using ECL detection kit.

2.6. Statistical analysis

Data were expressed as means ±SD from triplicates of at least three independent experiments. All analysis was done with SPSS (Statistical

analysis software package, USA). Statistical significance was established at a *P* value <0.05, and *P* value <0.01.

3. Results

3.1. Cytotoxicity of tacrine and PFF-A on HepG2 cells

To examine cytotoxicity of tacrine and PFF-A on HepG2 cells, serum-starved cells were treated with tacrine or PFF-A, and HepG2 cells were cultured for 24 hr. Tacrine induced cytotoxicity on HepG2 cells with dose-dependent pattern. The value of the 50% effective concentration (EC_{50}) of tacrine was estimated to be 0.3 mM (Fig. 2). However, viabilities of the HepG2 cells were not affected until 60 μM of PFF-A concentration (Fig. 3). The results suggest that tacrine induces hepatic cell death with 0.3 mM of EC_{50}, but PFF-A was non-cytotoxicity on HepG2 cells.

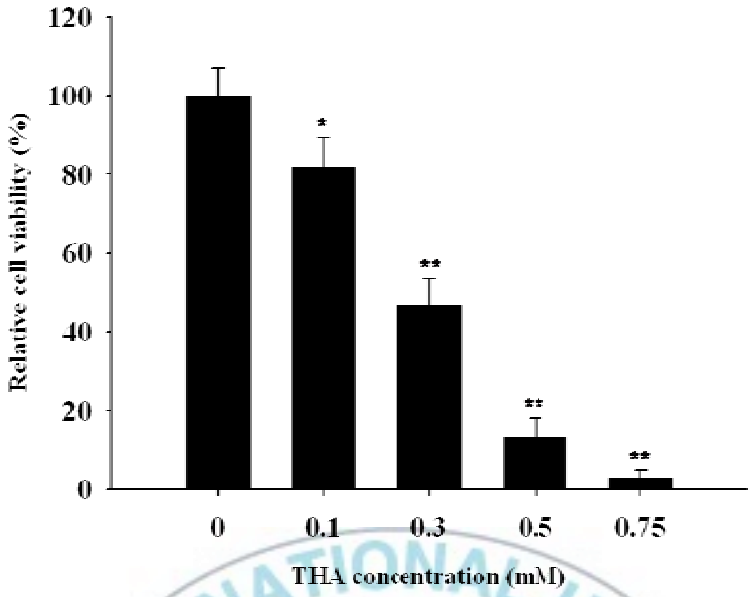

Fig. 2. Cytotoxicity of tacrine on HepG2. HepG2 cells preincubated without FBS for 24 hr at 37℃ were cultured with different concentration of tacrine for 24 hr at 37℃. Cell viability was determined by the MTS assay. Data was expressed as means ± SD from triplicates of at least three independent experiments. *P < 0.05 and *P < 0.01 versus tacrine-untreated hepatocytes. Tacrine was induced hepatotoxicity on HepG2 cell with dose-dependent pattern (EC_{50}, 0.3 mM tacrine).

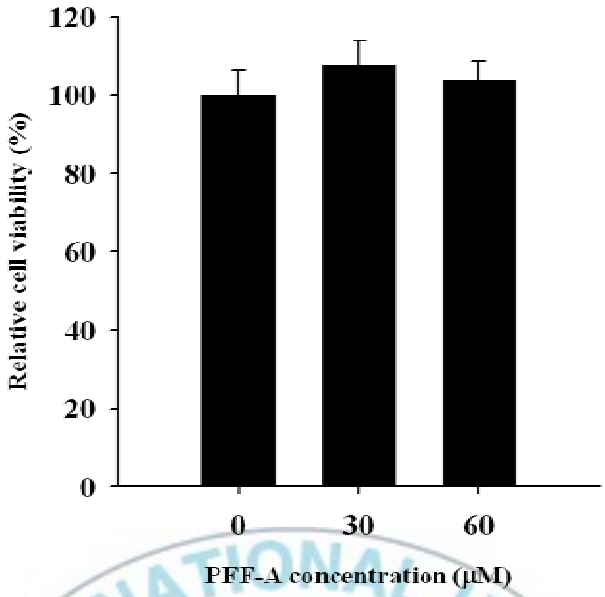

Fig. 3. Cytotoxicity of PFF-A on HepG2. HepG2 cells preincubated without FBS for 24 hr at 37℃ were cultured with different concentration of PFF-A for 24 hr at 37℃. Cell viability was determined by the MTS assay. PFF-A was not affected on the viability of HepG2 cells. Data was expressed as means ± SD from triplicates of at least three independent experiments.

3.2. Protective effect of PFF-A on tacrine-induced HepG2 cells

To examine hepatoprotective effect of PFF-A on tacrine-induced HepG2 cells, hepatocytes starved with FBS for 24 hr were co-treated by 0.3 mM of tacrine with various concentration of PFF-A (0-66 μM). After incubation for 24 hr, cells viability was measured by MTS assay (Fig. 4). PFF-A showed cytoprotective effect in tacrine-induced hepatic cells with dose-dependent pattern. The EC_{50} of PFF-A was determined to be 33.2 μM.

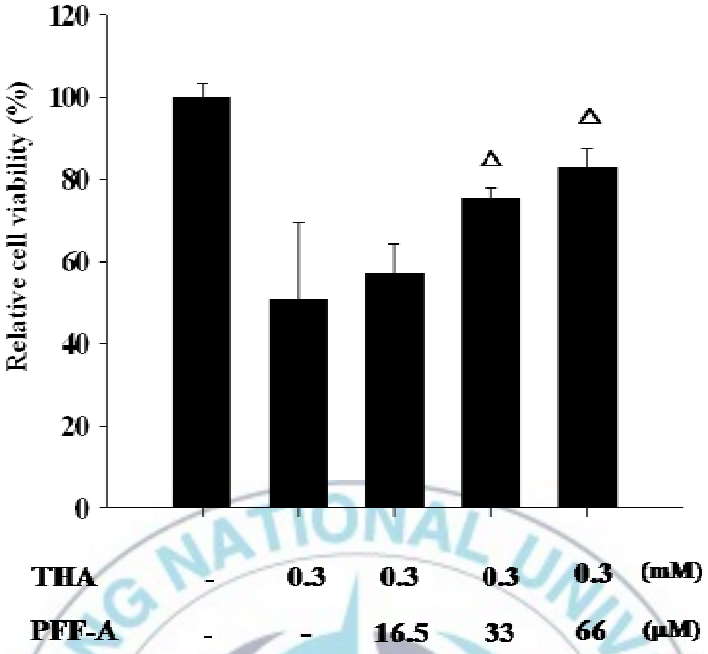

Fig. 4. Protective effect of PFF-A on tacrine-induced HepG2 cells. HepG2 cells starved with FBS for 24 hr were treated by 0.3 mM of tacrine with various concentrations of PFF-A (0-66 μM). After incubation for 24 hr, cell viability was measured by MTS assay. PFF-A showed a cytoprotective effect in tacrine-induced cells with dose-dependent pattern. The EC_{50} of PFF-A was 33.2 μM. Data was expressed as means ± SD from triplicates of at least three independent experiments. $^{\triangle}P < 0.05$ versus PFF-A-untreated hepatocyte cultures.

3.3.Determination of intracellular ROS on HepG2 cells

To investigate the production of intracellular ROS in tacrine-induced hepatocytes and inhibitory effects of PFF-A on the production of ntracellular ROS in tacrine-induced HepG2 cells, cells starved with FBS for 24 hr were treated with 0.3 mM of tacrine and various concentrations of PFF-A (0-33 μM). Intracellular ROS level was rapidly increased at 30 min after tacrine treatment and then the level of intracellular ROS was decreased after 1 hr, and tacrine triggers the generation of ROS on HepG2 cell for 24 hr continually (Fig. 5). However, the intracellular ROS level was not statistically significant up to 24 hr incubation time.

Intracellular ROS level was assayed at 30 min after tacrine treatment with different concentration. Cell viabilities with 0.1 and 0.3 mM tacrine treatment were reduced to 80 and 50%, respectively, compared to control group (Fig. 2), however, ROS levels were not different between 0.1 and 0.3 mM tacrine treatment (Fig. 6). Therefore, tacrine-induced HepG2 cell apoptosis might be resulted from the direct ROS damage and other reasons.

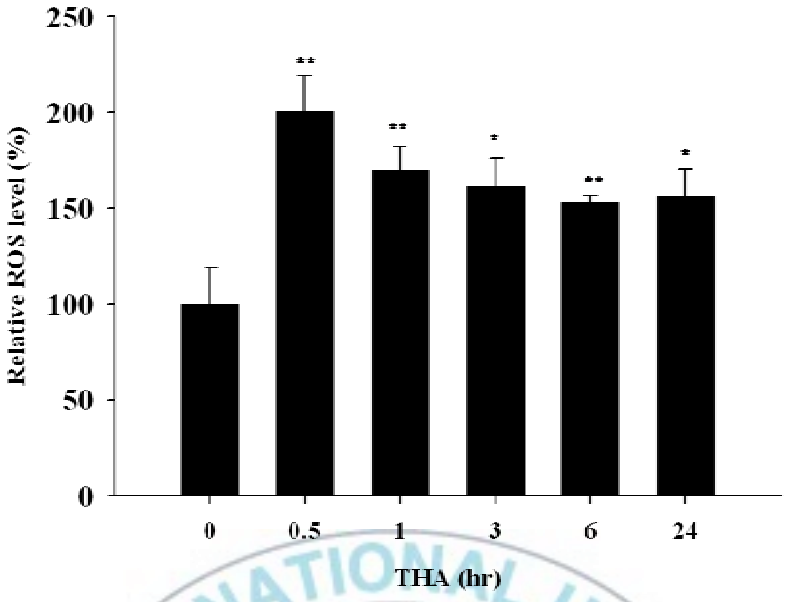

Fig. 5. Intracellular ROS level at different incubation time in tacrine-induced HepG2 cells. HepG2 cells starved with FBS for 24 hr were treated with 0.3 mM of tacrine. After incubation with tacrine for 30 min to 24 hr, ROS levels were measured by DCFH-DA with fluoroscencemetry. Data was expressed as means ± SD from triplicates of at least three independent experiments. *P < 0.05, **P < 0.01, versus tacrine-untreated hepatocyte cultures.

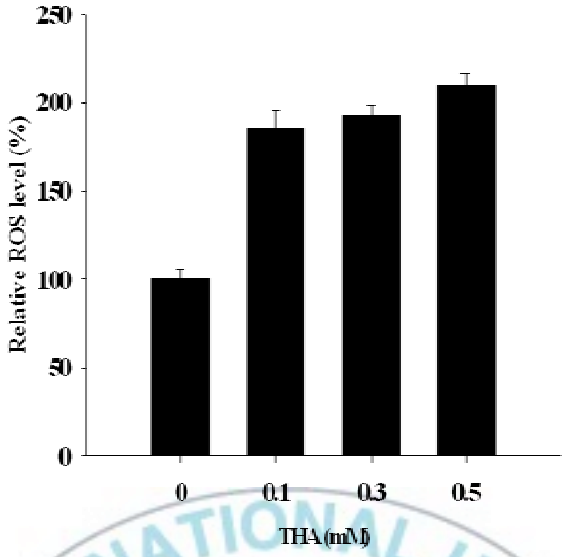

Fig. 6. Intracellular ROS level in different concentration of tacrine-induced HepG2 cells. HepG2 cells starved with FBS for 24 hr were treated with 0, 0.1, 0.3, and 0.5 mM of tacrine. After incubation with tacrine for 30 min, ROS levels were measured by DCFH-DA with fluoroscencemetry. Data was expressed as means ± SD from triplicates of at least three experiments.

Fig. 7 shows that PFF-A inhibits the production of intracellular ROS on tacrine-treated HepG2 cells. PFF-A treatment with 16.5 and 33 uM completely blocked the ROS production to control group on tacrine-induced HepG2 cells. In this result, 16.5 μM of PFF-A was only available to block the production of ROS induced by tacrine, but cell viability was not affected by same concentration of PFF-A in a previous result (Fig. 4). Aforementioned, tacrine-induced HepG2 cell death might be resulted from the activation of known apoptotic proteins and direct ROS production.

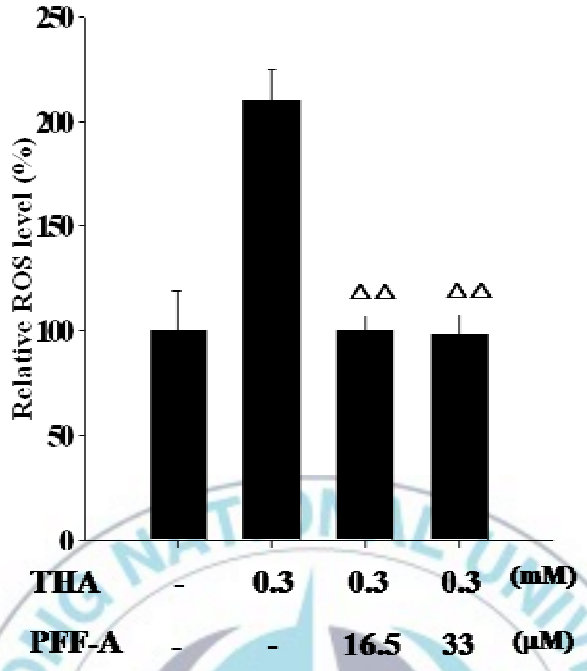

Fig 7. Effect of PFF-A on tacrine-induced intracellular ROS level. HepG2 cells starved with FBS for 24 hr were treated with 0.3 mM of tacrine absence or presence of 16.5 and 33 μM of PFF-A. After incubation with samples for 30 min, intracellular ROS levels were measured by DCFH-DA with fluoroscencemetry. Data was expressed as means ± SD from triplicates of at least three independent experiments. $^{\triangle\triangle}P < 0.01$ versus PFF-A-untreated hepatocyte cultures.

3.4. Tacrine induces Fas-related cell death signal transduction

To clarify tacrine affects protein signal transduction in HepG2 cells, Western blot analysis was conducted. Cell death signalings related with apoptosis such as Fas, cleaved caspase-3 and cleavage of PARP, were analyzed with Western blot. Fig. 8 shows tacrine triggers apopotsis via Fas activation on hepatocytes in time-dependent pattern. The augmentation of cleaved caspase-3 which is activated form of caspase-3 was detected at 8 hr and increased up to 24 hr. The fragmentation of PARP cleaved by cleaved caspase-3 was also detected from 8 hr to 24 hr.

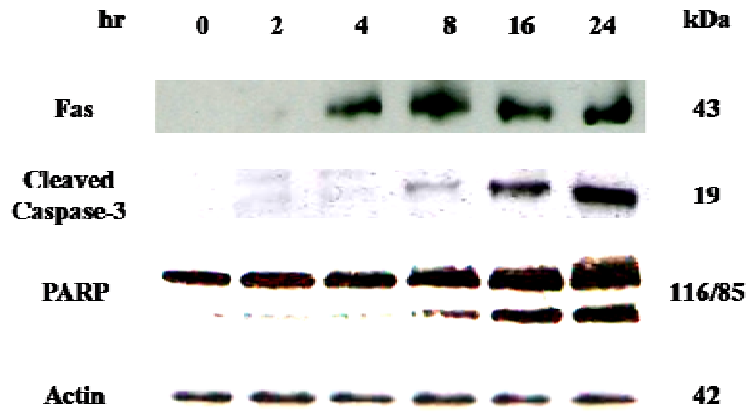

Fig. 8. Tacrine-induced Fas-related cell death signal transdcution. Cell death signaling related with apoptosis such as Fas, and their downstream proteins such as cleaved caspase-3, and PARP were analyzed with Western blot. HepG2 cells starved with FBS for 24 hr were treated with 0.3 mM of tacrine. Tacrine triggers Fas-mediated apopotsis on hepatocytes in time-dependent pattern.

3.5. PFF-A protects from tacrine-induced hepatic cell death by inhibiting of Fas-mediated apoptosis

Compared with tacrine-treated HepG2 cells, PFF-A has hepatoprotective effect on tacrine-induced cell death signaling proteins with dose-dependent manner (Fig. 9). Fas protein which indicates potential inducer of apoptosis, expressed at tacrine-treated HepG2 cells group. However, the Fas triggered by addition of tacrine dramatically decreased in 16.5 and 33 μM of PFF-A treatment. Because of the Fas protein reduction, down-stream proteins of Fas/FasL death signaling system such as caspase-8 and Bid are significantly changed in protein levels. The expression of caspase-8 induced by tacrine via Fas is decreased with PFF-A treatment at 24 hr, and Bid cleaved by caspase-8 is increased by PFF-A treatment at 24 hr. These changes eventually affect PARP, which plays a pivotal role for apoptosis in mammalian cells, to prevent from tacrine-induced hepatic cell death by decrease of cleavage level of PARP at 24 hr.

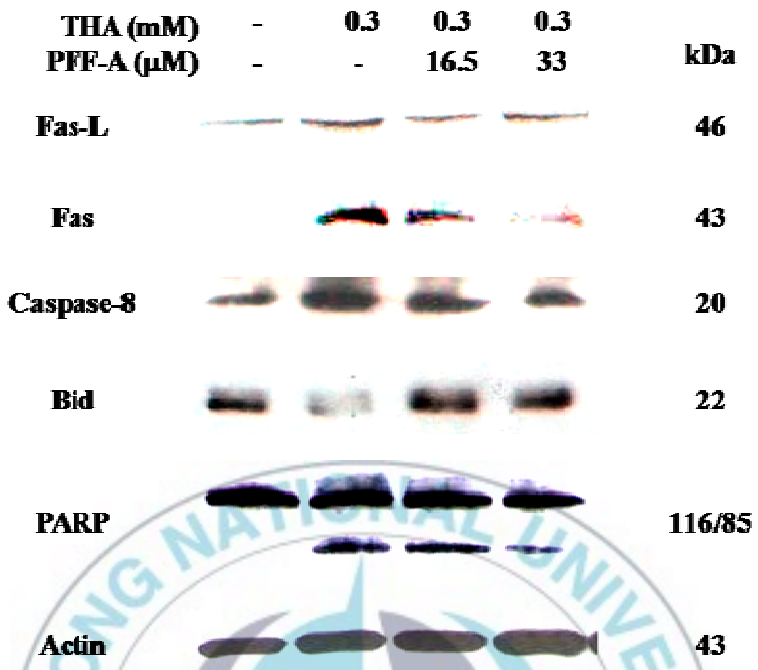

Fig. 9. Dose-dependent inhibitory effect of PFF-A on tacrine-induced signal trasnduction. 0.3 mM of tacrine was added to serum starved HepG2 cells with 0-33 μM of PFF-A for 24 hr to identify the effect of PFF-A on tacrine-induced HepG2 cells. PFF-A has a protection effect with dose-dependent manner on tacrine-induced HepG2 cells.

3.6. The role of pJNK in tacrine-induced HepG2 cells

The effect of tacrine treatment of HepG2 cells on the phosphorylation of JNK, one of the MAPKs proteins, was shown in Fig. 10a. In the time kinetic studies at 2, 4, 8, 16, and 24 hr, tacrine treatment induced a JNK phosphorylation beginning 2 hr and sustained until 24 hr after tacrine treatment on the cells. However, tacrine-induced pJNK decreased significantly with time dependent in the presence of 33 μM of PFF-A (Fig. 10b). In contrast with tacrine treatment, the level of JNK phosphorylation reduced continually for 24 hr on PFF-A co-treated HepG2 cells.

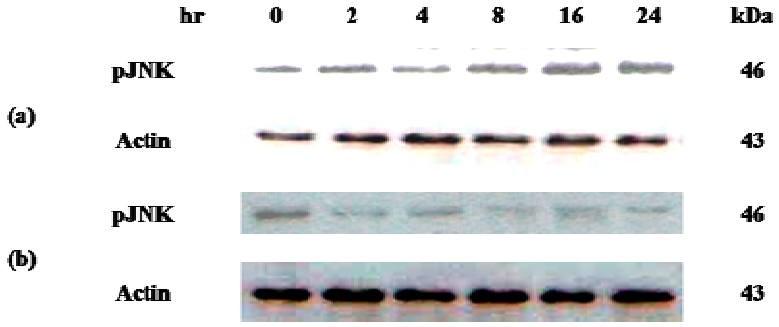

Fig. 10. Phosphorylation of JNK on HepG2 cells with time-dependent. 0.3 mM of tacrine was added to serum starved HepG2 cells with or without 33 μM of PFF-A for 24 hr to identify the alteration of pJNK on tacrine-induced HepG2 cells. (a) pJNK expreesion of HepG2 cells incubated with tacrine. (b) pJNK expression of HepG2 cells incubated with tacrine and PFF-A.

Among the MAPKs, the activation of pJNK is one of the pathway for apoptosis. To investigate the role of pJNK on tacrine-indcued HepG2 cells, SP600125, inhibitor of JNK phosphorylation, was pretreated on tacrine-treated HepG2 cells. Cells preincubated with 10 μM SP600125 for 1 hr were treated 0.3 mM of tacrine for 30 min, and cells incubated without SP600125 were co-treated 0.3 mM of tacrine with 0-33 μM of PFF-A for 24 hr. As shown in Fig. 11, we found that SP600125 did not affect on tacrine-induced ROS generation. Pre-treatment of SP600125 and co-treatment of PFF-A completely inhibited phosphorylation of JNK, however, that partially inhibited the expression of Fas and activation of caspase-3 on tacrine-induced HepG2 cells (Fig. 12).

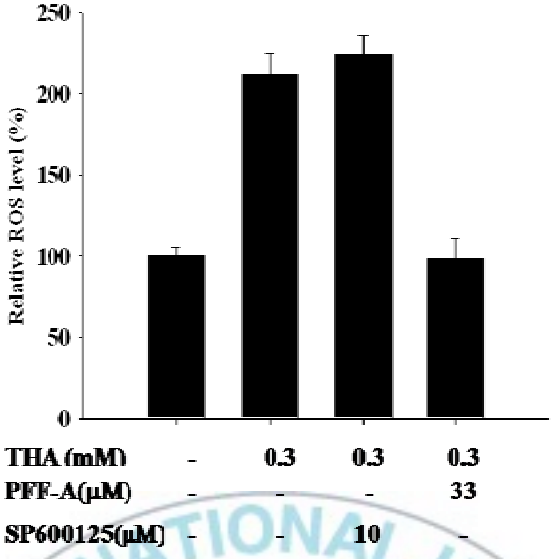

Fig. 11. The effect of SP600125 on tacrine-indcued ROS in HepG2 cells. HepG2 cells starved with FBS for 24 hr were pre-treated with 10 μM of SP600125 for 1 hr. After incubation with 0.3 mM of tacrine for 30 min, intracellular ROS levels were measured by DCFH-DA with fluoroscencemetry. Data was expressed as means ± SD from triplicates of at least three independent experiments.

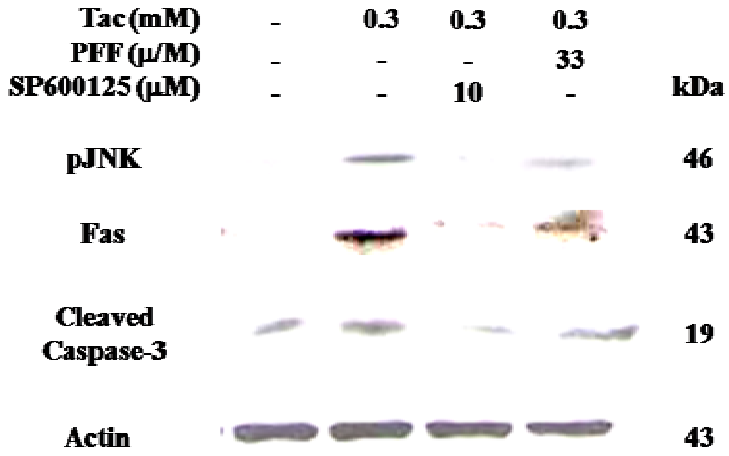

Fig. 12. Inhibitory effect of SP600125 and PFF-A on tacrine-induced JNK signal trasnduction. To identify the role of JNK pathway on tacrine-induced HepG2 cells, 0.3 mM of tacrine was added to serum starved HepG2 cells with or without 33 μM of PFF-A for 24 hr, and 10 μM of SP600125 was pre-treated to suerm starved HepG2 cells for 1 hr before 0.3 mM of tacrine treatment. The reducing expression level of pJNK leads to decrease Fas and cleaved caspase-3 proteins.

4. Discussion

Liver plays a pivotal role in metabolism of ingested agent including xenobiotics and drugs. In vivo and in vitro, liver or hepatocytes of mammalian are damaged by many drugs such as tacrine that is cholinesterase inhibitor used for therapeutic purpose of Alzheimer's diseases. In this result, tacrine induces apoptotic cell death in HepG2 cells with dose-dependent manner and EC_{50} value of tacrine was calculated at 0.3 mM (Fig. 2). Although tacrine suppresses acetylcholinesterase and butylylcholinesterase to increase Central Nerve System (CNS) acetylcholine levels of Alzheimer's disease patients [30-33] and protects from hydrogen peroxide-induced apoptosis by regulating expression of apoptosis-related genes in rat PC12 cells [34], recent studies related with cytotoxicity of tacrine on hepatocytes have suggested that liver cells of mammalians are influenced serious damages through metabolic activation of tacrine.

The mechanism of tacrine-induced hepatotoxicity has yet to be precisely elucidated, but it has been determined that tacrine alters intracellular glutathione concentrations in cultured hepatocytes, which suggests the involvement of reactive oxygen species (ROS) generation

affected lipid peroxidation, alterations of membrane fluidity in tacrine-induced cytotoxicity, and inhibition of in vivo mtDNA synthesis to progressively cause mtDNA depletion and apoptosis in liver of rat treated with tacrine during several weeks [35-37]. Fig. 5 shows generation of intracellular ROS induced by treatment of tacrine on HepG2 cells. With previous results of our cytotoxicity assays, these suggest that tacrine triggers death of hepatocytes through increasing ROS levels.

In addition, we found that tacrine activates Fas and triggers apoptosis in HepG2 cells via caspase-3 activation and PARP fragmentation (Fig. 8). Activation of Fas on the cell membrane by Fas ligand or agonist antibody results in the activation of caspase-8, the apical caspase in this pathway, and then caspase-8 activates caspase-3 through directrly or indirectly pathway[38,39]. Bid, one of the BH3-only Bcl-2 family members, is activated post-translationally via cleavage by caspase-8/FLICE in response to Fas or TNF receptor activation [40]. The truncated Bid (t-Bid) cleavage product, p15, acts at the mitochondria to cause cytochrome c release [41]. Cytochrome c released to cytosol activates procaspase-3, which presents as inactive proenzymes in cytosol, to active formation and active caspase-3 hydrolyzes PARP [42-45]. The specific cleavage of PARP at the sequence Asp-Glu-Val-Asp (DEVD)

has been reported to be a sensitive marker of caspase-3 activation in apoptosis [46]. In present result, the ROS induced by tacrine activates Fas and truncated Bid. The cleavage of Bid stimulated the release of mitochondrial cytochrome c which reduces activation of procaspase-3 to cleaved caspase-3. The 85 kDa fragment of PARP cleaved by activated caspase-3 begun to increase continually from 8 hr (Fig. 8).

PFF-A isolated from *E. stolonifera* did not affect on HepG2 cells, but it attenuates cell death induced by tacrine in HepG2 cells (Fig. 3, 4). When tacrine added with PFF-A on HepG2 cells, the intracellular ROS generation is completely blocked against tacrine treatment group (Fig. 7). We also found that expression of Fas/FasL system proteins induced by tacrine was decreased by treatment of PFF-A for 24 hr, because PFF-A inhibits tacrine-induced intracellular ROS levels on HepG2 cells in our previous results (Fig. 9). Fas-L and Fas activated by stimulus of tacrine in HepG2 cells were decreased by co-treatment of PFF-A with dose-dependent manner. Expression of other signal transductions such as caspase-8, cleavage of Bid, and PARP fragmentation were down-regulated by PFF-A. When 0.3 mM tacrine was treated with 33 μM of PFF-A on HepG2 cells, expression of Fas, and cleavage of Bid, also, were decreased with time-dependent manner.

Several compounds isolated from brown alga have been reported to

reduce intercellular ROS levels effectively. Choi et al. (1997) reported that methanolic extraction of *E. stolonifera*, its subsequent fractions, and its component, phloroglucinol and phlorotannin A, are useful as nitrite scavangers. Brown alga polyphenols have antioxidative properties, and phlorotannins extracted from *E. cava* were dose-dependent scavengers of DPPH radical [47,48]. Phlorotannins extracted from *E. stolonifera* inhibit total ROS generation [24], and eckol isolated from *E. cava* attenuates oxidative stress in lung fibroblast cells [25], and Kim et al. (2005) reported eckstolonol and phlorofucofuroeckol-A extracted from *E. stolonifera* among other seaweeds exert protective effects against tacrine-induced hepatoxicity [28]. Our evidence suggests that the intracellular events started from generation of ROS by tacrine lead to hepatic cell death sequentially. In the initial step, there is generation of ROS and activation of Fas-mediated protein signaling transduction. At this stage, PFF-A affecting in the production of ROS can prevent cell death through blocking these molecular events.

Interestingly, PFF-A has antioxdation property and we observed that the ROS induced by tacrine was blocked by 16.5 and 33 μM of PFF-A, but cell viability and apoptotic signal proteins were not inhibited effectively at 16.5 μM of PFF-A. Furthermore, the ROS induced by tacrine was generated similarly at 0.1, 0.3, and 0.5 mM of tacrine in

HepG2 cells. Although apoptosis initiates and executes by various extracellular stress including ROS, the apoptotic process is regulated via many intracellular signals such as the phosphorylation of JNK [49]. The activated JNK specifically phosphorylates the transcription factor c-Jun, and also regulates non-transcription factor such as member of Bcl-2 family, in response to a variety of extracellular stimuli [49]. Our results showed that tacrine induced phosphorylation of JNK and prolonged pJNK for 24 hr. In contrast with tacrine-treated group, the pJNK was inhibited and/or dephosphorylated by PFF-A co-treatment. In addition, we found that SP600125 showed high inhibiting effect of pJNK, cleaved caspase-3, and Fas receptor without alteration of tacrine-induced ROS in HepG2 cells. The role of JNK on ROS-activated hepatocytes death have previously been identified that it inhibits Fas-mediated apoptosis. The JNK may modulates early stage during Fas-mediated apoptosis such as enhancing the clustering of Fas receptor in response to Fas ligand [50]. Hence, our findings suggest that the hepatic programmed cell death on tacrine-induced HepG2 cells is co-activated through the Fas/FasL pathway and prolonged pJNK, and the PFF-A takes hepatoprotective effects via inhibiting the activation of Fas/FasL system with low expression of JNK decreased by reduction of ROS.

Based on our observations with other recently studies, compound

related with antioxidation effects is considered to prevent hepatocytes from tacrine-induced cytotoxicity because antioxidative action reduces formation or scavenging of free radicals. We suppose that tacrine stimulates hepatic cells death through Fas/FasL signal transduction activated by ROS, and PFF-A protects the cells from tacrine by inhibition pJNK and Fas down-stream proteins with reducing ROS level. It seems that the reduction effect of PFF-A caused by redox reaction directly and/or activation of some indirectly process including signal transduction of expression of scavenging enzyme such as catalase and superoxide dismutase (SOD). Although, in our report, the effect of PFF-A on expression of intracellular scavenging enzymes has not been reported yet, these findings provide a biochemical basis for the influence of PFF-A on the hepatoprotective effect.

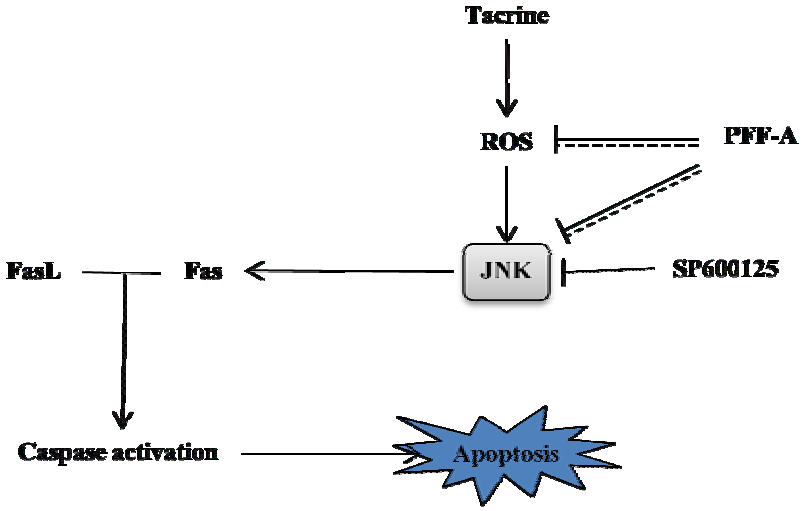

Fig. 13. The inhibitory effect of PFF-A on tacrine-induced HepG2 cells via Fas/FasL signal system and JNK..

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