PYP1-4 peptide from *Pyropia yezoensis* protects against acetaminophen-induced hepatotoxicity in HepG2 cells

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**Abstract.** Acetaminophen (APAP) is a widely used analgesic and antipyretic. It is safe at normal treatment doses; however, APAP overdose is a major cause of acute liver and kidney failure. A variety of methods to reduce the damage caused by APAP overdose have previously been evaluated. The protein-rich seaweed *Pyropia yezoensis* has antioxidant, anti-tumor and anti-inflammatory activities, and protects against cytotoxicity. However, little is known regarding the protective effects of *P. yezoensis* peptide against APAP-induced hepatotoxicity. The present study investigated the ability of *P. yezoensis* peptide (PYP1-4) to ameliorate the damage caused by APAP-induced hepatotoxicity using HepG2 as the model cell line in addition to the signaling pathways involved. Briefly, cell viability, nitric oxide, reactive oxygen species and apoptosis assays were performed in conjunction with western blot analysis and reverse transcription-quantitative PCR. First, the present study revealed the minimum toxic concentration of APAP (15 mM) and the resting concentration of PYP1-4 (0-500 ng/ml). Administration of PYP1-4 to APAP-induced cells decreased the nitric oxide and reactive oxygen species levels, and restored the levels of antioxidant-associated proteins (catalase, heme oxygenase 1, superoxide dismutase 2 and quinone oxidoreductase 1). PYP1-4 increased the translocation of nuclear factor, erythroid 2 like 2 to the nucleus and the activities of glycogen synthase kinase-3β, Akt and AMP-activated protein kinase. In addition, APAP induced apoptosis; however, PYP1-4 inhibited apoptosis bymodulating the levels of pro-apoptotic markers (Bad), anti-apoptotic markers (Bcl-2 and BH3 interacting domain death agonist), caspases and poly(ADP-ribose) polymerase 1. Subsequently, the insulin-like growth factor 1 receptor signaling pathway was investigated to determine whether PYP1-4 treatment restored the levels of cell growth-associated factors during APAP-induced hepatotoxicity. PYP1-4 treatment impacted the levels of components of the insulin receptor substrate 1/PI3K/Akt and Ras/Raf/ERK signaling pathways, and promoted cell survival. Therefore, the *P. yezoensis* peptide PYP1-4 may be useful for preventing APAP-induced hepatotoxicity.

**Introduction**

Acetaminophen (APAP) is an effective analgesic and antipyretic (1,2). APAP is safe at therapeutic doses, and higher doses can be provided to patients for short durations (3). However, the incidence of APAP poisoning is increasing (4). In the United States and in the United Kingdom, APAP overdose has been reported as a major cause of drug-induced liver failure (5,6). Since APAP overdose causes severe damage to liver and kidney cells in humans and experimental animals (7,8), a number of studies have focused on the prevention or treatment of APAP-induced liver failure (9,10).

The mechanism of APAP-induced hepatotoxicity has been established and extensively reviewed (11,12). The liver failure that follows APAP ingestion is not due to the drug itself, but to a toxic metabolite, N-acetyl-p-benzoquinone imine, produced by the cytochrome P450 group of enzymes in the liver. This metabolite is normally rendered harmless through its interaction with glutathione (GSH), an endogenous antioxidant (11,12). However, when this APAP metabolite is overproduced, GSH stores in the liver become depleted, the metabolite accumulates, and tissue injury occurs (13). As a result, APAP overdose stimulates the apoptotic and/or necrotic death signaling pathways in cellular models (14,15). Additionally, APAP overdose increases oxidative stress and reactive oxygen species (ROS) levels, decreases GSH levels, induces mitogen-activated protein kinase (MAPK) signaling pathways, and activates caspase cascades (15-17). Furthermore, APAP overdose leads to liver failure, promoting lipid peroxidation and transcriptional activation of inflammatory factors (11,18). Based on this mechanism, several inhibitors of APAP overdose-induced liver and kidney failure, such as N-acetylcysteine (NAC) have been developed (19).

Seaweeds have received increased research attention, since the majority contain polysaccharides, proteins,
vitamins and minerals with diverse biological activities (20). *Pyropia yezoensis*, a marine red alga, is cultured and consumed mainly in China, Japan and Korea (21). *P. yezoensis* produces free radicals and other potent oxidizing agents without causing serious photodynamic damage if exposed to adverse environmental conditions, such as a high light intensity or oxygen concentration (22,23). Therefore, *P. yezoensis* produces compounds that protect against external factors, including environmental pollutants, stresses and UV radiation (22,23). *P. yezoensis* has antioxidant (24,25), anti-inflammatory activities (28,29), and protects against neuronal senescence (30,31), photo-aging (22,23) and cytotoxicity (32,33).

A 14-kDa glycoprotein extracted from *P. yezoensis* reportedly protects against hepatotoxicity in rats with APAP-induced liver injury (33). After the protein is purified from the glycoprotein by protein sequencing and mass spectrometry, 10- and 7-kDa proteins are obtained (34). Treatment of the 10-kDa protein (protein ID PYP1; Rhod_EST AV429545) with digestive enzymes, including chymotrypsin, pepsin and trypsin, yields several peptides, which have been screened to identify those with protective effects (34).

Studies on the protective effects of *P. yezoensis* peptides in APAP-induced hepatotoxicity have produced inconclusive results (33,34). Therefore, the present study investigated the protective effects of *P. yezoensis* peptides on APAP-induced liver injury in HepG2 human liver cancer cells, as well as the underlying molecular mechanisms.

**Materials and methods**

**Peptide synthesis.** The peptide PYP1-4 (A-T-R-D-P-E-P-T-A-V-D-P-N) from *P. yezoensis* was commercially synthesized by Peptron Corporation and purified to >95% purity. PYP1-4 was purified using a Shimadzu Prominence high-performance liquid chromatography system with a C18 column (Capcell Pak; Shiseido Co., Ltd.), using the Class-VP software (version 6.14; Shimadzu Corporation). PYP1-4 was first dissolved in 0.1% trifluoroacetic acid/water at 1 mg/ml and 40 μl of the solution was then injected into the HPLC system. The HPLC system condition was as follows: Acetonitrile gradient, 10-40%; flow rate, 1 ml/min, temperature, 50°C; and UV detection, 220 nm. The molecular weight of PYP1-4 was 1,382 Da as determined using a Shimadzu Prominence high-performance liquid chromatography system with a C18 column (Capcell Pak; Shiseido Co., Ltd.), using the Class-VP software (version 6.14; Shimadzu Corporation). PYP1-4 was first dissolved in 0.1% trifluoroacetic acid/water at 1 mg/ml and 40 μl of the solution was then injected into the HPLC system. The HPLC system condition was as follows: Acetonitrile gradient, 10-40%; flow rate, 1 ml/min, temperature, 50°C; and UV detection, 220 nm. The molecular weight of PYP1-4 was 1,382 Da as determined using mass spectrometry (HP 1100 Series LC/MSD: Agilent Technologies, Inc.) using ionization mode (positive + H, 1,0079 Da; negative - H, -1,0079 Da) and multiple reaction monitoring (300-2,300 m/z). The synthesized peptides was reconstituted in water (10 mg/ml) and stored at -50°C.

**Cell culture.** HepG2 liver cancer cells (cat. no. HB-8065) were purchased from the American Type Culture Collection. The cells were cultured at 37°C with 5% CO2 and protected against photodynamic therapy by using a light microscope (magnification, x200; Eclipse TS100-F; Nikon Corporation).

**Nitric oxide (NO) assay.** The nitrite concentration in culture medium was determined spectrophotometrically as described previously by Lee et al (29). Briefly, cells were seeded in 48-well plates at 2x10^4 cells/well and incubated for 24 h at 37°C. The cells were treated with PYP1-4 (125, 250 or 500 ng/ml) and 15 mM APAP in SFM for 18 h at 37°C. Subsequently, 100 μl culture medium were transferred to a 96-well plate, and 100 μl Griess reagent (G4410; Sigma-Aldrich; Merck KGaA) was added. The plate was incubated for 10 min at 37°C, following which absorbance at a wavelength of 540 nm was measured using a FilterMAX F5 microplate reader.

**Intracellular ROS assay.** The intracellular ROS concentration was assayed using the ROS-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF-DA; cat. no. 35845; Sigma-Aldrich; Merck KGaA). Cells were seeded in 96-well plates at 2x10^4 cells/well and incubated for 24 h at 37°C. The cells were treated with PYP1-4 (125, 250 or 500 ng/ml) and 15 mM APAP in SFM for 18 h at 37°C. Subsequently, the cells were incubated with 10 μM DCF-DA at 37°C for 30 min. The fluorescence intensities of stained cells were measured using a FilterMAX F5 microplate reader at excitation and emission wavelengths of 485 and 535 nm, respectively.

**Apoptosis assay.** Apoptosis was assayed using the Muse<sup>®</sup> Annexin V and Dead Cell Assay Kit (cat. no. MCH100105; BD Biosciences). The cells were harvested and washed twice with PBS, and stained with FITC Annexin V and propidium iodide for 15 min at room temperature. The percentage of apoptotic cells was determined using Annexin V and dead cell software program of Muse<sup>™</sup> Cell Analyzer system (2013; EMD Milipore).

**Western blot analysis.** HepG2 cells were cultured for 18 h at 37°C in SFM containing 0, 125, 250 or 500 ng/mL PYP1-4 and 15 mM APAP. The cells were washed with PBS and lysed in RIPA lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM sodium chloride, 1% NP-40 and 0.25% sodium deoxycholate; pH 7.4) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride and 1 mM phenylmethanesulfonylfluoride) on ice for 30 min. The extracts were centrifuged at 12,000 x g for 10 min at 4°C, and the supernatants were used for western blot analysis. Protein concentration was measured using the Bicinchoninic Acid protein assay kit. Total protein (30 μg) was electrophoresed using a 10-15% acrylamide gel and transferred to PVDF transfer membranes (EMD Millipore). The membranes were blocked with 1% bovine serum albumin (BSA; GenDEPOT) in TBST (5 mM 850 KIM et al: P. yezoensis PEPTIDE PROTECTS AGAINST APAP-INDUCED HEPATOTOXICITY
Tris and 20 mM sodium chloride; pH 7.4; 0.1% Tween-20) and incubated with primary antibodies (dilution, 1:1,000) in 1% BSA-TBST with gentle agitation at 4˚C overnight. The membranes were washed twice for 15 min each in TBST, incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (dilution, 1:10,000) for 2 h at room temperature, and then washed. Immunoreactive bands were detected using an enhanced chemiluminescence substrate (Advanta Inc.) and visualized using the GeneSys imaging system (SynGene; Synoptics Ltd.). Differences in protein levels were determined by semi-quantifying western blot band densities using ImageJ software (version IJ.146r; National Institutes of Health). The primary antibodies used in the present study are as follows: Anti-catalase (CAT; cat. no. OAAB05216; rabbit), anti-superoxide dismutase 1 (cat. no. OASE00355; rabbit), anti-superoxide dismutase 2 (SOD2; cat. no. OASE00357; rabbit; Aviva Systems Biology Corporation), anti-heme oxygenase 1 (HO1; cat. no. sc-1796; goat), anti-quinone oxidoreductase 1 (QOR1; cat. no. sc-16464; goat), anti-nuclear factor, erythroid 2 like 2 (Nrf2; cat. no. sc-7148; rabbit), anti-phosphorylated-(p-)JNK (cat. no. sc-6254; mouse), anti-JNK (cat. no. sc-7345; mouse), anti-p-p38 MAP kinase (p38; cat. no. sc-7973; mouse), anti-p38 (cat. no. sc-7149; rabbit), anti-p-glycogen synthase kinase 3β (GSK3β; cat. no. sc-81496; mouse), anti-GSK3β (cat. no. sc-7291; mouse), anti-p-AMP-activated protein kinase (AMPK; cat. no. sc-33524; rabbit), anti-AMPK (cat. no. sc-74461; mouse), anti-Bcl-2 (cat. no. sc-492; rabbit), anti-Bcl-xL (cat. no. sc-7195; rabbit), anti-BH3 interacting domain death agonist (Bid; cat. no. sc-11423; rabbit), anti-poly (ADP-ribose) polymerase 1 (PARP; cat. no. sc-7150; rabbit), anti-caspase-9 (cat. no. sc-7885; rabbit), anti-caspase-3 (cat. no. sc-7148; rabbit), anti-Bad (cat. no. sc-7869; rabbit), anti-Bax (cat. no. sc-493; rabbit), anti-insulin-like growth factor 1 receptor (IGF-IR; cat. no. sc-390130; mouse), anti-epidermal growth factor receptor (EGFR; cat. no. sc-03; goat), anti-erb-b2 receptor tyrosine kinase 2 (ErB2B; cat. no. sc-284; rabbit), anti-erb-b2 receptor tyrosine kinase 3 (ErB3; cat. no. sc-285; rabbit), anti-insulin receptor substrate 1 (IRS-1; cat. no. sc-560; rabbit), anti-P13K (cat. no. sc-374534; mouse), anti-PTEN (cat. no. sc-7974; mouse), anti-pyruvate dehydrogenase kinase 1 (PDK1; cat. no. sc-28783; rabbit), anti-p-Akt (cat. no. sc-7985; rabbit), anti-Akt (cat. no. sc-8312; rabbit), anti-p-mTOR (cat. no. sc-293132; mouse), anti-mTOR (cat. no. sc-8319; rabbit), anti-p70S6 kinase (p70S6K; cat. no. sc-8418; mouse), anti-eukaryotic translation initiation factor 4E (eIF4E; cat. no. sc-514875; mouse), anti-SHC adaptor protein 1 (SHC; cat. no. sc-967; mouse), anti-AMPK (cat. no. sc-7291; mouse), anti-p-AMP-activated protein kinase (AMPK; cat. no. sc-33524; rabbit), anti-AMPK (cat. no. sc-74461; mouse), anti-Bcl-2 (cat. no. sc-492; rabbit), anti-Bcl-xL (cat. no. sc-7195; rabbit), anti-BH3 interacting domain death agonist (Bid; cat. no. sc-11423; rabbit), anti-poly (ADP-ribose) polymerase 1 (PARP; cat. no. sc-7150; rabbit), anti-caspase-9 (cat. no. sc-7885; rabbit), anti-caspase-3 (cat. no. sc-7148; rabbit), anti-Bad (cat. no. sc-7869; rabbit), anti-Bax (cat. no. sc-493; rabbit), anti-insulin-like growth factor 1 receptor (IGF-IR; cat. no. sc-390130; mouse), anti-epidermal growth factor receptor (EGFR; cat. no. sc-03; goat), anti-erb-b2 receptor tyrosine kinase 2 (ErB2B; cat. no. sc-284; rabbit), anti-erb-b2 receptor tyrosine kinase 3 (ErB3; cat. no. sc-285; rabbit), anti-insulin receptor substrate 1 (IRS-1; cat. no. sc-560; rabbit), anti-P13K (cat. no. sc-374534; mouse), anti-PTEN (cat. no. sc-7974; mouse), anti-pyruvate dehydrogenase kinase 1 (PDK1; cat. no. sc-28783; rabbit), anti-p-Akt (cat. no. sc-7985; rabbit), anti-Akt (cat. no. sc-8312; rabbit), anti-p-mTOR (cat. no. sc-293132; mouse), anti-mTOR (cat. no. sc-8319; rabbit), anti-p70S6 kinase (p70S6K; cat. no. sc-8418; mouse), anti-eukaryotic translation initiation factor 4E (eIF4E; cat. no. sc-514875; mouse), anti-SHC adaptor protein 1 (SHC; cat. no. sc-967; mouse), anti-AMPK (cat. no. sc-7291; mouse), anti-p-AMP-activated protein kinase (AMPK; cat. no. sc-33524; rabbit), anti-AMPK (cat. no. sc-74461; mouse), anti-Bcl-2 (cat. no. sc-492; rabbit), anti-Bcl-xL (cat. no. sc-7195; rabbit), anti-BH3 interacting domain death agonist (Bid; cat. no. sc-11423; rabbit), anti-poly (ADP-ribose) polymerase 1 (PARP; cat. no. sc-7150; rabbit), anti-caspase-9 (cat. no. sc-7885; rabbit), anti-caspase-3 (cat. no. sc-7148; rabbit), anti-Bad (cat. no. sc-7869; rabbit), anti-Bax (cat. no. sc-493; rabbit), anti-insulin-like growth factor 1 receptor (IGF-IR; cat. no. sc-390130; mouse), anti-epidermal growth factor receptor (EGFR; cat. no. sc-03; goat).}

**Reverse transcription-semi-quantitative PCR.** Total RNA was extracted from HepG2 cells using a QIAzol Lysis Reagent kit (Qiagen Sciences, Inc.). Reverse transcription was performed using AccuPower RT PreMix (Bioneer Corporation) according to the manufacturer's protocol. PCR amplification was performed using the template cDNA (1 ng). The reverse transcribed cDNA was amplified using a PCR premix kit (dNTP mix, nTaq Buffer and nTaq; Enzymix), and the following specific primer pairs (Cosmogenetech Co., Ltd.) were used: IGF-IR forward, 5'-ACAACATACGCCCTGTCTAC-3' and reverse, 5'-TGGCAGCAGCTTTGTTCTC-3'; EGFR forward, 5'-TGATTGCATCACGATTTGGA-3' and reverse, 5'-GCACCTGTAATAATGCCGGTGT-3'; ErBb2 forward, 5'-CTACGGCAGAGAACCAGAG-3' and reverse, 5'-ACA CCATTGCTTTCTCTTCC-3'; ErBb3 forward, 5'-GCGGCA CTTTTCTCTACTGG-3' and reverse, 5'-GGTCAGCCCAAC CAAAATCTC-3'; and β-actin forward, 5'-AAATCTGGCAGC ACACCTTCT-3' and reverse, 5'-ACAGCTGTGTGTTGCGTAC AG-3'. Reaction mixtures were subjected to initial denaturation at 95˚C for 3 min, followed by 34 cycles of 95˚C for 30 sec, 55-60˚C for 30 sec and 72˚C for 60 sec, then a final extension of 72˚C for 5 min. The products were normalized to β-actin as an internal control and separated by electrophoresis using a 1% agarose gel and stained with 0.5 µg/ml ethidium bromide for detection. Signal intensities were examined using a bio-imaging system (MiniBis Pro; DNR Bio-Imaging Systems, Ltd.). The software GeneTools version 4.03 (Syngene Europe) was used for densitometric analysis.

**Statistical analysis.** Results are presented as the mean ± SD of three independent experiments. The significance of differences among multiple means was assessed by one-way or two-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism software (version 7; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PYP1-4 protects against APAP-induced toxicity in HepG2 cells.** The present study assessed the effect of 0-500 ng/ml PYP1-4 on HepG2 cell viability. Treatment with PYP1-4 alone appeared to have significantly increased cell viability, but no significant differences between concentrations were observed (Fig. 1A). Subsequently, a survival rate of 60% was selected following treatment with 15 mM APAP for 18 h. The cell viability of the APAP overdose group was 61.5±2.4% compared with the control (Fig. 1B). Treatment with 15 mM APAP and 125, 250 and 500 ng/ml PYP1-4 significantly restored cell viability to 64.9±1.8, 69.9±3.0 and 75.9±1.4%, respectively, compared with the control. Microscopic observations confirmed these results (Fig. 1C).

**PYP1-4 decreases APAP-induced oxidative stress in HepG2 cells.** Oxidative stress is involved in APAP-induced liver failure, and liver tissue is damaged by various cytokines and high levels of NO following an APAP overdose (35). Griess reagent was used to investigate NO production in HepG2 cells treated with PYP1-4 and APAP overdose. The APAP overdose group exhibited a significantly higher NO level (130.5±9.9%) than the...
control group, whereas co-treatment with PYP1-4 significantly suppressed the NO level in a concentration-dependent manner (Fig. 2A). In the 500 ng/ml PYP1-4 group, NO production was reduced to 100.2±11.8% compared with the APAP overdose group (130.5±9.9%).

APAP-induced toxicity increases ROS levels and promotes oxidative stress (36-38). The present study investigated ROS levels in HepG2 cells using the fluorescent dye DCF-DA following treatment with PYP1-4 and APAP overdose. The ROS level was significantly higher in the APAP overdose group (184.6±16.6%) than in the control group (Fig. 2B). However, PYP1-4 co-treatment reduced the ROS level compared with that in the APAP overdose group, in a concentration-dependent manner (165.9±19.8 and 107.9±10.1% for 125, 250 and 500 ng/ml PYP1-4, respectively).

Subsequently, the levels of antioxidant enzymes, including CAT, HO1, SOD and NQO1 were investigated by western blotting. The APAP overdose group exhibited lower protein levels of CAT, HO1 and SOD2 than the control group, whereas PYP1-4 co-treatment significantly increased the CAT, HO1, SOD2 and NQO1 levels in a concentration-dependent manner (Fig. 2C and D).

To investigate the role of PYP1-4 in the modulation of MAPK signaling in APAP-induced cells, the phosphorylation levels of JNK and p38 were determined by western blotting. The phosphorylation levels were 2.7-fold (JNK) and 2.0-fold (p38) higher in the APAP overdose group than in the control group, whereas co-treatment with PYP1-4 significantly inhibited the phosphorylation of JNK and p38 compared APAP overdose group (Fig. 2C and D). p-JNK/JNK phosphorylation was significantly decreased in the PYP1-4 co-treatment groups compared with in the APAP group in a concentration-dependent manner (2.8-, 2.3- and 1.3-fold, respectively). Similarly, p-p38/p38 was significantly decreased (1.9-, 1.6- and 1.2-fold, respectively).

PYP1-4 increases Nrf2 expression and phosphorylation of GSK3β, Akt and AMPK in APAP-induced HepG2 cells. AMPK increases the inhibitory phosphorylation of GSK3β upstream of Akt (39) and phosphorylation of GSK3β stimulates Nrf2 (40,41). To identify the upstream effector of activation of Nrf2 by PYP1-4, the role of AMPK in PYP1-4-induced Akt/GSK3β phosphorylation and Nrf2 nuclear translocation was investigated in the present study. The APAP overdose group exhibited reduced HO1 and Nrf2 levels, as well as reduced ratios of p-GSK3β/GSK3β and p-AMPK/AMPK, compared with the control group (Fig. 3). However, PYP1-4 co-treatment groups significantly induced the expression and nuclear translocation of Nrf2, as well as the phosphorylation of GSK3β, Akt and AMPK in HepG2 cells.

PYP1-4 inhibits APAP-induced apoptosis. Toxic APAP doses reportedly induce apoptosis of murine hepatocytes (42,43). Therefore, the induction of apoptosis by APAP was investigated using FITC Annexin V assays in the present study. The apoptosis ratio was significantly higher in the APAP overdose group (43.2%) compared with the control group (4.77%; Fig. 4A). However, the cell survival rate was increased and the apoptosis ratio was significantly decreased in the PYP1-4 co-treatment groups compared with in the APAP group in a concentration-dependent manner (34.2, 26.6 and 20.2%, respectively).

To investigate the molecular mechanism by which PYP1-4 suppresses apoptosis, the present study examined its effect on the expression levels of Bcl-2-family proteins, which...
regulate apoptosis by controlling mitochondrial membrane permeability and cytochrome c release (44), in APAP-induced HepG2 cells. The levels of pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2, Bcl-xL, and Bid) Bcl-2-family proteins were examined. The PYP1-4 co-treatment groups exhibited lower Bad levels and higher Bcl-2 and Bid levels than the APAP overdose group, in a concentration-dependent manner (Fig. 4B and C).

Additionally, the present study demonstrated that PYP1-4 activated caspases. The PYP1-4 co-treatment groups exhibited increased expression levels of caspase-9 and caspase-3 compared with the APAP group in a concentration-dependent manner. Additionally, PARP cleavage in the PYP1-4 co-treatment groups was significantly decreased compared with that in the APAP group, in a concentration-dependent manner (Fig. 4B and C).

**PYP1-4 reverses the effects of overdose on the levels of growth-associated receptors.** PYP1-4 co-treatment reversed the effects of APAP overdose on apoptosis and survival. The present study assessed the protein and RNA levels of IGF-IR, EGFR, ErbB2 and ErbB3, based on the assumption that the increased cell survival was associated with effects on growth-associated receptors. PYP1-4 co-treatment groups increased the protein levels of IGF-IR and EGFR compared with APAP overdose (Fig. 5). However, the RNA levels of these receptors were unaffected, with the exception of ErbB3.

**PYP1-4 increases the levels of IRS-1/PI3K/Akt signaling pathway-associated proteins in APAP-induced cells.** IGF signaling affects cell survival, and the IGF-I protein level is elevated in HepG2 cells (45). PYP1-4 co-treatment restored APAP-induced apoptosis and the levels of growth factor
receptors (Figs. 4 and 5). Subsequently, the levels of proteins associated with the IRS-1/PI3K/Akt signaling pathway, one of the two major downstream IGF-IR signaling pathways (46), were assessed. PYP1-4 co-treatment groups significantly increased the protein levels of IGF-IR, IRS-1, PI3Kp85, PTEN, p70S6K and eIF4E in a concentration-dependent manner compared with the levels in the APAP overdose group (Fig. 6). In addition, PYP1-4 co-treatment groups significantly increased ratios of p-Akt/Akt and p-mTOR/mTOR compared with those in the APAP overdose group.

**Discussion**

Seaweeds have attracted attention from researchers due to their abundance of polysaccharides, proteins, vitamins, minerals and polyphenols (48,49). Seaweeds, including brown, green and red algae, possess anti-obesogenic, anticancer, antioxidant and anti-inflammatory activities due to various bioactive compounds (50). The red alga *P. yezoensis* is of increasing interest due to its rich sugars and protein content (51). Although numerous studies on have been investigated the polysaccharide and polyphenol constituents of *P. yezoensis* (22-24,26,28,31,32,52), studies on the proteins contained in this alga remain lacking (25,27,29,30,33,34).

APAP is safe at therapeutic doses; however, excessive doses cause serious hepatotoxicity in laboratory animals and humans, and are a major cause of liver and kidney failure (3,7,8). Therefore, methods to reduce the hepatotoxicity of APAP overdose are required.

Studies on seaweeds and APAP-induced hepatotoxicity have focused on *Sargassum* species (*Hizikia fusiformis*, syn. *Sargassum fusiforme*), red algae (*P. yezoensis*), green algae (*Ulva reticulata* and *Chlorella sorokiniana*) and sulfated polysaccharides (fucoxid) (33,53-56). In a previous study, prevention of APAP-induced hepatotoxicity is associated with a 14-kDa protein (PYP) of *P. yezoensis* (33). PYP may inhibit APAP-induced GSH depletion in rats. APAP also increases caspase-3 activity during apoptosis, DNA fragmentation and serum glutamic oxaloacetic transaminase/glutamic pyruvic transaminase levels, which are indicators of hepatic damage (33). Additionally, co-treatment with PYP and APAP reversed these effects to the levels in the control (33). Therefore, although further studies are required, there is evidence to support that PYP inhibits APAP-induced hepatotoxicity.
Based on these results, PYP has been purified from the 14-kDa protein using SDS-PAGE, automated protein sequencing and matrix assisted laser desorption/ionization quadrupole ion trap-time-of-flight mass spectrometry (34). The PYP fraction contains two proteins, PYP1 (10 kDa; an SDS-resistant dimer) and PYP2 (10 kDa) (34). Based on these results, the synthetic peptide PYP1 (1-20) corresponding to the N-terminal 20 residues of PYP1 (ALEGGKSSGGGEATRDPEPT) has been obtained (34). PYP1 (1-20) protects against APAP-induced apoptosis in HeLa (Chang Liver) cells, and has been determined to be the active fraction of PYP (34).

The present study investigated the protective effects of *P. yezoensis* peptides on APAP-induced hepatotoxicity. In a previous study, a total of 13 peptides were obtained by treating PYP1 (1-20) with trypsin, chymotrypsin and pepsin (34). These peptides were finally selected for PYP1-4 based on the cell viability assay results. The present study revealed that PYP1-4 at 0-500 ng/ml was non-toxic in HepG2 cells and reversed the effects of APAP-induced hepatotoxicity.

Activation of the Nrf2 signaling pathway serves an essential role in APAP-induced acute liver failure (57). Nrf2 is a redox-sensitive transcription factor and regulates the transcription of genes associated with protection against oxidative stress (58). In the cytoplasm, Nrf2 is typically present in the Nrf2-Kelch-like ECH-associated protein 1 (Keap1) complex (59). In response to oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus to induce the expression of genes encoding antioxidant enzymes (NQO1, glutathione S-transferase and HO1) by binding to the antioxidant response element in their promoters (60). Intracellular ROS...
accumulation disrupts the Nrf2-Keap1 interaction; oxidized Keap1 binds to the adapter protein GAP-associated tyrosine phosphoprotein p62 and releases Nrf2, which translocates to the nucleus and activates transcription of genes encoding

**Figure 5.** PYP1-4 treatment restores the levels of growth-associated factors in APAP-induced HepG2 cells. (A) HepG2 cells were incubated with 15 mM APAP with or without various concentrations of PYP1-4 for 18 h. The protein and RNA levels of growth-associated factors (IGF-IR, EGFR ErbB2, and ErbB3) were determined by western blot analysis and reverse transcription PCR. (B) Bands were normalized to β-actin as an internal control, and protein and RNA levels were graphed. Data are presented as the mean ± SD of three independent experiments, and were subjected to two-way ANOVA. *P<0.05 vs. the control group; †P<0.05 vs. the 15 mM APAP group. APAP, acetaminophen; EGFR, epidermal growth factor receptor; ErbB2, erb-b2 receptor tyrosine kinase 2; ErbB3, erb-b2 receptor tyrosine kinase 3; IGF-IR, insulin-like growth factor 1 receptor; PYP1-4, *P. yezoensis* peptide.

**Figure 6.** PYP1-4 restores the levels of IRS-1/PI3K/Akt signaling pathway proteins in APAP-induced HepG2 cells. (A) HepG2 cells were incubated with 15 mM APAP with or without various concentrations of PYP1-4 for 18 h. The levels of IRS-1/PI3K/Akt signaling pathway proteins (IGF-IR, IRS-1, PI3K, PTEN, PDK1, Akt, mTOR, p70S6K and elf4E) were determined by western blot analysis. (B) Bands were normalized to β-actin as an internal control, and the phosphorylated vs. total protein ratios were graphed. Data are the means ± SD of three independent experiments and were subjected to two-way analysis of variance. *P<0.05 vs. control group; †P<0.05 vs. 15 mM APAP group. APAP, acetaminophen; elf4E, eukaryotic translation initiation factor 4E; IGF-IR, insulin-like growth factor 1 receptor; IRS-1, insulin receptor substrate 1; p-, phosphorylated-; p70S6K, p70S6 kinase; PDK1, pyruvate dehydrogenase kinase 1; PYP1-4, *P. yezoensis* peptide.
antioxidant and detoxifying enzymes (61). Therefore, Nrf2 has potential as a therapeutic target for liver diseases, including APAP-induced hepatotoxicity (62). In present study, the antioxidant activity of PYP1-4 contributes to its protective effect against APAP-induced hepatotoxicity, and this protective effect is associated with the activation of the Nrf2/HO1/SOD2 signaling pathway.

Additionally, Nrf2 can be activated by post-transcriptional modification by kinases, including protein kinase C, PI3K and MAPK (63,64). AMPK activates the PI3K/Akt signaling pathway, and Akt activation is essential for the phosphorylation of GSK3β and may modulate oxidative stress (65). A heterotrimeric serine/threonine kinase, AMPK, senses the cellular energy status and regulates cell survival and death under oxidative stress (66). GSK3β is a constitutively activated Ser/Thr protein kinase that regulates glycogen metabolism, gene expression and cell death (67). Previously, based on evidence that GSK3β is a novel regulator of Nrf2, Nrf2 has been suggested to function in combination with the AMPK/Akt/GSK3β signaling network (40,41). Therefore, regulation of the Nrf2 signaling pathway by PYP1-4 may ameliorate APAP-induced acute liver failure by modulating the AMPK/Akt/GSK3β signaling network (40,41). Therefore, regulation of the Nrf2 signaling pathway by PYP1-4 may ameliorate APAP-induced acute liver failure by modulating the AMPK/Akt/GSK3β signaling pathway. In the present study, PYP1-4 increased Akt activity by phosphorylating GSK3β, and PYP1-4-induced Akt activation increased Nrf2 activity. In addition, the increased GSK3β phosphorylation caused by activation of AMPK protected against oxidative stress.

JNK phosphorylation and mitochondrial translocation increase mitochondrial dysfunction, and AMPK activation serves a crucial role in protecting mitochondria (68). In the present study, treatment with PYP1-4 activated AMPK, and inhibited the APAP-induced phosphorylation of JNK. These results suggested that PYP1-4 treatment protected against APAP-induced hepatotoxicity by inhibiting JNK phosphorylation. Resveratrol has been reported to protect mitochondria against oxidative stress by increasing phosphorylation of GSK3β by activating AMPK (68). In addition, esculentside A regulates Nrf2 activation via the AMPK/Akt/GSK3β signaling pathway (69). These results suggest that PYP1-4 treatment exhibits an antioxidant effect by activating Nrf2 via the AMPK/Akt/GSK3β pathway, thus protecting against APAP-induced hepatotoxicity (Fig. 3).

APAP-induced cell death remains controversial. The signal transduction pathways involved in apoptosis and necrosis exhibit a degree of overlap (70). In a previous study using ICR mice, 95% of APAP-damaged hepatocytes died due to necrosis in vivo (71); however, another study reported that APAP-induced hepatocytes (HuH7 cells) apoptosis serves a crucial role in liver failure (72). APAP-induced cell death has been hypothesized to be caused by necroptosis, which is characterized by features of necrosis and apoptosis (73). In the present study, APAP overdose increased apoptosis, whereas co-treatment with PYP1-4 resulted in a dose-dependent decrease in apoptosis.

Apoptosis can be initiated by intrinsic and/or extrinsic signaling pathways (73). Apoptosis of mammalian cells is regulated by Bcl-2 family proteins (44), which modulate mitochondrial membrane permeability and cytochrome c release. APAP induces metastasis of Bcl-2 family proteins (70), leading to the release of cytochrome c. Activation of apoptosis via the exogenous signaling pathway is mediated by the binding of an apoptotic ligand to a death receptor (74). These death receptors have intracellular domains that function as protein binding modules. Following recruitment and signaling of adapter molecules, cleavage and activation of pro-caspase-8, -9, -10 and -12 occur (75). This leads to the activation of caspase-3, -6 and -7, as well as the effector caspase, resulting in DNA fragmentation (75). In addition, APAP-induced hepatotoxicity occurs...
via matrix metalloproteinase degradation of cytochrome c and activation of caspase-8, -9 and -3 (76). Cleaved PARP is a marker of apoptosis; PARP is activated in cells undergoing stress and/or DNA damage, and is inactivated by cleavage of caspase-3 during programmed cell death (76). Therefore, the results of the present study suggested that PYPI-4 inhibits APAP-induced apoptosis via intrinsic (endogenous) and extrinsic (exogenous) signaling pathways (Fig. 4).

Several studies have investigated the mechanism by which IGF-IR protects against apoptosis (45,77,78). During apoptosis, the binding of wild-type IGF-IR suppresses cell death. A previous study has demonstrated that APAP-induced HeLa (Chang Liver) cells were restored to apoptosis following treatment with IGF-I (79). The present study demonstrated that the IGF-IR signaling pathway was affected by PYPI-4.

IGFs are synthesized in and secreted by adult and fetal hepatocytes, widely expressed in a number of cell types, essential for normal growth, development and differentiation, and mediate signals for apoptosis inhibition, mitogenesis and immobilization-independent growth (45,80).

IGF-IR-associated signaling pathways comprise the IRS-1/Pi3K/Akt and Ras/Raf/ERK signaling pathways (46,47). IGF-IR is autophosphorylated by intrinsic tyrosine kinase activity and promotes activation of downstream signaling molecules. The binding of activated IGF-IR and phosphorylated adaptor proteins such as IRS-1 then activate the Pi3K/Akt signaling pathway (81,82). IRS-1/Pi3K/Akt along with mTOR/p70S6K signaling activates translation initiation factors and inactivates regulatory factors (83). This signaling pathway is also involved in the Crosstalk with the Ras/Raf/ERK signaling pathways (84). In addition, Ras signaling is enhanced by upstream events such as the activation of IGF-IR (85). Ras continuously stimulates the MAPK-activating serine/threonine kinase Raf and induces cell growth through transcriptional activation of multiple targets (86).

MAPKs, including ERK, JNK and p38, are part of the IGF-IR signaling pathway and convert extracellular stimuli into a wide range of cellular responses. These proteins serve important roles in cell proliferation, differentiation, metabolism, survival and death (87,88), as well as in oxidative damage (77,78). JNK is primarily involved in apoptosis and is activated by oxidative damage, whereas ERK regulates cell growth and differentiation, is activated by oxidative damage, and acts as a cell death suppression signal to maintain homeostasis (89). Akt is a downstream target of the Pi3K/Akt signaling pathway and serves an important role in the inhibition of Pi3K-mediated cell proliferation (90). In the present study, PYPI-4 treatment of APAP-induced HepG2 cells induced growth and reduced oxidative damage and apoptosis in a dose-dependent manner on the IRS-1/Pi3K/Akt and Ras/Raf/ERK signaling pathways.

In conclusion, the present study revealed that PYPI-4 decreased APAP-induced oxidative damage, growth inhibition and apoptosis in HepG2 cells. Additionally, the IGF-IR signaling pathway contributed to the suppression of apoptosis and necrosis. These observations suggested that PYPI-4 exerts a hepatoprotective effect against APAP-induced oxidative damage and apoptosis. However, further research on the structure of PYPI-4 and on the signal transduction pathways involved in APAP-induced hepatotoxicity is required.
9. Lee WM: Acetaminophen and the U.S. Acute Liver Failure Study Group: Lowering the risks of hepatic failure. Hepatology 40: 6-9, 2004.
10. Knight TR, Fariss MW, Farhoud A and Jaeschke H: Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. Toxicol Sci 76: 229-236, 2003.
11. Hinson JA, Roberts DW and James LP: Mechanisms of acetaminophen-induced liver necrosis. Handb Exp Pharmacol 196: 369-405, 2010.
12. Yoon E, Babar A, Choudhary M, Kutner M and Pyrsopoulos N: Acetaminophen-induced hepatotoxicity: A comprehensive update. J Clin Transl Hepatol 4: 131-142, 2016.
13. James LP, Mayeux PR and Hinon JA: Acetaminophen-induced hepatotoxicity. Drug Metab Dispos 31: 1499-1506, 2003.
14. Zhao X, Cong X, Zheng L, Xu L, Yin L and Peng J: Dioscin, a natural glucoside from Fritillaria, shows remarkable protective effect against acetaminophen-induced liver damage in vitro and in vivo. Toxicol Lett 214: 69-80, 2012.
15. Liang YL, Zhang ZH, Liu XJ, Liu XQ, Tao L, Zhang YF, Wang H, Zhang C, Chen X and Xu DX: Melatonin protects against apoptosis-inducing factor (AIF)-dependent cell death during acetaminophen-induced acute liver failure. PLoS One 7: e51911, 2012.
16. Slitt AM, Dominick PK, Roberts JC and Cohen SD: Effect of ribose cytoine pretreatment on hepatic and renal acetaminophen metabolism and glutathione depletion. Basic Clin Pharmacol Toxicol 102: 487-494, 2008.
17. Yousef MI, Omar SA, El-Guendi MI and Abdelmegid LA: Potential protective effects of quercetin and curcumin on paracetamol-induced histological changes, oxidative stress, impaired liver and kidney functions and haematotoxicity in rat. Aquaculture 382-383: 2246-2261, 2017.
18. Yan M, Hoo Y, Yin S and Hu H: Mechanisms of acetaminophen-induced liver injury and its implications for therapeutic interventions. Redox Biol 17: 274-283, 2018.
19. Corcoran GB, Todd EL, Racz WJ, Hughes H, Smith CV and Mitchell JR: Effects of N-acetylcysteine on the disposition and metabolism of acetaminophen in mice. J Pharmacol Exp Ther 232: 857-863, 1985.
20. Shahidi F and Rahman J: Bioactive in seaweeds, algae, and microalgae. In: Food science and technology. AOCS Press, Illinois, USA, 2018.
21. Niwa K: Genetic analysis of artificial green and red mutants of Porphyra yezoensis Ueda (Bangiales, Rhodophyta). Mol Nutr Food Res 62: e1700469, 2018.
22. Mohibullah M, Bhuian MM, Hannan MA, Getachew P, Hong YK, Choi JS, Choi IS and Moon IS: The edible red alga Porphyra yezoensis promotes neuronal survival and cytoarchitecture in primary hippocampal neurons. Cell Mol Neurobiol 36: 669-682, 2016.
23. Choi JW, Kim IH, Kim YM, Lee MK and Nam TJ: Pyropia yezoensis glycoprotein regulates antioxidant status and prevents hepatotoxicity in a rat model of D-galactosamine/lipopolysaccharide-induced acute liver failure. Mol Med Rep 13: 3110-3114, 2016.
24. Hwang HJ, Kwon MJ, Kim IH and Nam TJ: Chemoprotective effects of a protein from the red alga Porphyra yezoensis on acetaminophen-induced liver injury in rats. Phytother Res 22: 1149-1153, 2008.
25. Choi YH, Yamaguchi K, Oda T and Nam TJ: Chemical and mass spectrometry characterization of the red alga Pyropia yezoensis chemoprotective protein (PYP): Protective activity of the N-terminal fragment of PYP1 against acetaminophen-induced cell death in chang liver cells. Int J Mol Med 35: 271-276, 2015.
26. Wallace JL: Acetaminophen hepatotoxicity: NO to the rescue. Br J Pharmacol 143: 1-2, 2004.
27. Liu WX, Jia FL, He YY and Zhang BX: Protective effects of 5-methoxypсорalen against acetaminophen-induced hepatotoxicity in mice. World J Gastroenterol 18: 2197-2202, 2012.
28. Olalere MT and Rocha BT: Acetaminophen-induced liver damage in mice: Effects of some medicinal plants on the hepato-protective defense system. J Ethnopharmacol 50: 319-327, 2008.
29. Lee KJ, You HJ, Park SJ, Kim YS, Chung YC, Jeong TC and Jeong HG: Hepatoprotective effects of Platycodon grandiflorum on acetaminophen-induced liver damage in mice. Cancer Lett 174: 73-81, 2001.
30. Toyosaki T, Yang X, Wuo D, Xing S, Bian F, Li W, Chi J, Bai X, Wu G, Chen X, et al: Salidroside ameliorates insulin resistance through activation of a mitochondria-associated AMPK/PI3K/Akt/GSK3β pathway. Br J Pharmacol 172: 3284-3301, 2015.
31. Mathur A, Rizvi F and Kakkar P: PHLP22 down regulation influences nuclear Nrf2 stability via Akt-I/Gsk3/Fyn kinase axis in acetaminophen induced oxidative renal toxicity: Protection accorded by morin. Food Chem Toxicol 89: 19-31, 2016.
32. Xing HY, Cai YQ, Wang XF, Wang LL, Li P, Wang GY and Chen HY: The cytoprotective effect of hyperoside against oxidative stress is mediated by the Nrf2-ARE signaling pathway through GSK-3β inactivation. PLoS One 10: e0145183, 2015.
33. Song E, Fu J, Xia X, Su C and Song Y: Bazhen decoction protects against acetaminophen induced acute liver injury by inhibiting oxidative stress, inflammation and apoptosis in mice. PLoS One 9: e0170455, 2014.
34. Sharma S, Singh RL, and Kakkar P: Modulation of Bax/Bcl-2 and caspases by probiotics and chemoprotective protein from Porphyra yezoensis induced apoptosis in primary hepatocytes. Food Chem Toxicol 49: 770-779, 2011.
35. Cory S, Huang DC and Adams JM: The Bcl-2 family: Roles in cell survival and oncogenesis. Oncogene 22: 8590-8607, 2003.
36. Yao NH, Yao DF, Dong ZZ, Yan XD, Chen J, Yao M, Wang L and Yan MJ: Effects of inhibited IGF-IR expression on proliferation and apoptosis of human hepatocellular carcinoma cell lines. Zhonghua Gan Zang Bing Za Zhi 21: 376-380, 2013 (In Chinese).
37. Kulik G, Klipper A and Weber MF: Antiangiopotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 17: 1395-1606, 1997.
38. Yu H and Rohan T: Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst 92: 1472-1489, 2000.
39. MacArtain P, Gill CI, Brooks M, Campbell R and Rowland IR: Nutritional value of edible seaweeds. Nutr Rev 65: 535-543, 2007.
40. Dawczynski CH, Schubert R and Jahreis G: Amino acids, fatty acids, and dietary fiber in edible seaweed products. Food Chemistry 103: 891-899, 2007.
41. Lee JC, Hou MF, Huang HW, Chang FR, Yeh CC, Tang YJ and Chang HW: Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties. Cancer Cell Invest 9: 13-55, 2013.
42. Qu W, Ma H, Pan Z, Luo L, Wang Z and He R: Preparation and antihypertensive activity of peptides from Porphyra yezoensis Ueda. J Food Sci 81: 16-20, 2016.
43. Yan Y, Chen CF, Yang Z, Shen H, Zhang W, He J, Wang L, et al: Salidroside ameliorates insulin resistance through activation of a mitochondria-associated AMPK/PI3K/Akt/GSK3β pathway. Br J Pharmacol 172: 3284-3301, 2015.
53. Hira K, Sultana V, Ara J and Haque SE: Protective role of Sargassum species in liver and kidney dysfunctions and associated disorders in rats intoxicated with carbon tetrachloride and acetaminophen. Pak J Pharm Sci 30: 721-728, 2017.

54. Balaji Raghavendra Rao H, Sathivel A and Devaki T: Antihepatotoxic nature of Ulva reticulata (Chlorophyceae) on acetaminophen-induced hepatotoxicity in experimental rats. J Med Food 7: 495-497, 2004.

55. Escapa C, Combrina RN, Paniagua S, García AI and Otero M: Paracetamol and salicylic acid removal from contaminated water by microalgae. J Environ Manage 203: 799-806, 2017.

56. Hong SW, Lee HS, Jung KH, Lee H and Hong SS: Protective effect of fucoidan against acetaminophen-induced liver injury. Arch Pharm Res 35: 1099-1105, 2012.

57. Isho K, Mizmura J and Yamamoto M: Discovery of the negative regulator of Nrf2, Keap1: A historical overview. Antioxid Redox Signal 13: 1665-1678, 2010.

58. Inoue H, Maeda-Yamamoto M, Nesumi A and Murakami A: Delphinidin-3-O-galactoside protects mouse hepatocytes from (-)-epigallocatechin-3-gallate-induced cytotoxicity via up-regulation of heme oxygenase-1 and heat shock protein 70. Nutr Res 32: 357-364, 2012.

59. Jaiswal AK: Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med 36: 1199-1207, 2004.

60. Kensler TW, Wakabayash N and Biswal S: Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 47: 89-116, 2007.

61. Jiang ZY, Xu LL, Lu MC, Chen ZY, Yuan ZW, Xu XL, Guo XK, Zhang XJ, Sun HP and You QD: Structure-activity and structural-property relationship and exploratory in vivo evaluation of the nanomolar Keap1-Nrf2 protein-protein interaction inhibitor. J Med Chem 58: 6410-6421, 2015.

62. Bataille AM and Manautou JE: Nrf2: A potential target for new therapeutics in liver disease. Clin Pharmacol Ther 92: 340-348, 2012.

63. Kong AN, Owuor E, Yu R, Hebar V, Chen C, Hu R and Mandlekar S: Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electronephil response element (ARE/EPEP). Drug Metab Rev 33: 255-271, 2001.

64. Nakaso K, Yano H, Fukuhara Y, Takeshima T, Wada-Isoe K and Nakashima K: PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by heme in human neuroblastoma cells. FEBS Lett 546: 181-184, 2003.

65. Horike N, Sakoda H, Kushiyama A, Ono H, Fujishiro M, Kamata H, Nishiyama K, Uchijima Y, Kurihara Y, Kurihara H and Asano T: AMP-activated protein kinase activation increases phosphorylation of glycolgen synthase kinase 3beta and thereby reduces AMPK-responsive element transcriptional activity and phosphenolpyruvate carboxykinase gene expression in the liver. J Biol Chem 283: 33902-33910, 2008.

66. Konrad D, Rudich A, Bilan PJ, Patel N, Richardson C, Witters LA, Klip A: Troglitazone causes acute mitochondrial membrane depolarisation and an AMPK-mediated increase in glucose phosphorylation in muscle cells. Diabetologia 48: 954-965, 2005.

67. Luo J: The role of Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. Cancer Lett 273: 194-200, 2009.

68. Shin SM, Cho UJ and Kim SG: Resveratrol protects mitochondria against oxidative stress through AMP-activated protein kinase-mediated glycolgen synthase kinase 3beta inhibition downstream of poly(ADP-ribose)polymerase-LKB1 pathway. Mol Pharm 76: 864-895, 2009.

69. Wang L, Zhuang S, Cheng H, Lv H, Cheng G and Ci X: Nrf2-mediated liver protection by esculetinoid A against acetaminophen toxicity through the AMPK/Akt/GSK3beta pathway. Free Radic Biol Med 101: 401-412, 2016.

70. Jaeschke H and Bajt ML: Intracellular signaling mechanisms of acetaminophen-induced liver cell death. Toxicol Sci 89: 31-41, 2006.

71. Ray SD, Mumaw VR, Raje RR and Fariss MW: Protection of acetaminophen-induced hepatoacellular apoptosis and necrosis by cholesteryl hemisuccinate pretreatment. J Pharmacol Exp Ther 279: 1470-1483, 1996.