Protective Effects of Panduratin A against Oxidative Damage of tert-Butylhydroperoxide in Human HepG2 Cells

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Received February 9, 2005; accepted March 22, 2005

The protective effect of panduratin A, isolated from \textit{Kaempferia pandurata} Roxb. (Zingiberaceae), against tert-butylhydroperoxide (t-BHP)-induced cytotoxicity was investigated in a human hepatoma cell line, HepG2. The tetrazolium dye colorimetric test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay) was used to monitor cytotoxicity. Lipid peroxidation [malondialdehyde (MDA) formation] and intracellular glutathione level were estimated by fluorometric methods. Intracellular reactive oxygen species (ROS) formation was measured using a fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Panduratin A significantly reduced the cell growth inhibition caused by t-BHP. Furthermore, panduratin A ameliorated lipid peroxidation as demonstrated by a reduction in MDA formation, and attenuated glutathione (GSH) depletion in a dose-dependent manner. It was also found that panduratin A reduced intracellular ROS formation caused by t-BHP. These results strongly suggest that panduratin A has significant protective ability against oxidative damage caused by reactive intermediates.

Key words panduratin A; tert-butylhydroperoxide; oxidative damage; HepG2 cell

Reactive oxygen species (ROS) act as subcellular messengers in such complex cellular processes as mitogenic signal transduction, gene expression, and regulation of cell proliferation. Excessive production of ROS may, however, cause oxidative stress, loss of cell function, and ultimately apoptosis or necrosis. Fortunately, cells possess antioxidant molecules which can neutralize ROS before they induce damage on vital components. However, oxidative stress occurs when antioxidant systems are overwhelmed by ROS. Oxidative stress is considered to play a prominent role in the causation of many diseases including aging, cardiovascular disease, cancer and liver injury.

\textit{t}-Butylhydroperoxide (t-BHP) is an organic hydroperoxide that can be metabolized to free radical intermediates, which can subsequently initiate lipid peroxidation, affect cell integrity, and form covalent bonds with cellular molecules resulting in cell injury. t-BHP causes leakage of lactate dehydrogenase (LDH) and formation of malondialdehyde (MDA) in hepatocytes, and also mediates DNA base damage in mammalian cells. These phenomena are similar to the oxidative stress occurring in the cell and/or tissue.

Because of its unique metabolism and relationship to the gastrointestinal tract, the liver is an important target of the toxicity of drugs, xenobiotics, and oxidative stress. Therefore, primary importance is to understand the role of antioxidants which can reduce the oxidative stress induced by reactive intermediates from various chemicals and drugs. We have investigated hepatoprotective drugs, based on the measurement of cytotoxicity of t-BHP against human-liver-derived HepG2 cells. HepG2 cells have been suggested to be a useful \textit{in vitro} model for investigation of the metabolism and toxicity of drugs compared with animal cells, since HepG2 cells retain many of the specialized functions which are characteristics of normal human hepatocytes, including the synthesis and secretion of plasma proteins.

\textit{Kaempferia pandurata} Roxb. is a perennial herb of the family Zingiberaceae cultivated in some tropical countries, including Thailand and Indonesia. Its rhizome has been used as a food ingredient, an aphrodisiac, and a folk medicine for the treatment of colic disorder, fungal infections, dry cough, rheumatism, and muscular pains. Panduratin A (Fig. 1A) is a chalcone derivative isolated from \textit{K. pandurata}. It was recently reported that panduratin A possessed significant topical anti-inflammatory activity in murine macrophages, and in the TPA-induced ear edema in rats.

The objective of the current study was to examine the ability of panduratin A to protect hepatocytic injury induced by t-BHP and to characterize the mechanisms involved. For this purpose, we have studied the cell viability, lipid peroxidation, glutathione (GSH) level, and ROS level of t-BHP-treated HepG2 cells in the absence or presence of panduratin A. In all experiments, silybin (Fig. 1B) was used as a positive control. Silybin is the main component of silymarin extracted from the milk thistle plant, \textit{Silybum marianum}, which has been used for centuries as a natural remedy for liver diseases.

Fig. 1. Chemical Structure of Panduratin A and Silybin

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MATERIALS AND METHODS

Plant Material Rhizomes of *Kaempferia pandurata* (Rostr.) Schilth. (syn. *Boesenbergia rotunda*, Zingiberaceae) was collected in 2000 from the Biofarmaka Research Center of Bogor Agricultural University in Indonesia, and identified by Dr. N. I. Baek, Institute of Life Science, Kyunghee University, Yongin, South Korea. The sample was dried, ground to powder, and deposited at 4°C in the Department of Biotechnology, Yonsei University, Seoul, South Korea. Panduratin A was isolated from the ethyl acetate fraction of *K. pandurata* by successive silica gel chromatography and recycling preparative HPLC using a JAI GEL W-252 column, as we have previously reported.12 The chemical structure of panduratin A (Fig. 1A) was identified by a variety of spectroscopic analyses, including 1H-NMR, 13C-, and FAB-MS spectra.

Reagents Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY, U.S.A.). DCFH-DA was purchased from Molecular Probes (Eugene, OR, U.S.A.). The other reagents used in this experiment were all from Sigma (St. Louis, MO, U.S.A.).

Cell Culture The human hepatoma cell line, HepG2 (KCLB No. 58065) was purchased from the Korea Cell Line Bank (Seoul, Korea). HepG2 cells were cultured in DMEM containing 10% heat-inactivated FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in 5% CO2. The culture medium was changed twice every week, and the cells were subcultured at a 1:4 ratio once a week.

Cell Viability Assay The tetrazolium dye colorimetric test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test) was used to determine the viability of HepG2 cells. The MTT assay is based on ability of functional mitochondria to catalyze the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan, the concentration of which can be measured spectrophotometrically.15) HepG2 cells were first cultured in 96-well microplates (1.5×104/ml) for 24 h, washed twice using PBS, and pretreated with different concentrations of panduratin A or silybin. After 30 min incubation, t-BHP solution was added to the wells, and the cells were reincubated. After 24 h incubation, MTT reagent (2 mg/ml) was added to each well, and the plate was incubated at 37°C for an additional 4 h. The media were then removed, and the intracellular formazan product was dissolved in DMSO. The absorbency of each well was then measured at 570 nm, and the percentage viability was calculated.

Measurement of MDA and GSH Level Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acid, was measured to determine the extent of lipid peroxidation. The MDA level was determined by the thiobarbituric acid method16) and the amount of MDA equivalents peroxidation. The MDA level was determined by the thiobarbituric acid, was measured to determine the extent of lipid hyde (MDA), formed from the breakdown of polyunsaturated viability was calculated.

The concentration of which can be measured spectrophotometrically,15) HepG2 cells were first cultured in 96-well microplates (1.5×104/ml) for 24 h, washed twice using PBS, and pretreated with different concentrations of panduratin A or silybin. After 30 min incubation, t-BHP solution was added to the wells, and the cells were reincubated. After 24 h incubation, MTT reagent (2 mg/ml) was added to each well, and the plate was incubated at 37°C for an additional 4 h. The media were then removed, and the intracellular formazan product was dissolved in DMSO. The absorbency of each well was then measured at 570 nm, and the percentage viability was calculated.

**Measurement of MDA and GSH Level** Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acid, was measured to determine the extent of lipid peroxidation. The MDA level was determined by the thiobarbituric acid method16) and the amount of MDA equivalents was calculated from a standard curve prepared using malondialdehyde bis (dimethyl acetal). Intracellular glutathione (GSH) was measured using o-phthalaldehyde (OPT) as the fluorescent reagent according to the method of Hissin and Hilf.17) The GSH concentration was determined using a standard curve with known amounts of GSH. The cells were preincubated with panduratin A or silybin for 30 min and treated with t-BHP. The cells were harvested after incubation for 3 h and 5 min to measure MDA and GSH level respectively.

**Measurement of Intracellular ROS** Production of reactive oxygen species (ROS) was monitored spectrofluorometrically by the 2’,7’-dichlorofluorescein diacetate (DCFH-DA) method.18,19) DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to the nonfluorescent DCFH, which can be rapidly oxidized to the highly fluorescent DCF in the presence of ROS.19) After the cells were incubated with panduratin A or silybin for 30 min and treated with t-BHP, DCFH-DA was added to the culture plates at a final concentration of 5 μM and incubated for 4 h at 37°C in darkness. DCF fluorescence intensity was detected with emission wavelength at 530 nm and excitation wavelength at 485 nm using CytoFluor Series 4000 multiwell fluorescence plate reader (PerSeptive Biosystems Inc., Framingham, MA, U.S.A.).

**Statistical Analysis** Each experiment was performed at least in triplicate. Results are presented as the mean±standard deviation (S.D.). Statistical analysis was performed using one-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffe’s test (SPSS 12.0). The criterion for statistical significance is expressed as *p<0.05.

RESULTS

**The Cytotoxicity of Panduratin A in HepG2 Cells** The cytotoxicity of panduratin A in HepG2 cells was evaluated using MTT test. Panduratin A did not present any cytotoxic effect at concentrations ranging from 1 to 15 μM (data not shown). At a higher concentration of 17.5 μM, panduratin A significantly inhibited the growth of HepG2 cells. Thus, 1—15 μM of panduratin A was used for all subsequent experiments for testing its possible protection against t-BHP toxicity.

**Effect of Panduratin A on t-BHP-Induced Cell Growth Suppression** The possible protection by panduratin A against t-BHP-induced loss of viability was evaluated by preincubating the cells with or without panduratin A or silybin for 30 min, followed by treatment with 250 μM t-BHP for 24 h. As shown in Fig. 2, incubation with t-BHP for 24 h resulted in only 47.33±1.63% viability in HepG2 cells. Treatment of panduratin A reduced the cell growth suppression caused by t-BHP and increased cell viability in a dose-dependent manner. The cell viability was restored up to 81.28±1.46% by pretreating of 15 μM of panduratin A, which was almost comparable to 77.61±2.87% at the same concentration of silybin.

**Effect of Panduratin A on t-BHP-Induced Lipid Peroxidation and GSH Depletion** Treatment of t-BHP increases lipid peroxidation in HepG2 cells, which plays a central role in the development of toxicity. The increased level of MDA equivalents in t-BHP-injured cells was partially restored by concomitant treatment with panduratin A and silybin (Table 1). Similar inhibitory effect of panduratin A on t-BHP-induced GSH depletion was also measured, and pretreatment of panduratin A or silybin significantly restores the GSH level reduced by t-BHP treatment (Table 1).

**Effect of Panduratin A on ROS Overproduction Induced by t-BHP** The intracellular ROS level was evaluated...
by a converting reaction of DCFH-DA to DCF. Dose-dependent reaction of panduratin A on ROS level in t-BHP-treated cells is shown in Fig. 3A. Increased DCF fluorescence intensity caused by t-BHP dropped significantly from 665 ± 11.79 in cells treated with t-BHP only to 170 ± 30.62 in cells to which 15 μM panduratin A was added. The dose-response study demonstrated that panduratin A (10—15 μM) has a stronger reductive effect on ROS level than silybin. The time-course of the reductive effect of panduratin A on t-BHP-induced ROS overproduction is presented in Fig. 3B. A decrease in ROS production was observed in the cells pretreated with panduratin A at as little as 30 min incubation.

**DISCUSSION**

Recently, much attention has been focused on the protective function, especially antioxidative effect, of naturally occurring compounds and on the mechanisms of their actions. Phenolic compounds, which are widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living system.20,21 Panduratin A (Fig. 1A) is a cyclohexenyl chalcone derivative isolated from Kaempferia pandurata. Chalcones are a group of phenolic compounds belonging to the flavonoid family, and widely occur in nature as pigments. This category of flavonoids displays a broad spectrum of bioactivities such as anticancer, antifungal, antibacterial, anti-inflammatory, analgesic, and antioxidative activities.22—24 In a previous study, panduratin A showed significant anti-inflammatory activity,12,13 however, antioxidative activity of panduratin A has not yet been examined. This study aimed to investigate the protective effects of panduratin A against oxidative stress and the mechanisms involved in the human hepatoma cell line, HepG2.

tert-Butylhydroperoxide (t-BHP) is often used as a model compound to induce oxidative stress in cell systems by producing free radical intermediates, increase of which might be a primary factor for hepatotoxicity. In the cytotoxicity assays of this study, t-BHP induced strong inhibition on cell growth, and pretreatment with panduratin A (10—15 μM) significantly protected the HepG2 cells against oxidative damage caused by t-BHP as indicated by the reduced cell growth suppression in a dose-dependent manner (Fig. 2).

As shown in Table 1, the increased MDA level indicated that t-BHP caused oxidative damage to hepatic cell membranes. Lipid peroxidation is initiated by active oxygen species attacking unsaturated fatty acids and is propagated by...
a chain reaction cycle involving lipids, peroxy radicals, and lipid hydroperoxides. Peroxidation of the cell membrane phospholipids and accumulation of lipid peroxides are expected to alter the membrane fluidity and permeability, consequently leading to disruption of membrane structure and function. The results of the present study suggest that panduratin A is capable of ameliorating hepatocyte lipid peroxidation caused by t-BHP.

It is well established that intracellular glutathione (GSH), the most important biomolecule protecting against chemically induced oxidative stress, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction. There are two distinct pathways of t-BHP metabolism; one via cytochrome p-450, and the other by GSH-peroxidase converting t-BHP to t-butanol and oxidized GSH. These metabolic pathways could increase cellular reactive metabolites, which may attack membrane phospholipids, proteins, and nucleic acids. Thus, antioxidants which can inhibit free radical generation are important in terms of protecting the liver from chemical-induced damage by stabilizing antioxidant systems in the cell. Our study showed that t-BHP stimulated reactive oxygen species (ROS) overproduction and reduced GSH level in HepG2 cells. The data further showed that cells treated with panduratin A displayed a dose-dependent reduction of t-BHP-induced ROS generation (Fig. 3A) and this reductive effect is enhanced with time (Fig. 3B). Moreover, GSH depletion was recovered when the cells were preincubated with panduratin A (Table 1). These results present that the protection afforded by panduratin A against t-BHP-induced hepatotoxicity might be related to its ability to scavenge free radicals.

In summary, the present study suggested the protective effect of panduratin A on cytotoxicity induced by t-BHP in human hepatoma cells. The t-BHP toxicity is associated with elevated ROS level, lipid peroxidation, and disruption of intracellular antioxidant systems. Treatment of panduratin A protected against all these alterations induced by t-BHP, probably acting mainly to quench radical species. This effect was comparable to silybin, which was used as a positive control. The present findings suggest that panduratin A may be used as a natural antioxidant to protect against oxidative damage caused by toxic chemicals. The investigation of exact mechanisms and further in vivo experiments are needed in order to evaluate the possibility of application as a natural antioxidant.

Acknowledgement This work was supported by the Ministry of Commerce, Industry and Energy for the Regional Innovation System program in 2004.

REFERENCE