Inhibition of Human 20S Proteasome by Ginsenosides from *Panax ginseng*

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Panax ginseng C. A. Meyer (Araliaceae) has been used in traditional herbal medicine and health food for over 2000 years.[1] The study of ginseng has a long history, and in the West, the efficacy of ginseng was known by the 18th century.[1] A variety of ginsenosides extracted from *P. ginseng* especially show diverse biological activities for the promotion of health with undefined effects on cardiovascular, immune, and central nervous systems.[1,2] Recently there has been a renewed interest in investigating the pharmacological effects of ginseng using biochemical and molecular biological techniques. Pharmacological effects of ginseng have been demonstrated in the central nervous, cardiovascular, endocrine, and immune systems.[2] In addition, ginseng and its constituents have been ascribed to antineoplastic, antistress, and antioxidant activity.[2] Ginseng contains many active components, and there are numerous studies that ginseng does have beneficial effects.[3]

The ubiquitin protease pathway (UPP) has emerged recently as having a promising biological role in cancer therapy. UPP is responsible for degrading the majority of intracellular proteins in eukaryotes.[4] Ubiquitin is a small 76 amino acid protein conserved in all eukaryotic cells with a molecular weight of 8.6 kDa. When polyubiquitin is attached to target proteins, tagged proteins are selected for destruction by cytoplasmic organelles called proteasomes.[5] The eukaryotic 26S proteasome is a proteolytic cellular apparatus, which consists of two subunits: the 20S core particle and 19S regulatory particle (19S cap).[6] The 20S core particle is a multicatalytic protease with a cylindrical structure composed of four stacked rings. The two outer rings complex with the 19S regulatory particles, forming a narrow channel through which only denatured proteins can pass.[7] The catalytic chamber is formed by the two inner rings, each of which contains three well-characterized peptidase activities-chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolase-like hydrolytic active sites.[8] Proteins are degraded by the core particle in a progressive manner, generating peptides of 3-25 amino acids in length.[9]

UPP plays an important role in selective protein degradation and regulates cellular events, including cell-cycle progression, apoptosis, and inflammation.[10] Since proteasomes interact primarily with endogenous proteins, inhibition of proteasomes may block the signaling action of transcription factor NF-*k*B and, thus inhibit the completion of the cell cycle and mitotic proliferation of cancerous cells, leading to cell death by apoptosis.[10] The potential of specific proteasome inhibitors to function as anticancer agents is now of considerable interest in the drug discovery process.[11] Preclinical evaluation has shown that cancer cells are more sensitive to the proapoptotic effects of proteasome inhibition than normal cells.[12] Therefore, several groups of proteasome inhibitors such as peptide aldehydes, peptide boronates, nonpeptide inhibitors, peptide vinyl sulfones, and epoxyketones, have been developed and are now widely used as research tools to study the role of the UPP in various cellular processes.

Currently, only two proteasome inhibitors, bortezomib and NPI-0052, have been reported in clinical trials.[13,14] Bortezomib synthesized in 1995 was the first clinically used proteasome inhibitor.[14] NPI-0052 was derived from the fermentation of *Salinospora tropica*, a new marine Gram-positive actinomycete.[14] NPI-0052 appears to be a more effective inducer of apoptosis than bortezomib in lymphocytes of chronic lymphocytic leukemia cells.[14] These two proteasome inhibitors, however, have undesirable side effects such as fatigue, nausea, vomiting, peripheral neuropathy, anemia, diarrhea, and constipation.[15] Therefore, there has been an intensive request for developing new proteasome inhibitors as antitumor agents having little or no side effects, especially from natural products.

Natural products have been vigorously investigated for working as a proteasome inhibitor and an antitumor agent. The natural product proteasome inhibitors include NPI-0052, lactacystin, withaferin A, celastrol, and gliotoxin, which were isolated from *Salinospora tropica*,[14] *Streptomyces* sp.,[16] *Withania somnifera*,[17] *Tripterygium wilfordii*,[18] and *Toxoplasma gondii*,[19] respectively. As part of an attempt to discover 20S proteasome inhibitors from natural products, ten ginsenosides isolated from red ginseng were screened for the 20S proteasome inhibitory activity in this study. For more than 2000 years, it has been known that *P. ginseng* has a low toxicity, which is an ideal characteristics for antitumor agent.

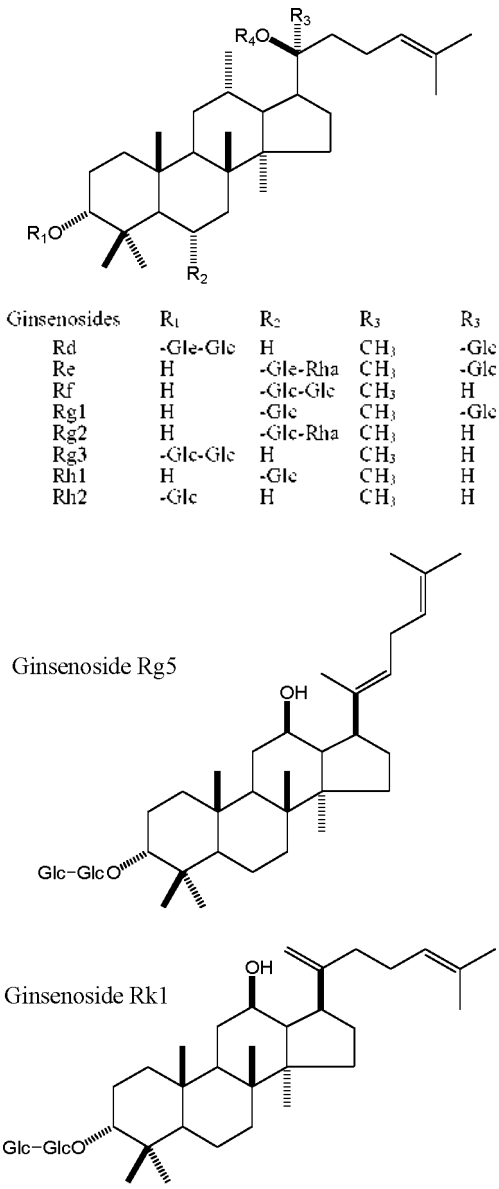
To the best of our knowledge, there has been only one report on proteasome inhibitory activity of ginsenosides.[20] The ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 efficacy in that study were evaluated on the 26S proteasome purified from the pig red blood cells (RBC). In this study, we used the 20S proteasome purified from human erythrocytes as our target and evaluated the efficacy of ginsenosides Rd, Re, Rf, Rg1, Rg2, Rg3, Rg5, Rh1, Rh2, and Rk1. Unlike a typical protease, the 20S proteasome ensures that virtually all peptide bonds within a protein substrate are susceptible to cleavage by processing within several proteolytic chambers.[21] We only checked the chymotrysin-like activity of 20S proteasome,

considering that ginsenosides seem to inhibit only chymotrypsin-like activity of proteasome in the previous study.[20]

To investigate the proteasome inhibitory activity of ginsenosides, commercially available 20S proteasome purified from human erythrocytes was used in this study. All ginsenosides were isolated using high-speed counter current chromatography as well as preparative HPLC.[22] Their structures were determined by spectroscopic analysis using ^{1}H-NMR, ^{13}C-NMR, and MS. Each ginsenoside was dissolved in DMSO to make a stock solution to a final concentration of 5 mM. Epoximicin, a natural proteasome inhibitor, was used as the positive control. It is a rapid, potent and irreversible inhibitor of the 20S proteasome chymotrypsin-like activity. Suc-Leu-Leu-Val-Tyr linked to aminomethylcoumarin (Suc-LLVY-AMC) was used as a substrate to investigate the chymotrypsin-like activity of the 20S proteasome. First, 150 μM of each ginsenosides was added to the 20S proteasome and incubated at 37 °C for 10 min; also at the same time, 0.5 μM of epoximicin was added to 20S proteasome and incubated at 37 °C for 10 min for the positive control. The substrate Suc-LLVY-AMC was then added to the reaction mixture containing the 20S proteasome and incubated for 5 min. The released fluorogenic AMC was measured to determine the chymotrypsin-like activity of the 20S proteasome.

Most of the ginsenosides are dammarane-type saponins which are classified into two types, protopanaxadiols and protopanaxatriols. Ginsenosides Rg3, Rk1, Rd, Rh2, and Rg5 belong to protopanaxadiols while ginsenosides Rf, Rg1, Re, Rh1, and Rg2 belong to protopanaxatriols, as shown in Fig. 1. The inhibitory effects of these compounds on the 20S proteasome are shown in Table 1. Ginsenoside Rd had the highest inhibitory activity on chymotrypsin-like activity of the 20S proteasome with an IC_{50} of 33.5 μM. Other protopanaxadiols also exhibited much higher inhibitory activity than the protopanaxatriols. Protopanaxadiols Rg3, Rk1, and Rg5 showed potent inhibitory activities with IC_{50} of 79.7, 81.6, and 132.8 μM, respectively and Rh2 showed moderate inhibitory activity with an IC_{50} of 201.0 μM. All protopanaxatriol-type ginsenosides Rf, Rg1, Re, Rh1, and Rg2 did not exhibit any inhibitory activity. These biological structure-activity relationships (SARs) strongly suggest that both glycosylation at C-3-OH and non-oxidation at C-6 on ginsenosides might be important for the inhibition of the chymotrypsin-like activity of the 20S proteasome. The structure-activity relationships also indicate that ginsenosides with two molecules of glucose linked to C-3-OH have a more potent inhibitory activity than that with one molecule of glucose linked to the same position.

Previously, Chang *et al.* reported that ginsenoside Rd inhibited the activity of 26S proteasome from pigs' RBC with its IC_{50} at 109.9 μM.[20] In our study, ginsenoside Rd showed more dramatic inhibitory activity on 20S proteasome from human erythrocyte with its IC_{50} at 33.5 μM than 26S proteasome from pigs' RBC, almost three times more effective. This result could be interpreted by two possibilities, one as that proteasomes from human erythrocyte might be more severely inhibited by Rd than those from pigs' RBC, or the other as that 20S proteasome itself might be more severely repressed by Rd than 26S proteasome. There are more than



Ginsenosides	R_1	R_2	R_3	R_3
Rd	-Glc-Glc	H	CH_3	-Glc
Re	H	-Glc-Rha	CH_3	-Glc
Rf	H	-Glc-Glc	CH_3	H
Rg1	H	-Glc	CH_3	-Glc
Rg2	H	-Glc-Rha	CH_3	H
Rg3	-Glc-Glc	H	CH_3	H
Rh1	H	-Glc	CH_3	H
Rh2	-Glc	H	CH_3	H

Figure 1. Chemical structures of ginsenosides.

Table 1. The inhibitory activities of the 20S proteasome inhibition by ginsenosides from *Panax ginseng*.

Ginsenoside	Protopanaxadiol					protopanaxatriol				
	Rd	Rg3	Rg5	Rh2	Rk1	Re	Rf	Rg1	Rg2	Rh1
IC_{50} (μM)[a]	33.5 ± 7.3	79.7 ± 8.7	132.8 ± 10.3	201 ± 11.5	81.6 ± 5.8	> 300	> 300	> 300	> 300	> 300

[a]IC_{50} (50% inhibition concentrations) were calculated from a log dose inhibition curve and expressed as the mean ± SD of triplicate experiments. Epoximicin was used as the positive control (IC_{50} = 65 nM).

92% amino acid and nucleotide similarities between human and pig 20S proteasome.[23,24] however, the small differences in amino acids could determine the degree of sensitivity to ginsenoside Rd. 20S proteasome is the catalytic core particle with peptidase activity while 26S proteasome comprises of 20S catalytic core particle and 19S regulatory particle, therefore, the access of Rd into the catalytic core particle might be easier than 26S proteasomes. These two possibilities could work separately, or together to induce the higher inhibition effect of Rd on 20S proteasome from human erythrocyte than on 26S proteasome from pigs' RBC. However, two lessons should be addressed that eventually human proteasomes are the target for developing anticancer agents and our data clearly show that Rd inhibit the human 20S proteasomes at low concentrations.

Conclusively, we show that ginsenoside Rd has the highest inhibitory activity for the 20S human proteasome. Ginsenoside Rd induces apoptosis of cancer cells through down-regulating *Bcl-2* expression, up-regulating *Bax* expression, and activating the caspase-3 pathway.[25] Our study and a previous study[20] strongly suggest that ginsenoside Rd is a potent anticancer agent with mild side effects by inducing apoptosis through down-regulating the chymotrypsin acitivity of proteasomes. In addition, ginsenoside Rg3, Rg5, Rk1 also showed potent inhibitory activities although they were not as potent as Rd. Protopanaxadiol-type ginsenosides are much more potent inhibitors against the 20S proteasome than protopanaxatriol-type ginsenosides since the five protopanaxatriol-type ginsenosides did not show any inhibitory activity. These results suggest that both glycosylation at C-3 and non-oxidation at C-6 in ginsenosides might play an important role in its inhibitory activity.

Experimental Section

Ginsenosides. Ginsenosides Rd, Re, Rf, Rg1, Rg2, Rg3, Rg5, Rh1, Rh2, and Rk1 were isolated by high-speed counter-current chromatography as well as HPLC as previously described.[22]

20S Proteasome. The purified, human erythrocyte 20S proteasome from the commercially available 20S Proteasome Assay Kit (BIOMOL International, LP.) was used and proteasome inhibition was determined using the kit.

20S Proteasome Inhibition Assay. The inhibition assay was done as follws.[26] The assay buffer was added to the blank and control and the diluted solution of the positive control was added to the inhibitor wells. The enriched proteasome fraction was diluted to a final assay concentration of 50 μg/mL using assay buffer. 0.03% sodium dodecyl sulfate was used to activate the 20S proteasome's chymotrypsin-like activity. This diluted fraction was then added to each well, and then, the plate was preincubated for 10 min at 37 °C to allow the inhibitor and enzyme to interact. The enzymatic reaction was started by adding Suc-LLVY-AMC substrate to a final concentration of 10 μM. The chymotrypsin-like enzymatic activity of the proteasome was determined by measuring the generation of free AMC using a fluorescent plate reader (FluorOptima, BMG LabTech Ltd., UK), capable of excitation at a wavelength of 355 nm and detection of emitted light at 460 nm. Epoximicin was used as the positive control (IC_{50} 65 nM), which is a rapid, potent and irreversible inhibitor of the 20S proteasome chymotrypsin-like activity. It can also inhibits the proteasome's trypsin-like and peptidyl-glutamyl peptide hydrolase activities, but at 100- and 1,000-fold slower rate, respectively.

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