**Cytoprotective Effect of 20(S)-Rg3 on Benzo[a]pyrene-Induced DNA Damage**

Po Ying Poon, Hoi Hin Kwok, Patrick Y. K. Yue, Mildred S. M. Yang, Nai Ki Mak, Chris K. C. Wong, and Ricky N. S. Wong

Department of Biology, Faculty of Science, Hong Kong Baptist University, Kowloon, Hong Kong SAR, China

Received March 14, 2011; accepted September 28, 2011

**ABSTRACT:**

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon ubiquitously existing in the environment. Its metabolites have been shown to cause DNA damage and cellular dysfunction in humans. *Panax ginseng* C.A. Meyer is a Chinese medicinal herb, and ginsenosides are the main active constituent of ginseng. Accumulating evidence had indicated that ginseng extract and ginsenosides possess cytoprotective effects. In this study, the protective effect of ginsenosides on BaP-induced DNA damage in human dermal fibroblasts (HDFs) and HepG2 cells was investigated. The genotoxic effect of BaP was measured by the comet assay. Results showed that tail moment was increased in BaP-treated cells, but cotreatment of ginsenoside 20(S)-Rg3 can significantly decrease BaP-induced DNA damage. A downstream mechanistic study revealed that 20(S)-Rg3 increased the gene expression of an important phase II detoxifying enzyme NAD(P)H:quinone oxidoreductase 1. The effect was also associated with the activation of protein kinase B (Akt) and nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2). These results indicated that 20(S)-Rg3 might protect HDFs from BaP-induced DNA damage through the activation of the phosphatidylinositol 3-kinase/Akt/Nrf2 pathway. Our results also demonstrated that 20(S)-Rg3 is a functional ligand of pregnane X receptor (PXR), a nuclear receptor that mediates the induction of drug clearance pathways. Subsequent knockdown of PXR expression by small interfering RNA confirmed the involvement of PXR on the protective effects of 20(S)-Rg3 against BaP-induced DNA damage. In summary, ginsenoside 20(S)-Rg3 can protect against BaP-induced genotoxicity in human cells, suggesting that ginseng may serve as a natural cytoprotective agent against environmental carcinogens.

**Introduction**

Benzo[a]pyrene (BaP) is a ubiquitous environmental contaminant found in smoke of incomplete combustion, diesel engine exhaust, fuel exhaust condensates, charbroiled food, and cigarette smoke, which is known to be mutagenic and carcinogenic. After absorption, BaP exerts its toxic effect through transformation by cytochrome P450 enzymes inside the cell to become reactive intermediates in causing DNA damage (Briédé et al., 2004). BaP metabolites can lead to different pathways of cytotoxicity, such as damage to macromolecules and impairment in cell functions, and may finally cause carcinogenesis. Herbal substances and dietary phytochemicals are suggested as potential cytoprotective agents to maintain cellular functions (Jeong et al., 2004).

Ginseng is a Chinese traditional medicinal herb that has been used to promote longevity and immunity in China for thousands of years. Studies using different in vitro and in vivo models suggested that intake of ginseng may reduce the risk of several types of cancer (Nakata et al., 1998). Current research indicates that the most important component of ginseng is ginsenosides because they account for most of the pharmacological effects (Attele et al., 1999). Ginsenosides are classified into three major categories by their chemical characteristics. They are 20(S)-protopanaxadiol (Rb1, Rg3, and Rh2), 20(S)-protopanaxatriol (Re, Rf, and Rg1), and oleanolic acid derivatives (De Smet, 2002).

The cytoprotective effect of ginsenosides has been demonstrated in various studies. Ginsenoside Rg3 exerts a cytoprotective effect toward DNA damage and cell apoptosis by reducing oxidative stress (Zhang et al., 2008). Kwok et al. (2010) showed that ginsenoside 20(S)-protopanaxatriol protects endothelial cells against oxidative stress by regulating potential cytoprotective agents to maintain cellular functions (Jeong et al., 2004).

This work was supported in part by the Central Allocation Group of the Research Grant Council, Hong Kong SAR [Grant HKBU1/06C].

**Abbreviations:** BaP, benzo[a]pyrene; Nrf2, nuclear factor erythroid 2-related factor 2; Erk, extracellular signal-regulated kinase; NQO1, NAD(P)H:quinone oxidoreductase 1; PXR, pregnane X receptor; GST, glutathione transferase; UGT, UDP-glucuronitransferase; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; SR12813, tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; HDF, human dermal fibroblast; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PCR, polymerase chain reaction; FRET, fluorescence resonance energy transfer; siRNA, small interfering RNA; siPXR, siRNA targeting human PXR; DME, drug-metabolizing enzyme; SULT, sulfotransferase.

http://dx.doi.org/10.1124/dmd.111.039503.
the intracellular redox status. The underlying protective mechanisms may be due to the activation of intracellular survival signaling pathways. Akt is a central node that activates various downstream targets such as nuclear factor erythroid 2-related factor 2 (Nrf2) (Kim et al., 2010). Nrf2 is an important transcriptional factor of several detoxifying phase II enzymes, for example, NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1. Phase II enzymes, such as NQO1, can facilitate conjugation of BaP metabolites to increase their hydrophilicity so as to enhance their excretion from cells (Köhle and Bock, 2007).

On the other hand, pregnane X receptor (PXR), an orphan nuclear receptor, serves as a sensor of xenobiotics that mediates the induction of phase II enzymes and phase III transporter gene expression (Xu et al., 2005) for detoxification of potentially harmful substances. It was demonstrated that PXR protects HepG2 cells from BaP-induced DNA damage. In PXR-overexpressing liver cells, the mRNA levels of glutathione transferase (GST) M1, GSTA1, GSTA2, UDP-glucuronosyltransferase (UGT) 1A6, and breast cancer resistance protein are up-regulated on challenge with BaP (Naspinski et al., 2008). PXR can be activated by different herbal compounds such as Ginkgo biloba (Yeung et al., 2008) and St. John’s wort (Moore et al., 2000). A study demonstrated that G. biloba extracts induce the expression of multiple drug-metabolizing enzymes and transporters in cells through PXR (Li 2008).

**FIG. 1. Genotoxicity of BaP.** HDFs were treated with different concentrations of BaP for 24 h. In the comet assay, 200 cells were analyzed for each treatment group. Distribution of tail moment (A) and mean tail moment (B) in HDFs exposed to different concentrations of BaP are shown. C, cell viability was determined by the MTT method after 24 h of treatment. Results are expressed as the mean ± S.D. from at least three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
et al., 2009). Ginsenosides can also exert their effect through activation of nuclear receptors by acting as phytochemicals (Lee et al., 1997). Previous study illustrated that ginsenoside Rb1 can increase phase II enzymes through an estrogen receptor-related PI3K/Akt/Nrf2-dependent pathway (Hwang and Jeong, 2010).

In this study, we examined the cytoprotective mechanism of ginsenosides toward BaP-induced DNA damage in human cells and the possible involvement of the PI3K/Akt/Nrf2 signaling and PXR pathway.

Materials and Methods

Chemicals and Reagents. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, Opti-MEM, penicillin/streptomycin, phosphate-buffered saline (PBS), and trypsin-EDTA were supplied by Invitrogen (Carlsbad, CA). BaP, dimethyl sulfoxide (DMSO), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), 7-ethoxyresorufin, dicoumarol, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate (SR12813), propidium iodide, anti-actin, and L-sulforaphane were purchased from Sigma-Aldrich (St. Louis, MO). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), agarose, and Triton X-100 were purchased from USB (Cleveland, OH). Ginsenosides (purity >98%) were obtained from Fleton (Chengdu, China). Antibodies of PXR (N-16) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PXR antibody was obtained from Novus Biologicals, Inc. (Littleton, CO). Polyclonal antibodies, such as anti-Akt, phospho-Akt (Ser473), Erk, and phospho-Erk (Thr202/Tyr204) were obtained from Cell Signaling Technology (Danvers MA).

Cell Culture and Treatment. Neonatal human dermal fibroblasts (HDFs) were obtained from Walkersville, Inc. (Walkersville, MD) and human hepatoma cells (HepG2 cells) were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured in a 100-mm² tissue culture dish with DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator with 5% CO2 at 37°C. Cells were starved with serum-free DMEM overnight before drug treatment. Cells were then treated with BaP in the presence or absence of ginsenosides in DMEM (DMSO ≤0.1%).

MTT Proliferation Assay. HDFs were seeded at a density of 2 × 10⁴ cells/well in a 96-well plate overnight. After drug treatment for 24 h, MTT (20 µl) was added to each well and incubated for 3 h. Then, the medium was discarded, and 100 µl of DMSO was added to dissolve the formazan salts in each well. The OD was measured with the microplate spectrophotometer.

![Figure 2](https://example.com/figure2.png)

**FIG. 2.** Effect of ginsenosides on BaP-treated cells. Cells were cotreated with ginsenosides (10 µM) and BaP (10 µM) for 24 h. A, viability of HDFs was determined by the MTT method. **, p < 0.01; †, p < 0.05. B, DNA damage in HDFs was determined by the TUNEL assay after treatment for 24 h. Region M1 indicates the percentage of TUNEL-positive cells. C, comet images (100× magnification, top panel) and the quantitative measurement of tail moment (bottom panel). *, p < 0.05; †, p < 0.05. Results are expressed as the mean ± S.D. from three independent experiments.
(Infinite F200; Tecan, Männedorf, Switzerland) at 540 and 690 nm. Cell viability was calculated according to the equation (OD_sample − OD_blank/ OD_control − OD_blank) × 100%.

Alkaline Single-Cell Gel Electrophoresis Assay ( Comet Assay ). HDFs and HepG2 cells were plated in six-well plates at a density of $2 \times 10^5$ cells/well. After drug treatment for 24 h, cells were harvested by trypsination, cell suspensions were then mixed with agarose (0.65%), and the mixture was poured onto an agarose-precipitated semisolid microscopic slide followed by covering the agarose gel with coverslips. The coverslips were removed gently after the agarose gel was set at room temperature. The cells were lysed in ice-cold lysis solution (pH 10.0) at 4°C for 1 h and then incubated in electrophoresis buffer (pH 3.5) for 30 min and subsequently subjected to electrophoresis at a constant current of 250 mA for 6 min. The slides were then rinsed in neutralizing buffer (pH 7.5) and immersed in water at 4°C until observation. The DNA was stained with propidium iodide (10 µg/ml) for 30 min. The comet image was captured using an inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a cool charge-coupled device camera (Apogee Electronics Corp., Santa Monica, CA). Tail moment of 200 comets of each individual treatment group was analyzed using CometScore (version 1.5; TriTek, Sudernduck, VA) and at least three independent experiments were performed.

TUNEL. A TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. In brief, after drug treatment, HDFs were fixed with 4% paraformaldehyde in PBS at room temperature for 1 h. After fixation, cells were washed with PBS and were permeabilized by 4% ice-cold paraformaldehyde. Cells were then incubated with a TUNEL reaction mixture for 1 h at 37°C. The labeled cells were assayed using a flow cytometer (FACSorter; BD, Franklin Lakes, NJ) with excitation at 488 nm and emission at the FL-1 channel. The percentage of TUNEL-positive cells was analyzed by CELL-Quest software (BD Biosciences, San Jose, CA).

Western Blotting. After drug treatment, HDFs were lysed with CytoBuster Extraction reagent (Novagen, Madison, WI) containing Protease Inhibitor Cocktail Set III (1:200, v/v) (Calbiochem, San Diego, CA). The cell lysate was centrifuged at 20,800g for 15 min at 4°C. After centrifugation, supernatant was collected, and protein content was determined by the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The membrane was blocked with nonfat milk in Tris-buffered saline-Tween 20 for 1 h at room temperature, followed by probing with diluted primary antibodies for 3 h at room temperature. The membrane was further incubated with secondary antibodies for 1 h at room temperature. Finally, protein bands were revealed by exposure to medical X-ray film after incubation with chemiluminescence substrate (Bio-Rad Laboratories).

Immunofluorescence Staining. HDFs were seeded at a density of $1 \times 10^4$ cells on a coverslip in a 24-well plate overnight. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were then permeabilized with Triton X-100 (0.2%) in PBS for 15 min. The cells were incubated with primary antibody (1:200 dilution) overnight at 4°C. After washing with PBS, the cells were incubated with secondary antibodies (PE antibody 1:250 dilution) for 2 to 3 h in the dark at room temperature. Nuclei were visualized with DAPI (0.5 µg/ml staining). The washed coverslip was mounted on the slides using DAKO fluorescence mounting medium (Vector Laboratories, Burlingame, CA) in the dark, and images were captured using an Olympus FX1000 confocal scanning laser microscope (Olympus, Essex, UK).

Real-Time Reverse Transcriptase-Polymerase Chain Reaction. Total RNAs were extracted with TRizol reagent (Invitrogen) and reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Amplification of cDNA with custom oligonucleotides as primers was carried out on a PcrMaster Mix (PerkinElmer) in a final volume of 20 µl containing 1 µl of cDNA and primers. The PCR reactions were performed using an iCycler (Bio-Rad Laboratories). Real-time PCR was performed in the following thermal profile: 95°C, 10 min, 1 cycle; 95°C, 30 s, 55°C, 1 min, and 72°C, 1 min, 40 cycles. The primers were as follows: glyceraldehyde-3-phosphate dehydrogenase forward primer 5′-ATCAGCAATTGCTCCTGCAAC-3′ and reverse primer 5′-TGTTGAGCTCCTCAGGAG-3′; and NQO1 forward primer 5′-CAAATCCTGGAGGTGCAAATTT-3′ and reverse primer 5′-GGTTGTCAGTTGGAGAAGAGC-3′ (Invitrogen). Real-time PCRs were conducted using an iCycler iq real-time PCR detection system (Bio-Rad Laboratories). The data were then normalized using the expression levels of glyceraldehyde-3-phosphate dehydrogenase mRNA.

**X**

**PXR Competitive Binding Assay.** Binding affinity of ginsenosides with PXR was determined by the LanthaScreen PXR (SRX) Competitive Binding Assay (Invitrogen) according to the manufacturer’s instructions. Serial dilutions of SR12813 (0.03 nm-100 µM) and 20(S)-Rg3 and 20(R)-Rg3 (0.01-50 µM) were used to compete with a fluorescent PXR ligand for binding to human PXR. The fluorescence resonance energy transfer (FRET) signal was then measured by a spectrophotometer (Infinite F200). The binding curves were fitted with a two-state one-site competition model by Prism software (GraphPad Software Inc., San Diego, CA).

Small Interfering RNA Transfection Assay. Three pairs of small interfering RNAs (siRNA) targeting human PXR (siPXR) (Ambion, Austin, TX) were used. HepG2 cells were seeded into 24-well plates at a density of 7.5 × 10^4 cells/well overnight before transfection, and then siRNA (20 nM) and siLentFect transfection reagent (1 µl) (Bio-Rad Laboratories) were mixed in 50 µl of Opti-MEM and incubated for 15 min before transfection. Nontargeting siRNA (20 nM) (Dharmacon RNA Technologies, Lafayette, CO) was used in parallel with the siPXR. Drug treatment was performed 24 h after transfection. The sequences of siPXR are as follows: sense 5′-GCUCAAUCAUGGAUCUGT-3′, antisense 5′-AUUCAUC-UAGAUG-CAC-3′, sense 5′-UAGCACC-UUUCUCCAAU-3′, antisense 5′-AGUGG-AGAGAGUUGAGC-3′, sense 5′-UAGCAUCUCUUAAUGAAGC-3′, and antisense 5′-UAGCAGAUGAAGGUCAG-3′.

**Statistical Analysis.** Each experiment was repeated at least three times. Data are expressed as means ± S.D. Statistical analyses were performed by Newman-Keuls post hoc test and one-way ANOVA, respectively.
Results

Genotoxicity of Benzo[a]pyrene. The effect of BaP on DNA damage and viability of HDFs was determined by the comet assay and MTT viability assay, respectively. After 24 h of treatment, BaP (0.1–50 μM) was found to induce DNA damage and cell death in a dose-dependent manner (Fig. 1). BaP at the concentrations of ≥10 μM induced significant DNA damage (Fig. 1, A and B) and cell death in HDFs (Fig. 1C) compared with controls.

Protective Effects of Ginsenosides on BaP-Induced DNA Damage in HDFs. Ginsenosides have previously been shown to possess a cytoprotective effect (Kwok et al., 2010). Therefore, the protective effect of ginsenosides [Rg1, Rb1, 20(S)-Rg3, and 20(R)-Rg3] (Supplemental Fig. 1) on BaP-induced HDF DNA damage was investigated. As shown in Fig. 2A, treatment of HDFs with BaP (10 μM) resulted in approximately 30% cell death. In the presence of 20(S)-

<table>
<thead>
<tr>
<th>15 mins</th>
<th>30 mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>-</td>
</tr>
<tr>
<td>20(S)-Rg3</td>
<td>-</td>
</tr>
</tbody>
</table>

![Fig. 4. Activation of Akt and Erk by 20(S)-Rg3.](image)

DNA damage was assessed by the comet assay, and cell viability was measured by MTT viability assay. The effects of BaP and 20(S)-Rg3 on DNA and cell viability were dose-dependent. The protective effect of 20(S)-Rg3 was observed at concentrations of ≥10 μM.

![Graphs showing phosphorylation levels of Akt and Erk.](image)
Rg3 (10 μM), the cytotoxic effect of BaP was totally abolished. However, other ginsenosides, such as Rg1, Rb1, or 20(R)-Rg3 showed no significant protection against BaP-induced cell death. The cell viabilities of HDFs treated with 20(S)-Rg3, 20(R)-Rg3, Rg1, and Rb1 alone are approximately 124, 105, 98, and 91%, respectively (Supplemental Fig. 2).

BaP-induced DNA strand breakage was then determined by the TUNEL assay. The percentage of TUNEL-positive cells in the control and BaP treatment group was 2.39 and 15.96%, respectively. Among the different ginsenosides, 20(S)-Rg3, 20(R)-Rg3, Rg1, and Rb1, 20(S)-Rg3 was the most effective in reducing BaP-induced DNA strand breakage from 15.96% (BaP treatment group) to 3.07% (Fig. 2B). 20(R)-Rg3 and Rb1 was found to decrease TUNEL-positive cells to 12.76 and 9.77%, respectively, whereas Rg1 did not exert similar protection against DNA strand breakage (Fig. 2B). The cytoprotective effect was further confirmed by the comet assay. BaP alone increased the mean tail moment and in the presence of 20(S)-Rg3, tail moment was significantly reduced in the HDFs, whereas other ginsenosides did not show a similar cytoprotective effect (Fig. 2C).

**Fig. 5.** Inhibitory effect of PI3K on 20(S)-Rg3-induced Nrf2 translocation in HDFs. Cells were pretreated with LY294002 (10 μM) for 1 h, followed by 20(S)-Rg3 (10 μM), BaP (10 μM), and l-sulforaphane (SFN) (20 μM) treatments for 2 h. For immunofluorescent staining of HDFs, cells were stained with Nrf2 antibody, followed by the phycoerythrin-conjugated anti-rabbit secondary antibody, and then the nuclei were counterstained with DAPI. Scale bar, 20 μm.
Role of PI3K in 20(S)-Rg3-Mediated Cytoprotection. The PI3K/Akt signaling pathway has a major role in regulating cell survival. LY294002, a specific inhibitor of PI3K (IC_50 = ~0.31 μM for PI3Kβ), is widely used to study the involvement of the PI3K/Akt mechanism. Therefore, it was used to determine the role of PI3K in the protective mechanism of 20(S)-Rg3 in HDFs. From the result of the comet assay, the mean tail moment of BaP alone was 16 ± 1.5, and it was reduced to 0.3 ± 0.3 after cotreatment with BaP and 20(S)-Rg3 (Fig. 3). However, pretreatment of HDFs with LY294002 before BaP and 20(S)-Rg3 cotreatment resulted in a mean tail moment of 14.7 ± 1.4, and the value was very similar to that for BaP-treated HDFs (16 ± 1.5). This observation suggested that the PI3K/Akt pathway may be involved in the cytoprotective effect of 20(S)-Rg3.

Effect of Benzo[a]pyrene and 20(S)-Rg3 on the Activation of Akt and Erk. Results from Western blotting analysis indicated that 20(S)-Rg3 induced phosphorylation of both Akt (Ser473) and Erk (Thr202/Tyr204). BaP alone exerted no effect on the phosphorylation activation of Akt and Erk. 20(S)-Rg3 was able to activate Akt and Erk in a time-dependent manner, and the peak of phosphorylation of Akt and Erk was at approximately 30 min (Fig. 4).

Role of PI3K/Akt Pathway in Nrf2 Activation. Nrf2 is an important transcriptional factor that induces the expression of phase II enzymes. Thus, the effects of 20(S)-Rg3 and BaP on the nuclear translocation of Nrf2 were examined. Cells treated with 20(S)-Rg3 alone or cotreated with BaP and 20(S)-Rg3 showed a significant increase in Nrf2 staining in the nucleus (Fig. 5). Less immunoreactivity of Nrf2 staining was observed in the nucleus of control cells treated with BaP alone or DMSO. This result clearly indicated that 20(S)-Rg3 could induce the nuclear translocation of Nrf2. To further address the role of the PI3K/Akt pathway in 20(S)-Rg3-induced Nrf2 translocation, LY294002 was used to block the PI3K → Akt pathway. After pretreatment with LY294002 (10 μM) for 1 h, 20(S)-Rg3-induced Nrf2 translocation was abolished (Fig. 5). This observation indicates that 20(S)-Rg3-induced Nrf2 translocation is PI3K/Akt-dependent. t-sulforaphane, a phase II enzyme inducer that has been shown to disrupt the Nrf2-Keap1 complex and allow free translocation of Nrf2 into the nucleus was used as a positive control to demonstrate the nuclear translocation of Nrf2.

Effect of 20(S)-Rg3 on NQO1 mRNA Expression. NQO1, an important phase II enzyme responsible for conjugation of metabolites to enhance BaP elimination, is one of the transcriptional targets of Nrf2. It was revealed that cotreatment of 20(S)-Rg3 (10 μM) with BaP in HDFs could increase NQO1 gene expression by approximately 2-fold compared with BaP alone (Fig. 6). Hence, NQO1 expression is possibly induced by Nrf2 translocation upon 20(S)-Rg3 treatment and may subsequent enhance the export of BaP metabolites.

Time-Resolved FRET Competitive Binding Assay of PXR with Rg3 and SR12813. Previous studies indicated that ginsenosides may bind on different nuclear receptor such as glucocorticoid receptor or estrogen receptor to exert diverse physiological responses; however, little is known about the effect of ginsenosides on PXR. PXR, a sensor of xenobiotics involved in drug clearance pathways, can be activated by a wide spectrum of steroids and pharmaceutical agents. Because PXR is barely expressed in HDFs (Supplemental Fig. 3) (Chang et al., 2008; Naspiniski et al., 2008), we used human hepatoma HepG2 cells, which express a high level of PXR, as a cellular model to study the possible involvement of PXR in 20(S)-Rg3-induced protection against BaP. Here we examine the effects of 20(S)-Rg3 on PXR. First, to test the specific binding affinity of Rg3 to PXR, a PXR competitive ligand binding assay was performed (Fig. 7). The binding affinity of 20(S)-Rg3 was found to be comparable to that of the potent synthetic PXR ligand SR12813. The data suggested that 20(S)-Rg3 is a high-affinity PXR agonist.

Role of PXR in 20(S)-Rg3-Mediated Protection. The involvement of PXR in the cytoprotective effect of 20(S)-Rg3 against BaP-induced DNA damage was confirmed by decreasing the expression of PXR using siRNA. Depletion of PXR by specific siRNA in HepG2 cells was verified by Western blot analysis (Fig. 8A). Similar to HDFs, DNA damage induced by BaP (10 μM) can also be reduced by 20(S)-Rg3 in HepG2 cells (Fig. 8B). However, knockdown of PXR abolished the protective effect of 20(S)-Rg3 against BaP-induced DNA damage. This observation suggested that PXR is also involved in the protective mechanism of 20(S)-Rg3.

Discussion

BaP, a typical environmental pollutant present mainly in cigarette smoke, barbecued meat, and smoked food, has been implicated in increasing the risk of cancer. Humans are constantly exposed to BaP through skin and gastrointestinal absorption. Skin is the largest organ of the human body that protects the body from chemicals and fungal,
bacterial, and viral pathogens. Dermis is the structural layer of skin, but it is also one of the sites for drug metabolism, because skin cells express drug-metabolizing enzymes (DMEs), which are essential for metabolism and removal of xenobiotics. With direct contact (i.e., skin painting) or skin lesions, xenobiotics can penetrate into the skin and down to the dermis layer where fibroblasts are found. On the other hand, liver plays a central role in detoxification of endobiotics and xenobiotics in the human body. Ingested xenobiotics in food are absorbed by the digestive tract and then delivered to the liver through the portal blood (Malhi et al., 2010). Liver cells express high level of DMEs, which can facilitate the elimination of xenobiotics such as BaP from the body, thus protecting the body from toxicity. Failure to remove xenobiotics and their subsequent accumulation may result in genetic damage, finally leading to cell mutagenicity and cancer induction.

In human cells, BaP undergoes sequential metabolic reactions catalyzed by cytochrome P450s (i.e., CYP1A1) and epoxide hydrolase to produce primary reactive metabolites (epoxides and hydroxy metabolites), which are further metabolized to diols, diol epoxides, and quinones. Diole epoxide (i.e., BP-7,8-diol-9,10-epoxide) is an ultimate reactive metabolite that can generate DNA adducts and DNA breaks. Furthermore, reactive oxygen species that may be produced during the autooxidation of 6-hydroxy-BaP to quinones, superoxide, hydroxyl radicals, and hydrogen peroxide through the redox cycle can lead to oxidative DNA adducts (Miller and Ramos, 2001) and disrupt cellular redox balance.

The results of this study show that BaP significantly induced DNA damage (Fig. 1, A and B) and cell death (Fig. 1C). DNA damage caused by BaP may due to the formation of ultimate carcinogenic metabolites metabolized by phase I enzymes. Benzyl[a]pyrene diol epoxide is one of the major final metabolites that can cause damage to DNA and disrupt cell functions.

Ginseng is a traditional Chinese medicine. The most important pharmacological components in ginseng are ginsenosides, which have various bioactivities as revealed in many systems, such as antioxidation (Kwok et al., 2010) and cytoprotection (Chan et al., 2004). In the present study, we demonstrated the cytoprotective effect of ginsenosides. Among the 30 types of well known ginsenosides, Rb1 is the most abundant ginsenoside under the 20(S)-protopanaxadiol category in American ginseng (Panax quinquefolius), and Rg1 is one of the most abundant ginsenosides among the 20(S)-protopanaxatriol category in Chinese ginseng (Panax ginseng) (Court et al., 1996). In addition, Rg3 is one of the major ginsenosides found in red ginseng (Wang et al., 2007). Ginsenoside Rg3 exists as stereoisomer pair, 20(S)-Rg3 and 20(R)-Rg3, which differ in the position of hydroxyl group on the chiral center at carbon-20. Park et al. (2008) showed that 20(S)-Rg3 and 20(R)-Rg3 exhibited differential pharmacological effects.

20(S)-Rg3 was found to be the most effective ginsenoside to protect cells against the challenge of BaP in our present study. 20(S)-Rg3 showed protection against cell death (Fig. 2A) and DNA damage (Fig. 2, B and C) after treatment with BaP. Much evidence has already shown that 20(S)-Rg3 has anticarcinogenic (Yun et al., 2001) and antiapoptotic activity (Min et al., 2006). Our present results indicate that only 20(S)-Rg3 can possess a major cytoprotective effect toward DNA damage and cell death. PI3K/Akt is regarded as a cell survival regulator, which regulates diverse physiological responses including proliferation and cell survival (Liao and Hung, 2010). PI3K can be activated by growth factor receptor tyrosine kinases or G protein-coupled receptors (Liao and Hung, 2010). Our previous finding also demonstrated that ginsenosides can activate the PI3K/Akt pathway (Sengupta et al., 2004). In the present study, 20(S)-Rg3 can rapidly activate the phosphorylation of Akt (Fig. 4) and protect cells from BaP-induced DNA damage and cell death. Ginsenoside Rg1 has been shown to activate mitogen-activated protein kinases and Akt through interacting with insulin-like growth factor-1 and fibroblast growth factor-2 signaling (Lu et al., 2009). Thus, 20(S)-Rg3 may activate...
PI3K/Akt in a receptor tyrosine kinase-dependent manner. Activated Akt can lead to different cellular signaling, such as promoting cell survival by inhibition of the phosphorylation of pro-apoptotic protein Bad (Manning and Cantley, 2007), which can prevent BaP-induced cell death in HDFs.

Nrf2 is a master transcriptional factor of phase II detoxifying and antioxidant enzymes (Hsieh et al., 2009). It has been shown that activation of Akt can induce phosphorylation of Nrf2 and leads to nuclear translocation from cytoplasm (Wang et al., 2008). The translocated Nrf2 can promote phase II detoxifying and antioxidant enzyme gene transcription to facilitate the xenobiotic elimination process. It is believed that a series of DMEs would be modulated in our system as well. By using LY294002, an inhibitor of PI3K, we showed that 20(S)-Rg3-induced Nrf2 translocation can be abolished, indicating the involvement of the PI3K/Akt.

DMEs take part in the metabolism and elimination of xenobiotics (Meyer, 1996). DMEs include phase I and phase II metabolizing enzymes and phase III transporters. Gene expression of phase II enzymes, such as GST P1, UGT1A1, sulfotransferase (SULT) 1A1, and NQO1, can be induced by Nrf2. They may enhance the hydrophilicity and excretability of the BaP metabolites (Köhle and Bock, 2007). Therefore, we further investigated the involvement of DMEs in 20(S)-Rg3-mediated detoxification. In our study, we demonstrated the increase in NQO1 gene expression in HDFs after treatment with 20(S)-Rg3; this indicated that the detoxification action of 20(S)-Rg3 may be associated with NQO1 induction. NQO1 detoxifies quinones to quinols (Ross, 2004); then quinols can be further conjugated by UGTs and SULTs and subsequently excreted through multidrug resistance-associated proteins (Xu et al., 2005). Even though we have not demonstrated the involvement of UGTs and SULTs, we predicted that the detoxifying action of 20(S)-Rg3 would enhance further conjugation.

Studies have demonstrated that PXR can directly regulate phase II enzymes and phase III transporter gene expressions (Nakata et al., 2006). PXR (NR1I2), also known as steroid and xenobiotic receptor or pregnane-activated receptor, is an orphan nuclear receptor. It is an important component of the body’s adaptive defense mechanism against toxic substances. It serves as a sensor of xenobiotics that mediates induction of drug clearance pathways to ensure rapid detoxification of potentially harmful substances. It has been demonstrated that ginsenosides can act as phytochemicals to exert their effect through activation of nuclear receptors (Lee et al., 1997). Of interest, a binding study showed that 20(S)-Rg3 but not 20(R)-Rg3 can act as a functional ligand of PXR (Fig. 7). Activated PXR may translocate into the nucleus and eliminate BaP metabolites by regulating various DMEs. The dashed and solid arrows indicate the two different possible pathways that are activated by 20(S)-Rg3.

FIG. 9. The cytoprotective mechanism of 20(S)-Rg3 against BaP-induced DNA damage. 20(S)-Rg3 induces Akt phosphorylation through the activation of PI3K and subsequently activates Nrf2 to translocate from cytosol into the nucleus. Nrf2 translocation can regulate the expression of the phase II enzyme, NQO1, which may enhance conjugation of BaP metabolites to facilitate the elimination process. 20(S)-Rg3 can also protect cells from BaP-induced DNA damage through activation of PXR. 20(S)-Rg3 acts as a functional ligand of PXR. Activated PXR may translocate into the nucleus and eliminate BaP metabolites by regulating various DMEs. The dashed and solid arrows indicate the two different possible pathways that are activated by 20(S)-Rg3.
the stereoisomer of the natural compound is critical in their pharmacological action. Upon PXR binding, gene expression of DME in different phases may be induced. To validate the role of PXR in this process, we used human hepatic cells expressing PXR as a study model. By means of RNA interference, the intrinsic PXR was artificially depleted, and the results indicated that the protective effect of 20(S)-Rg3 is critically related to PXR. This finding is in agreement with previous results showing that PXR is involved in inducing different DMEs for cytoprotection.

For example, activation of PXR by G. biloba terpenoids induces the expression of multiple hepatic DMEs and transporters and thus may protect against the toxicity of polycyclic aromatic hydrocarbons, such as BaP (Li et al., 2009).

A pharmacokinetic study on human subjects using high-performance liquid chromatography-mass spectrometry demonstrated that after intake of 20(S)-Rg3 (60 mg), the plasma concentration of 20(S)-Rg3 ranged from 2.5 to 1000 ng/ml (0.003–1.278 μM) (Wang et al., 1999; Zhao et al., 2010), which is relatively lower than the concentration used in the present study (10 μM). However, as herbal tonics with low toxicity, ginseng and ginsenosides are consumed regularly to improve vitality in Eastern countries. As a result, the concentration of 20(S)-Rg3 in plasma may gradually increase to a level close to that in the present study. However, further in vivo studies are required to elucidate the beneficial effects of ginseng against the toxic effect of environmental contaminants.

In conclusion, 20(S)-Rg3, one of the prevalent effective components of ginseng, was shown to inhibit BaP-induced DNA damage through PI3K/Akt/Nrf2 and PXR pathways (Fig. 9). We uncovered a potential protective effect of ginsenoside against exposure to environmental contaminants; this warrants further study of the cytoprotective effect of ginsenoside with long-term consumption.

Authorship Contributions

Participated in research design: Poon, Kwok, Yue, Yang, and R.N.S. Wong.

Conducted experiments: Poon.

Contributed new reagents or analytic tools: C.K.C. Wong.

Performed data analysis: Poon.

Wrote or contributed to the writing of the manuscript: Poon, Kwok, Yue, Mak, and R.N.S. Wong.

References


Panax ginseng, is a functional ligand of glucocorticoid receptor.
