Taxifolin Exerts Cytoprotective Effect by Activation of Nrf2-ARE Signaling Pathway in HepG2 cells

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Background: Oxidative stress contributes to various deleterious health effects including aging, cardiovascular disease, chronic inflammation, neurodegenerative disease and cancer. Cells have developed adaptive cytoprotective response by up-regulation of the antioxidant defense systems including Nrf2-ARE signaling pathway. Nrf2 (Nuclear factor erythroid 2-related factor 2) protein activated by oxidative stress and electrophiles is contributed to the cytoprotective effect by induction of antioxidant gene expression through the binding with cis-acting consensus sequence so-called antioxidant response element (ARE). Phytochemicals are rich in antioxidant activity and some effects are produced by induction of antioxidant genes through Nrf2-ARE.

Methods: Our study have screened for inducers of Nrf2-ARE signaling pathway, using HepG2 cells transiently transfected with plasmid containing ARE-luciferase reporter and plasmid with Renilla-luciferase reporter. The cytotoxicity assay was performed by sulforhodamine B assay.

Results: Taxifolin was able to induce the Nrf2-ARE in ARE-luciferase reporter assay stronger than other tested phytochemicals and did not toxic to HepG2 cells. Taxifolin showed cytoprotective effect in a model of doxorubicin-induced cytotoxicity. When HepG2 cells were pretreated with taxifolin 18 h, the cytotoxicity of doxorubicin was greatly decreased.

Conclusion: The study showed that inducer of Nrf2-ARE pathway including taxifolin could provide health benefit in protection against oxidative injury.

Key words: Nrf2, Antioxidant, Cytoprotective effect, Doxorubicin, reporter assay

ศรีนครินทร์เวชสาร 2557; 29 (suppl):122-25. ♦ Srinagarind Med J 2014 ;29 (suppl):122-25.

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Introduction

Nrf2 is a transcription factor involved in the induction of antioxidant enzymes such as glutamate–cysteine ligase catalytic subunit (GCLC), heme oxygenase-1 (HO-1), and glutathione-*S*-transferases (GSTs) which contribute to cytoprotection against oxidative stress and electrophilic agents in cells[1,2]. In normal condition, Nrf2 is mainly located in the cytoplasm and is ubiquitinated by Keap1 and degraded by proteasomal system. Under oxidative stress, Nrf2 is released from KEAP1, a repressor protein, and translocated to the nucleus, where it binds to the consensus sequence nucleotide sequence, antioxidant response elements (ARE), and up-regulates a numerous cytoprotective gene[3]. It is well established that activation of Nrf2-ARE signaling system plays roles in protection against oxidative injury and cancer chemoprevention[4]. There are number of reports shown that many of phytochemicals are able to induce Nrf2-mediated antioxidant defense system such as curcumin and green tea catechin[5,6].

In this study, we screened the effect of several phytochemicals on Nrf2-ARE signaling pathway using the ARE-luciferase reporter assay. We found that most

of compounds could induce Nrf2-ARE signaling pathway. Taxifolin was further tested for its cytoprotective effect in a model of doxorubicin-induced cytotoxicity.

Methods

Cell culture

Human hepatoma (HepG2) cells (ATCC) were used in this study. Cells were cultured in DMEM media containing 10% fetal bovine serum (Hyclone) supplemented with 1% non-essential amino acids (In Vitrogen), 12.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.3, 100 U/mL penicillin, 50 μg/mL gentamicin. The cells were subcultured every 2-3 days before confluence using 0.25% trypsin–EDTA. The medium was changed after an overnight incubation.

ARE- luciferase reporter gene activity.

Cells were seeded at a density of 1 x 10^5 cells/well in 24-well plates. After incubation for overnight, the cells were transiently transfected with plasmid PGL4.7 containing 4xARE-luciferase and plasmid PGL4.74 (hRluc/TK) containing Renilla luciferase reporter using lipofectamine 2000 (In Vitrogen) as transfecting reagent, for 8 h in serum free and without antibiotics medium. Then, cells were treated for 24 h with tert-butylhydroquinone (t-BHQ), a known Nrf2-ARE inducer used as control, silibinin, kaemferol, quercetin, chrysin, epigallocatechin gallate (EGCG) and taxifolin dissolved in DMSO and diluted in fresh serum-free media (a solution giving a final concentration of 0.1% v/v DMSO). For control experiments, vehicle alone (0.1% v/v DMSO in serum-free media) was used. The cells were washed, lysed and measured the signals using dual luciferase assay kit (Promega). The relative luciferase activity was normalized to Renilla luciferase activity. The experiment was carried out in triplicate and expressed as the mean ± SEM.

Cytotoxicity Assay

HepG2 cells were treated with different concentrations of taxifolin for 24 h. The cell viability was assessed by the the sulphorhodamine B (SRB) assay. In study of the cytoprotective effect of taxifolin, HepG2 cells were pretreated with 10 μM taxifolin for 18 h before addition of doxorubicin (Doxo) at varied concentrations and incubation for another 24 h. The cytotoxicity of the cells was assayed by the SRB method. The method in brief, cultured cells were washed once with phosphate-buffered saline (PBS) and fixed with 100μl of ice-cold trichloroacetic acid for 1 h and stained with 50 μl of 0.4% SRB in 1% acetic acid for 30 min. Fixed cells were rinsed several times with 1% acetic acid, and protein-bound dye was dissolved with 200 μl of 10 mM Tris base solution for determination of absorbance with a microplate reader with filter wavelength of 540 nm.

Results and discussion

We evaluated in the effect of several phytochemical compounds on Nrf2-ARE signaling pathway using a cell-based ARE-reporter assay in HepG2 cell line. We discovered that most of phytochemical compounds could induce ARE-driven luciferase gene expression. Moreover, taxifolin (dihydroquercetin) was identified as potent Nrf2 inducers (Fig. 1). To study the cytotoxic effect of taxifolin in HepG2, the cells were incubated with taxifolin at varied concentrations for 24 h. The result showed that taxifolin was not substantially toxic to HepG2 cells (Fig. 2). Taxifolin is a member of flavanonol compound found in milk thistle and citrus fruits. It was known to possess antioxidant and anti-inflammatory effects[7]. Consistent with our screening, taxifolin was supposed to activate the Nrf2-mediated antioxidant defense genes for cytoprotection. The following experiment was carried out using doxorubicin, a highly toxic anticancer agent, exerting cytoxicity by oxidative damage[8], as a model to evaluate cytoprotective effect of taxifolin. In the present study, HepG2 cells were pretreated with taxifolin 10 μM for 18 h before addition of doxorubicin at varied concentrations and incubation for another 24 h. Doxorubicin showed to be highly toxic in HepG2 (Fig. 3) with median inhibitory concentration (IC50) value of 0.4 μM, whereas the combination treatment of taxifolin and doxorubicin significantly preventive cells death from doxorubicin (Fig. 3). The IC50 of combination of taxifolin with doxorubicin in HepG2 was increased by 8 folds higher when compared with doxorubicin alone (Table 1). In addition, the maximal cytotoxic effect (Emax) of the combination was

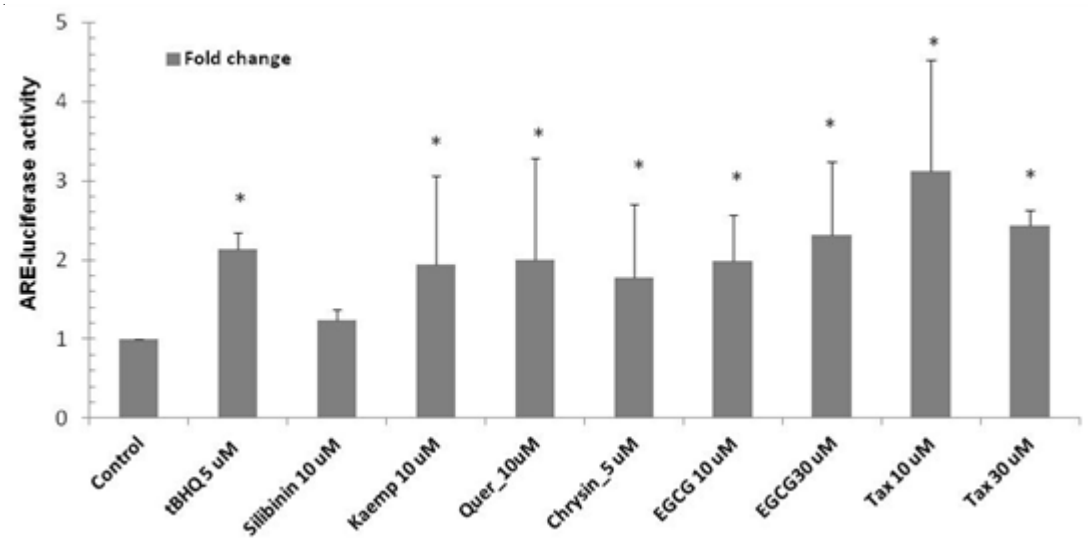

Fig. 1 Antioxidant response element (ARE) reporter assay for various compounds. HepG2 cells were transfected with plasmid PGL4.7 containing ARE-luciferase and plasmid PGL4.74 (hRluc/TK) containing Renilla luciferase reporter for 8 h and cells were treated with tested agents for 24 h. The cells were washed, lysed and measured the signals using dual luciferase assay kit. Values are fold change from the control and expressed as mean ± SEM, each from three experiments. * significantly different from Control

Table 1 Protective effect of taxifolin in doxorubicin-induced cytotoxicity in HepG2 cells

	Conditions	E_{max} (%)[a]	IC50 (μM)
HepG2	Doxorubicin alone	69.3 ± 6.2[b]	0.4 ± 0.2
	Doxorubicin plus taxifolin	30.7 ± 16	2.9 ± 2.7

[a] Emax: maximal cancer cell killing effect (%), IC50: median inhibitory concentration

[b] Each value represents the mean ± SEM, each from three experiments

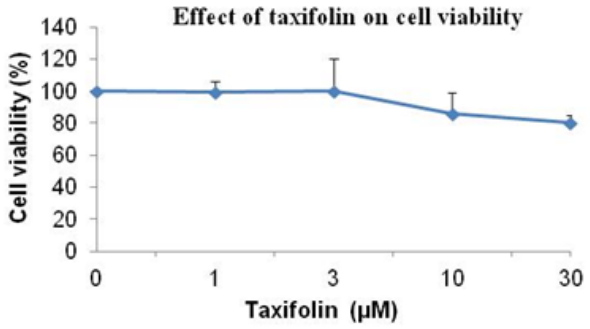

Fig. 2 Effect of taxifolin on cell viability. HepG2 were cultured with taxifolin at varied concentrations for 24 h. The cell viability was assessed by the SRB method. Values are mean ± SEM, each from three experiments.

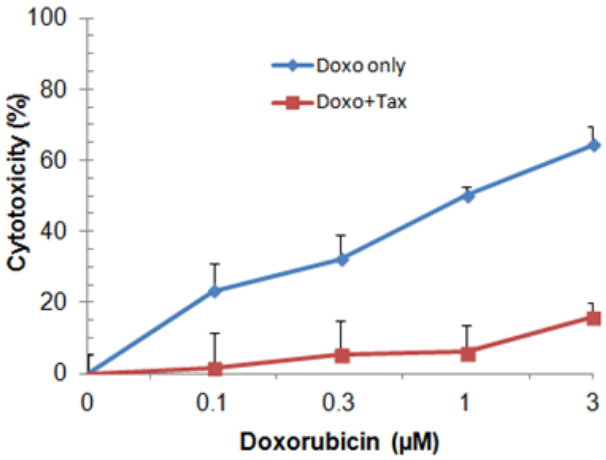

Fig. 3 Cytoprotective effect of taxifolin against doxorubicin. HepG2 cells were pretreated with taxifolin (Tax: 10 μM) for 18 h before addition of doxorubicin (Doxo) at varied concentrations and incubation for another 24 h. The cytotoxicity of the cells was assayed by the SRB method. Values are mean ± SEM, each from three experiments.

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apparently decreased. These results suggest that taxifolin can alleviate oxidative damage by exerting effect through activation of Nrf2-ARE signaling pathway, thereby up-regulated the antioxidant enzymes.

Conclusion

The data demonstrated that the cytoprotective effect of taxifolin against doxorubicin by activating Nrf2-ARE signaling pathway. Moreover, phytochemical inducers of Nrf2-ARE including taxifolin may provide health benefit to consumers of various vegetables.

Acknowledgment

This works was supported by Research Fund from Khon Kaen University (563603) and Thailand Research Fund. PS was supported by the Royal Golden Jubilee Ph.D. Program

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