Induction of Chemoprotective Phase 2 Enzymes by Ginseng and its Components

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- *Panax* spp
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- panaxytriol

Abstract

Phase 2 detoxification enzymes protect against carcinogenesis and oxidative stress. Ginseng (*Panax* spp.) extracts and components were assayed for inducer activity of NQO1 (quinone reductase), a phase 2 enzyme, in Hepa1c1c7 cells. Ginseng extracts were analyzed for ginsenosides and panaxytriol. Korean red *Panax ginseng* extracts demonstrated the most potent phase 2 enzyme induction activity (76 900 U/g dried rhizome powder and 27 800 U/g for two similar preparations). The ginsenoside-enriched HT-1001 American ginseng (*Panax quinquefolius*) extract was the next most potent inducer, with activity of 15 900 U/g, followed by raw American ginseng root with activity of 8700 U/g. Neither a polysaccharide-enriched extract of American ginseng nor a commercial white *Panax ginseng* preparation showed any inducer activity. Pure ginsenosides showed no inducer activity. Protopanaxadiol and protopanaxatriol, deglycosylated ginsenoside metabolic derivatives, showed potent induction activity (approximately 500 000 U/g each). Synthetic panaxytriol was over 10-fold more potent (induction potency 5 760 000 U/g). There was no correlation between ginsenoside content and phase 2 enzyme induction. The most potent inducing red ginseng extract also had the highest panaxytriol content, 120.8 µg/g. We found that ginseng induced NQO1 and that polyacetylenes are the most active components.

Abbreviations

CD:	concentration to double induction activity
HPLC:	high performance liquid chromatography
MTT:	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
NADPH:	nicotinamide adenine dinucleotide phosphate (reduced)
NQO1:	NAD(P)H: quinone acceptor oxidoreductase 1 or quinone reductase
Nrf2:	nuclear erythroid 2 p45-related factor 2

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Introduction

Ginseng is a widely used herbal and nutritional supplement. It was ranked as the third most popular herbal product in America, with up to $300 million in sales [1] in 1997. It is of particular interest as a niche product, providing $46 million annually to rural harvesters in the U.S.A. (J. Hunter-Cevera, University of Maryland Biotechnology Institute, Baltimore, MD, personal communication). It has been used in Asia for over 2000 years and has generated the most extensive body of scientific literature of any medicinal herb. In Chinese medicine, ginseng is used to increase the body's resistance to physical, chemical, and biological stress.

Ginseng is the common name for at least 10 species of the genus *Panax*, all of which are indigenous to Asia (Asian, Japanese and Sanqi) and North America. Dried ginseng root is traditionally used for medicinal purposes in East and South-East Asia. Ginsenosides, which are plant steroids of the saponin class, are thought to be the pharmacologically active components of ginseng based on a variety of *in vitro* activities reviewed by Attele et al. [2]. Other ingredients, such as polysaccharides (ginsenans) and peptidoglycans (panaxans), may also stimulate pharmacological effects. The most commonly used ginseng species are those classified as Asian ginseng (*Panax ginseng*), which can be consumed in raw form (white) or steamed (red), American

ginseng (*Panax quinquefolius*) and Sanqi ginseng (*Panax notoginseng*).

Numerous studies have evaluated the cancer preventive effects of ginseng [3]. Studies with rodent models using ginseng extract or component supplementation have reported reductions in chemically-induced cancer incidence in a variety of models [4]. A case-control study of 1987 pairs of humans showed a large reduction in risk (odds ratio 0.5, 95% confidence interval 0.44–0.58) of developing some forms of cancer in subjects taking ginseng. Reductions in the risk of developing lung, gastrointestinal tract, liver, pancreatic and ovarian cancers were also reported [5]. A cohort study involving 4634 subjects in South Korea reported a 60% reduction in the incidence of cancers in ginseng consumers (odds ratio 0.4, 95% confidence interval 0.28–0.56), as well as demonstrating a dose-response relationship [6].

Evidence is very strong that phytochemicals derived from certain plants, such as sulforaphane from broccoli, can protect against cancer development, and that one of the principal mechanisms is induction of phase 2 detoxification enzymes. These enzymes modulate carcinogen metabolism and neutralize reactive electrophiles which may be ultimate carcinogens or mutagens [7]. Phase 2 enzymes have been described as indirect antioxidants and induction of these enzymes can provide sustained protection against oxidative damage [8].

We postulate that ginseng may exert its cancer chemopreventive activity in part by inducing phase 2 enzymes. This hypothesis is bolstered by the fact that ginseng administration induces the antioxidant enzymes superoxide dismutase and glutathione peroxidase in rats [9]. Ginseng has been shown to have indirect antioxidant activities beyond its direct free radical scavenging ability [10]. Cell lines incubated with pure ginsenosides, Rg3, Rg5 and Rh2, obtained from Korean red ginseng, had a 2- to 3-fold increase in activation of nuclear erythroid 2-related factor 2 (Nrf2), as measured by the antioxidant response element luciferase reporter assay (Thimmulappa R, Johns Hopkins University, Baltimore, MD, personal communication). Induction of phase 2 enzymes is mediated through the antioxidant response element pathway [11], and thus we postulated that ginseng extracts might induce phase 2 enzymes mediated by this pathway.

Materials and Methods

Materials

All solvents (dimethyl sulfoxide, acetonitrile, dimethylformamide) were HPLC grade and obtained from J.T. Baker. Cell culture media and sera were from Gibco-BRL. Bioassay and other reagents were from Sigma-Aldrich.

Ground dry American ginseng (*Panax quinquefolius*) root was obtained through the American Ginseng Board, from APN Laboratories (batch 10405, capsule containing 500 mg American ginseng extract and 2 mg of vegetable magnesium stearate). Ginsenoside-enriched heat-processed American ginseng extract (Remember-fX®, batch 425, capsule containing 100 mg of HT-1001 extract and 150 mg of fillers), HT-1001 standardized to contain 20–50% total ginsenoside content, and polysaccharide-enriched American ginseng extract (Cold-fX®, batch 139, capsule containing 100 mg of CVT-E002 extract and 90 mg of fillers), CVT-E002 standardized to contain 80% poly-furanosyl-pyranosyl-saccharides, were purchased from CV Technologies, Inc. Korean red *Panax ginseng* extracts were obtained from Ginseng Science, Inc. (Sun Ginseng®) and Aipop Co. White *Panax ginseng,* standardized to 4% total ginsenoside content (Ginsana®,G115) was obtained from Pharmaton. *Panax notoginseng* capsules were obtained from Holley Pharmaceuticals. Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rg1, pseudoginsengoside F11, notoginsenoside R1, protopanaxadiol, protopanaxatriol, sulforaphane, β-naphthoflavone and rifampin (all > 95% purity) were purchased from LKT Laboratories.

Panaxytriol (> 95% pure) was synthesized by two of the authors (HY, SD), as previously described [12]. β-Glucosidase (almond source) was obtained from Sigma-Aldrich.

Phase 2 enzyme inducer assay

Ginseng extracts were prepared in a mixture of equal parts of dimethyl sulfoxide, acetonitrile, dimethylformamide and water. Pure ginsenosides and panaxytriol were dissolved in dimethyl sulfoxide. Phase 2 enzyme inducer activity was measured using a rapid assay of activity of representative phase 2 enzyme in a cell line. This assay was originally developed by Prochaska and colleagues [13] and has been further refined [14]. Briefly, Hepa 1c1c7 murine hepatoma cells were grown in 96-well microtiter plates. Serial dilutions of the extracts or compounds which were assayed were added into the wells. After 48 hours, the cells were lysed and the activity of NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1 or quinone reductase) was assayed by the addition of a reaction mixture containing an NADPH-generating system, menadione and MTT. NQO1 catalyzes the reduction of menadione to menadiol by NADPH, and MTT is reduced non-enzymatically by menadiol, resulting in the formation of a blue color that can then be quantitated [15]. The reaction was stopped after 5 minutes by adding dicoumarol, a potent inhibitor of NQO1. NQO1 activity and protein content of the lysate were measured in duplicate plates with an optical microtiter plate absorbance reader at 490 nm and 550 nm, respectively. The concentration of the extract or compound which doubles the inducer activity is designated as the concentration of doubling (CD). One unit of inducer activity is defined as the amount that, when added to a single microtiter well, doubles the NQO1 specific activity. Sulforaphane and β-naphthoflavone were used as positive controls in each bioassay.

Analysis of ginsenoside and panaxytriol content

The ginseng powders were extracted and then assayed for ginsenoside components by high performance liquid chromatography (HPLC) analyses in our laboratory. In brief, authentic calibration standards and quality assurance samples for each of 6 ginsenosides were dissolved in methanol : water (30 : 70). The calibrators, quality assurance samples, and unknowns were separated by HPLC using an Alliance 2690 separations module with a 996 photodiode array detector (Waters Corp.). A Beckman Ultrasphere 5 µm, 250 × 4.6 mm column was used for separation, and compounds were monitored at 198 nm. The mobile phase consisted of solvent A (10 mM potassium dihydrogenphosphate : acetonitrile 80 : 20), solvent B (acetonitrile : water 85 : 15) and solvent C (acetonitrile). A gradient elution profile was employed for the run. The Waters Empower Pro Chromatography Software (version 5.00.00.00, Waters Corp.) was used for data collection and analysis. Using the above method, we obtained calibration curves for the 6 ginsenosides, and each had inter- and intraday precision and accuracy of > 85%. The contents of each capsule were oven-dried and separated into 100-mg aliquots using a calibrated analytical balance. The aliquots were then sonicated at 65 °C with 3.3 mL of 40% methanol for 30 min, centrifuged (1500 G for 10 min), and the supernatant was filtered (1500 × *g* for 20 min) using a Centricon Plus-20 filter (Fisher Scientific). This step was repeated two

Table 1 Phase 2 inducer activity and ginsenoside content of ginseng extracts.

Sample description	Inducer activity[1]	Ginsenoside concentrations[2] in commercial preparations						
		Rb1	Rb2	Rc	Rd	Re	Rg1	Total
Panax ginseng								
▶ Korean red (Aipop®)	27800	1.35	0.89	1.29	0.72	0.78	0.35	5.37
▶ Korean red (Sun®)	76900	0.50 ± 0.00	0.27 ± 0.01	0.70 ± 0.05	0.33 ± 0.00	0.48 ± 0.003	0.18 ± 0.00	2.46 ± 0.07
▶ Ginsana G115	ND	0.84	0.45	0.73	0.45	0.51	0.32	3.29
American ginseng								
▶ ground root	8700	1.61 ± 0.20	0.02 ± 0.00	0.23 ± 0.01	0.34 ± 0.020	0.97 ± 0.04	0.14 ± 0.00	3.32 ± 0.26
▶ REMEMBER-fX® (HT-1001)	15900	0.94 ± 0.30	0.03 ± 0.01	0.34 ± 0.12	0.30 ± 0.113	0.74 ± 0.06	0.11 ± 0.01	2.47 ± 0.62
▶ COLD-fX® (CVT-E002)	ND	0.65 ± 0.05	0.03 ± 0.05	0.26 ± 0.02	0.14 ± 0.013	0.37 ± 0.00	0.06 ± 0.00	1.50 ± 0.08
Panax notoginseng	2000	4.80 ± 0.73	0.10 ± 0.01	0	1.20 ± 0.208	2.03 ± 0.11	5.25 ± 0.10	13.38 ± 1.16

[1] Inducer activity expressed as U/g, ND = not detected. [2] Concentrations of ginsenosides are expressed as % weight/weight, mean ± SD; analytical techniques are described in the text

additional times using 3.3 and 3.4 mL of 30% methanol. 40 µL of the resulting solution were used for HPLC.

The ginseng powders were also assayed for panaxytriol content by gas chromatography-mass spectrometry (GC/MS) analyses in our laboratory. In brief, 1 mL of methanol was added into 0.1 g of ginseng powder and incubated at room temperature overnight. After centrifugation for 10 mins at 3500 rpm (Eppendorf 5424) at room temperature, the supernatant was collected and transferred into 15 mL falcon tubes. The same procedure was repeated for three times and the supernatants combined. The extract was dried with nitrogen gas and the residue was reconstituted in 1 mL of *n*-hexane. The hexane extract was then centrifuged for 3 min at 3500 rpm at room temperature. 200 µL of supernatant was aliquoted into microfuge tubes and 2 µL of 100 µg/mL internal standard (panaxytriol ketone) was added. The mixture was vortexed and transferred into a glass vial for GC/MS analysis. GC/MS analysis was performed on an Agilent 6890 N GC coupled to a 5975 inert XL mass selective detector (MSD) (Agilent Technologies). A DM-1 capillary column (cross-linked methyl siloxane) (30 m × 0.25 mm × 0.25 µM film thickness) (Dikma Technologies) was used. Purified helium gas (purity 99.9999%; Soxal) was used as the carrier gas at a constant column flow rate of 1 mL/min. Standards and samples were injected using an Agilent 7683B series autosampler into a 4 mm ID focus liner (SGE). The injection volume was 1.0 µL. Samples were injected in split mode with a split ratio of 10 : 1, and a split flow of 10 mL/min. The mass spectrometer was operated in EI mode. The quantitative ion at m/z = 118.0 and m/z = 101.9 were selected for panaxytriol ketone, the internal standard (IS), and panaxytriol, the analyte, respectively.

Statistical methods

Simple pairwise correlations (using the Pearson method) were performed between inducer activity of the ginseng powders and the concentrations of individual ginsenosides, and with the total ginsenoside and panaxytriol content, with and without stratifying for ginseng type. Data were analyzed using Stata version 8.2 (Stata Corp.).

Results

The NQO1 inducer activities of ginseng extracts are summarized in **Table 1**. The Korean red ginseng extracts demonstrated the most potent phase 2 enzyme induction activity, with CD values of 87 µg/mL (76900 U/g sample) for Sun Ginseng® and 240 µg/mL (27800 U/g sample) for the Aipop® sample. The ginsenoside-enriched HT-1001 American ginseng extract was the next most potent, with a CD value of 420 µg/mL (15900 U/g), followed by ground American ginseng root with a CD value of 767 µg/mL (8700 U/g). Neither the polysaccharide-enriched extract of American ginseng nor the white *Panax ginseng* extract (Ginsana G115 extract) yielded any inducer activity.

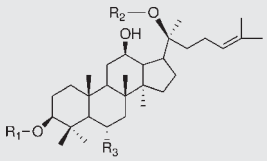
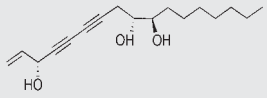
The phase 2 enzyme inducer activity of ginseng components is summarized in **Table 2**. None of the 6 pure ginsenosides that are most abundant in ginseng (Rb1, Rb2, Rc, Rd, Re, Rg1) yielded detectable induction activity. Pseudoginsenoside F11 and notoginsenoside R1, found mainly in American ginseng and *Panax notoginseng*, respectively, also did not demonstrate any induction activity. However, protopanxadiol and protopanaxatriol, which are deglycosylated derivatives of the ginsenosides, yielded potent induction activity, with CD values of 29 µM (499000 U/g) and 28 µM (499000 U/g), respectively. To determine if the aglycones of these ginsenosides have inducer activity, we incubated ginsenoside Rb1 with 1 U/mL of β-glucosidase over 48 hours at 37 °C. The resultant metabolites did not exhibit any induction activity.

Panaxytriol was over 10-fold more potent as an inducer than either protopanxadiol or protopanaxatriol, and it had an average CD of 3.85 µM, or 5760000 U/g. For comparison, the reported CD value of sulforaphane was 0.2 µM (188000000 U/g), and chlorophyll a was 250 µM (24600 U/g) [16].

Table 1 also shows the ginsenoside content of the various ginseng extracts tested. The *Panax notoginseng* extract had the highest content of total ginsenosides, while the polysaccharide enriched American ginseng COLD-fX® extract contained the least. There was no correlation between total ginsenoside content and phase 2 enzyme inducer activity (R^2 = 0.07, p = 0.58). There were also no correlations between concentrations of individual ginsenosides and inducer activity (not shown).

The panaxytriol content of the most potent phase 2 enzyme inducing extract Sun ginseng® was 120.8 µg/g. The two next most potent inducers, the Aipop® red ginseng extract and HT-1001 also contained panaxytriol, 9.42 µg/g and 9.38 µg/g, respectively. In contrast, the ginseng extract that did not induce phase 2 enzymes, COLD-fX® had very low levels of panaxytriol at 0.616 µg/g. The correlation was significant (R^2 = 0.92, p = 0.04).

Table 2 Phase 2 inducer activity and structures of ginsenosides and panaxytriol.

Component	Structures			Inducer activity[1]
Ginsenosides				
	R_1	R_2	R_3	
Rb1	-glc[2 → 1]glc	-glc[6 → 1]glc	-H	not detected
Rb2	-glc[2 → 1]glc	-glc[6 → 1]α-L-arabinopyranose	-H	not detected
Rc	-glc[2 → 1]glc	-glc[6 → 1]α-L-arabinofuranose	-H	not detected
Rd	-glc[2 → 1]glc	-glc	-H	not detected
Re	-H	-glc	-O-glc[2 → 1]rhamnose	not detected
Rg1	-H	-glc	-O-glc	not detected
Protopanaxadiol	-H	-H	-H	499000
Protopanaxatriol	-H	-H	-OH	499000
Panaxytriol				5760000

[1] Inducer activity expressed as U/g. glc = glucose

Discussion

There is accumulating evidence that phase 2 enzymes do not operate at their full capacity, can be activated in many tissues, and that induction of these enzymes is an effective and sufficient strategy for protecting against cancer and many other types of toxicities, including oxidative stress [17]. Therefore, it is important to identify safe and cost-effective inducers of phase 2 enzymes.

Ginseng is widely consumed as a dietary supplement and a food to promote health in a variety of formulations. We evaluated various ginseng sources which are commercially available, and identified those that induce phase 2 enzymes *in vitro*. This activity may contribute to the cancer chemoprotective and antioxidant effects observed by others in animal models, and in epidemiological studies of ginseng effects [18].

Substantial phase 2 enzyme inducer activity was found in some, but not all, ginseng preparations. Although previous reports have attributed pharmacological effects of ginseng extracts, including antineoplastic and immunomodulatory effects, to sterols known as ginsenosides [2], the phase 2 enzyme induction activity that we observed is unlikely to be attributable to any of the 6 most abundant ginsenosides. When we tested these as pure compounds, they had no direct activity nor were their metabolites active after we treated the parent ginsenoside Rb1 with β-glucosidase. HPLC analyses of each of the extracts revealed that there was no correlation between the concentration of major ginsenosides and phase 2 enzyme inducer activity. Although protopanaxadiol and protopanaxatriol demonstrated potent inducer activity, these aglycones are not found in significant amounts in ginseng extracts [19], and thus could not account for the phase 2 enzyme inducer activity of the extracts we tested.

Of the extracts tested, the Korean red ginseng extracts exhibited the most potent phase 2 enzyme activity, although they did not contain the highest content of ginsenosides. This could be a result of chemical modifications that are by-products of steam-processing [20]. The ginsenoside-enriched HT-1001 American ginseng extract yielded more potent activity than raw American ginseng, also suggesting that heat-processing may have produced biologically active compounds which may be more potent phase 2 enzyme inducers. This is consistent with previous studies of heat-processed ginseng components, which showed that steaming ginseng alters the composition of ginsenosides and increases endothelial relaxation, antioxidant [21] and antiproliferative activities [22].

Ginseng does, however, contain small amounts of other compounds which may be very potent phase 2 enzyme inducers. For example, compounds such as polyacetylenes [23] may be potent electrophiles which activate Nrf2. Consistent with predictions based on structural considerations of other phase 2 enzyme inducers, we have shown that panaxytriol, a polyacetylene, was a very potent inducer of NQO1.We have shown that the ginseng extracts which induce phase 2 enzymes also had higher concentrations of panaxytriol. Polyacetylenes have been found to be cytotoxic in a variety of cancer cell lines [24–26], and the ginseng polyacetylene evaluated in this study was toxic to Hepa1c1c7 cells at concentration greater than 50 µM (data not shown). However, these compounds have little toxicity in rodent models [27], and thus may have a promising role in cancer chemoprevention. Due to the over 10-fold greater potency of these compounds weight-by-weight compared to ginsenosides, we view this as an intriguing observation which now requires further investigation.

We have also demonstrated that a component of ginseng, panaxytriol, that was derived from total chemical synthesis, has potent phase 2 enzyme induction activity. Further understanding of the induction of phase 2 enzymes by various ginseng preparations and components may help identify a role for these widely used nutritional supplements in public health and disease prevention. We recently completed a clinical study of one of the American ginseng extracts tested in this set of experiments (HT-1001), and showed a reduction in oxidative stress markers in healthy volunteers consistent with induction of antioxidant phase 2 enzymes. However, there was no induction of UDP-glucuronosyl transferase 2B7, a phase 2 enzyme, as indicated by metabolism of a probe drug, zidovudine [28].

Both American and Korean red ginseng extracts induced phase 2 enzyme activity in an *in vitro* cytoprotective enzyme induction system. The inducer effects are, however, not likely to be due to the ginsenoside content of the extracts since individual ginsenosides had no detectable inducer activity. However, panaxytriol, a polyacetylene, was a potent phase 2 enzyme inducer, which suggests that the polyacetylenes present in ginseng could be responsible for a substantial proportion of the inducer activity of the extracts we tested. Alternatively, since the most potent ginseng extracts had undergone steam- or heat-processing during manufacturing, this could have altered their chemistry to produce modified ginsenosides, or other novel NQO1-inducing compounds. Additional research is required to identify other ginseng constituents that are produced during commercial processing, and to identify the components responsible for phase 2 enzyme induction. Additional clinical studies will be required to determine if ginseng extracts and components are bioavailable and induce cytoprotective enzymes in humans, since their contribution to cancer chemopreventive effects could well be a key to the widely reported health benefits of this plant.

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Conflict of interest statement: ASA has received American ginseng capsules from the Ginseng Board of Wisconsin for her American ginseng-indinavir pharmacokinetic and pharmacodynamic interaction study. None of the other authors report any competing interests. Mention of specific commercial ginseng products does not constitute endorsement or recommendation of those products by the authors.

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