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Role of Ginsenoside Rd in Inhibiting 26S Proteasome Activity

TSUI-LING CHANG,*,† HSIOU-YU DING,*,§ AND YI-WEN KAO†

Department of Biological Sciences and Technology, National University of Tainan, No. 33, sec. 2, Shu-Lin Street, Tainan, Taiwan, and Institute of Cosmetics Science, Chia Nan University of Pharmacy and Science, 60 sec, 1 Erh-Jen RD, Jen-Te, Tainan, Taiwan

Drugs targeting 26S proteasome as antitumor agents are considered to be important for cancer therapy. Although the active components are yet to be identified, for more than 1000 years, the low-toxicity *Panax ginseng* has been used in traditional herbal medicine for either treating or preventing cancer. Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 are distinct components that can be isolated from *P. ginseng* C.A. Meyer. In this study 26S proteasome was purified from pig red blood cells, and the activity of the seven isolated ginsenosides was analyzed by proteolysis assay. It was found that ginsenoside Rd inhibited 52.9% the chymotrypsin-like activity of 26S proteasome with an IC_{50} value of 107.5 μM when Suc-LLVY-AMC was used as a substrate. Ginsenoside Rd displayed a mixed type inhibition of 26S proteasome when analyzed by Lineweaver−Burk plots of the inhibition kinetics. Unlike ginsenoside Rd, the other ginsenosides showed low inhibitory effect of the chymotrypsin-like activity of 26S proteasome. Seven ginsenosides did not inhibit the trypsin-like and caspase-like activities of 26S when Ac-RLR-AMC or Z-LLE-AMC was used as substrate. These results suggest that ginsenoside Rd is a potential drug for cancer prevention due to its specific 26S proteasome inhibitory effect and known low toxicity. Furthermore, both 3-*O*-Glc_2-Glc and 20-*O*-β-Glc positions of the ginsenoside may play a role in the inhibitory property of the chymotrypsin-like activity in 26S proteasome.

KEYWORDS: Ginsenoside Rd; MG 132; *Panax ginseng* C.A. Meyer; 26S proteasome; proteasome inhibitor

INTRODUCTION

Panax ginseng has been used in traditional herbal medicine and health food for more than 1000 years. It has known toxicity (*1*) but with undefined effects on the cardiovascular, immune, and central nervous systems (*2*); current commercial interest is based upon the purported benefits of ginseng for cancer prevention (*3*). Its active ingredients, ginsenosides, are steroidal saponins and have been found to have anticancer properties against tumor cell lines and tumor growth (*4*). Ginsenoside Rd, 20(*S*)-dammarane-3-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-20-*O*-β-D-glucopyanosyl-(1→6)-xylopyranosyl-12β-ol, inhibited 20−90% growth of human lung cancer cell line H838 (p53wt) and prostate cancer cell line PC3 (p53 null) (*5*). However, there is no study on the effects of *P. ginseng* on the ubiquitin−proteasome system.

The ubiquitin−proteasome pathway (UPP) is responsible for degrading the majority of intracellular proteins in eukaryotes (*6*). Its regulatory role is an energy-dependent molecular machine to catalyze proteolysis of ubiquitin-modified proteins in normal cellular function (*6*).

Ubiquitin is a 76 amino acid protein of 8.6 kDa conserved small protein present in all eukaryotic cells. When polyubiquitin is attached to target proteins, tagged proteins are selected for destruction by cytoplasmic organelles called proteasomes (*7*). The eukaryotic 26S proteasome is a proteolytic cellular apparatus, which consists of two subunits: the 20S core particle and the 19S regulatory particle (19S cap, PA700) (*8*−*10*). The 20S core particle is a cylindrical structure composed of four stacked rings and a multicatalytic protease. The two outer rings (called α rings) complex with the 19S regulatory particles, forming a narrow channel through which only denatured proteins can pass. The catalytic chamber is formed by the two inner β rings, each of which contains three well-characterized peptidase activities—chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolase-like (PGPH) hydrolytic active sites (*11*). Proteins are degraded by the core particle in a progressive manner, generating peptides of 3−25 amino acids in length (*12*).

In addition to its role in the removal of damaged proteins, recent experimental data have shown that cancers, cardiovascular disease, and type 2 diabetes may activate the UPP system (*13*−*17*). The accumulation of ubiquitin (Ub) has been documented in various cancers, which suggests that disturbance of the protein

* Authors to whom correspondence should be addressed [(T.-L.C.) telephone (886) 6-2602587, fax (886) 6-2606153, e-mail tsuiling@mail.nutn.edu.tw; (H.-Y.D.) telephone (886) 6-2664911, fax (886) 6-2670324, e-mail hsiou221@yahoo.com.tw].

† National University of Tainan.

§ Chia Nan University of Pharmacy and Science.

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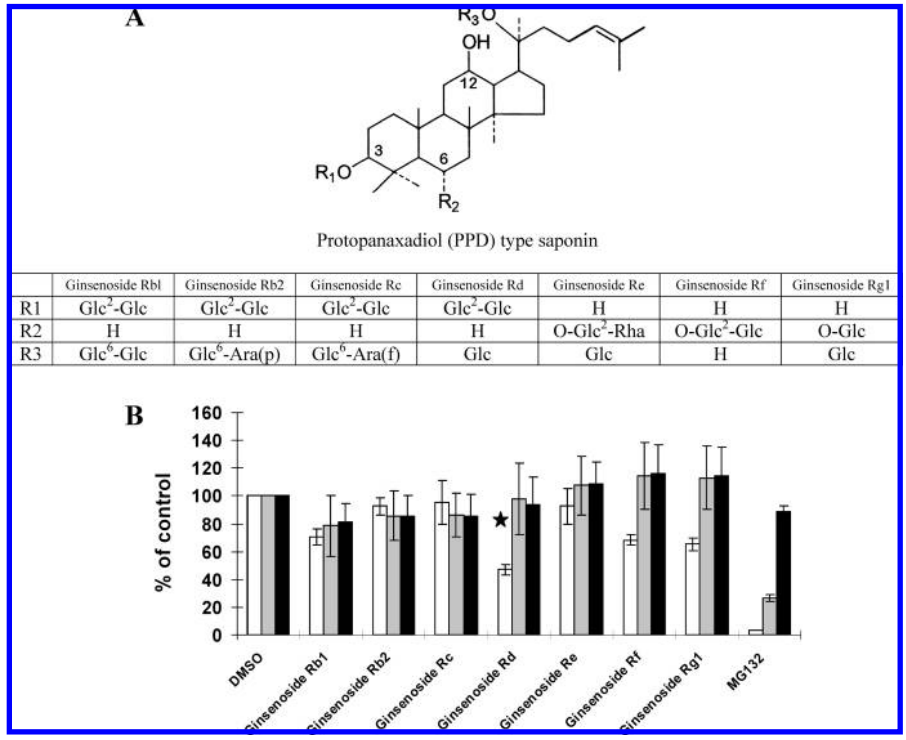

	Ginsenoside Rbl	Ginsenoside Rb2	Ginsenoside Rc	Ginsenoside Rd	Ginsenoside Re	Ginsenoside Rf	Ginsenoside Rg1
R1	Glc^2-Glc	Glc^2-Glc	Glc^2-Glc	Glc^2-Glc	H	H	H
R2	H	H	H	H	O-Glc^2-Rha	O-Glc^2-Glc	O-Glc
R3	Glc^6-Glc	Glc^6-Ara(p)	Glc^6-Ara(f)	Glc	Glc	H	Glc

Figure 1. Comparison of the percentage of chymotrypsin-like, caspase-like, and trypsin-like activities of 26S proteasome inhibited by 100 μM ginsenosides and MG 132: (**A**) chemical structures of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 isolated from *Panax ginseng*; (**B**) chymotrypsin-like (white bars), caspase-like (gray bars), and trypsin-like (black bars) activities of 26S proteasome (26S proteasome with DMSO = 100%). Data are reported as mean ± SEM. Each sample is the mean of three determinations. The star (★) indicates values where difference from control value (white lane 1) was statistically significant at $P < 0.005$. Glc, glucose; Rha, rhamnose; Ara(p), arabinosylpyranose; Ara(f), arabinosylfuranose.

degradation process could have profound effects on tumor growth. We know that the levels of free ubiquitin (FUb) and multiubiquitin chain (MUC) were up-regulated in colorectal cancer (*14*). Tumor cells seem to have much higher proteasome activity than do nonmalignant cells, as demonstrated in K562 human myelogenous leukemia cells (*15*). Overexpression of ubiquitin-specific peptidases (USPs) and proteasome subunits (PSs) were found in breast cancer tissue (*16*). Furthermore, changes of proteasomal proteins were found in 10 different tumor cell lines (*17*). In view of the published experimental findings, it is reasonable to assume that the UPP may provide potential therapeutic targets for the treatment of cancer and other disorders. A platform for drugs targeting proteasomes as antitumor agents is a very important subject for pretreatment of diseases (*18*). Therefore, several groups of proteasome inhibitors, 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 (*18*).

Two proteasome inhibitors have been reported currently in clinical trial. Bortezomib was the first of the proteasome inhibitors to be used clinically (*19*). It appears that proteasome inhibitor NPI-0052 is a more effective inducer of apoptosis than bortezomib in lymphocytes of chronic lymphocytic leukemia cells (*20*). The combination of proteasome inhibitors bortezomib and NPI-0052 triggers in vivo synergistic cytotoxicity in multiple myeloma (*21*), but these two clinical proteasome inhibitors have their undesirable side effects, whereas the low-toxicity Chinese herbal medicines including *P. gingseng* have been used for more than 1000 years in the treatment and possible prevention of malignancy through the inhibition of 26S proteasomes.

In this study, we have found that isolated ginsenoside Rd is a potent in vitro inhibitor on the chymotrypsin-like activity of 26S proteasome, and this finding should provide a pharmacological rationale for further study on the treatment and prevention of neoplastic diseases.

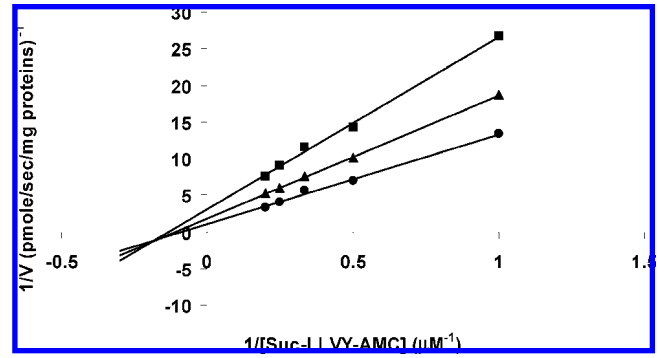

Figure 2. Lineweaver−Burk plot of ginsenoside Rd [0 (●), 50 μM (▲), 100 μM (■)] effect on 26S proteasome activity when the concentration of the substrate Suc-LLVY-AMC was 0.5, 1, 2, 3, 4, or 5 μM. Each point represents the mean of three independent determinations.

MATERIALS AND METHODS

Ginsenosides. Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 were purchased from Extrasynthese (Genay, France). Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 were dissolved in dimethyl sulfoxide (DMSO) to 10 mM stock solutions.

Purification of 26S Proteasome from Pig Red Blood Cells (RBCs). Fresh pig blood was collected at a local abattoir with sodium citrate as anticoagulant. Plasma and white blood cells (WBCs) were removed from blood samples by centrifugation (500*g* for 10 min). RBCs were resuspended 1:1 (v/v) with cold phosphate-buffered saline (PBS) and centrifuged at 3700*g* for 17 min at 4 °C. 26S proteasomes were purified from pig RBCs as described previously (*22*).

Proteolysis Measurement. Proteasome activity was assessed using synthetic peptide substrates linked to the fluorometric reporter aminomethylcoumarin (AMC). The peptide activity of pig 26S proteasome

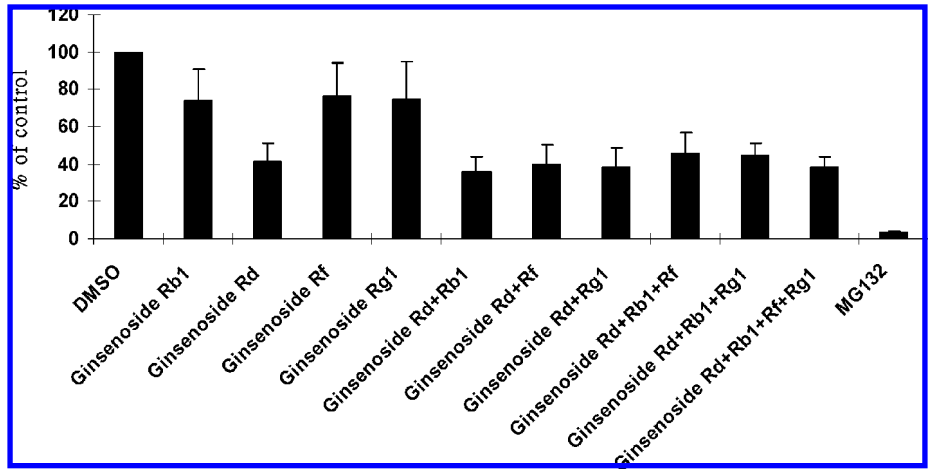

Figure 3. Comparison of percentage of chymotrypsin-like activity of 26S proteasome inhibited by combined ginsenosides and MG 132. Control is the percentage of the chymotrypsin-like activities of 26S proteasome (26S proteasome with DMSO = 100%). Data are presented as mean ± SEM. Each sample is the mean of three separate determinations.

toward three different fluorogenic peptides was measured by incubation with 1 μL of each ginsenoside or MG132 in 30 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1 mM ATP, and 0.5 mM DTT with 50 μM Suc-Leu-Leu-Val-Tyr-AMC (Bachem), Ac-Arg-Leu-Arg-AMC (Biomol), or Z-Leu-Leu-Glu-AMC (A.G. Scientific, Inc.) for 15 min at 37 °C. The final concentration of 26S proteasomes in the assays was 385 μg/mL (or 160 nM assuming a molecular mass of 2.4 MDa). AMC hydrolyzed from the peptides was quantified in a BioTek FL800 plate reader using 360 nm excitation and 460 nm emission wavelengths at 37 °C for 15 min. Enzymatic activity was normalized for protein concentration and expressed as percent of activity of control (26S proteasome with 1 μL of DMSO). Each measurement was carried out using at least three independent experiments.

Michaelis−Menten Kinetics of Ginsenoside Rd. One microliter of ginsenoside Rd was mixed with 160 nM 26S proteasome in 100 μL of reaction buffer [30 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, and substrates (Suc-Leu-Val-Tyr-AMC)]. The concentration of Suc-Leu-Val-Tyr-AMC was from 1 to 5 μM. The velocities of reactions were measured without and with inhibitor (50 and 100 μM ginsenoside Rd). The following procedures used the same protocol as described above.

Statistical Analysis. Data presented are the mean of three different experiments ± standard error (SE) for each proteolysis measurement. We used Student's unpaired *t* test to characterize the differences between two sets of values. A standard $p < 0.005$ was set as the significance level.

RESULTS AND DISCUSSION

Effects of Ginsenosides on 26S Proteasome Activity. Proteasome inhibitors possess the ability to inhibit cell proliferation and induce apoptosis, and these properties render these agents as possible anticancer candidates. Chinese medicine historically used ginsenosides for the treatment of cardiovascular diseases, inflammation, different body pains, and internal and external bleeding due to injury. To investigate the inhibition of ginsenosides in 26S proteasome function, intact 26S proteasomes were purified from pig RBCs by using cell lyses, precipitated by 40% saturated ammonium sulfate, and separated by DEAE Affi-Gel Blue column (*22*). Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 were dissolved in DMSO to 10 mM stock solutions. MG 132 is a chymotrypsin-like and caspase-like proteasome inhibitor of 20S proteasome for laboratory use (*18*). First, we exposed pig 26S proteasomes to 100 μM each ginsenoside and MG 132 for 5 min and then incubated them with synthetic peptide substrates linked to the fluorometric reporter AMC that measure the chymotryptic-like, caspase-like, and tryptic activities of the 26S proteasome. The chemical structures of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 are shown in **Figure 1A**. All seven ginsenosides are 20(*S*)-dammarane. The inhibitory effects of these compounds on 26S proteasome are illustrated in **Figure 1B**. The results showed that ginsenoside Rd produced maximal inhibition (52.9%) of the chymotryptic activity of the proteasome (**Figure 1B**). We next compared the caspase-like and tryptic-like activities of the 26S proteasome, whereas all seven ginsenosides have no significant inhibitory effect on these 26S proteasomes enzymatic activities (**Figure 1B**). The biological structure−activity relationships (SAR) for saponins present in *P. ginseng* showed that ginsenoside with both 3-*O*-Glc_2-Glc and 20-*O*-β-Glc positions of saponin is the best inhibitor of the seven ginsenosides for chymotrypsin-like activities. Ginsenoside Rc has almost no inhibition on 26S proteasome activity. Ginsenoside Rd inhibited the chymotrypsin-like activity of 26S proteasome with an IC_{50} value of 107.5 μM as based on linear curve fitting.

Ginsenoside Rd significantly inhibits HeLa cell proliferation and induces in vitro cell apoptosis through down-regulating Bcl-2 expression and activating the caspase-3 pathway (*23*). Our study showed that ginsenoside Rd is the best one that inhibited 52.9% of the chymotryptic activity of 26S proteasome preparation when compared to other ginsenosides (**Figure 1B**). PS-341 has been shown to inhibit the chymotryptic activity (β5*) yet actually increase tryptic activity (*24*). Unlike PS-341, ginsenoside Rd does not increase caspase-like and tryptic-like activities. Protein degradation in vitro and in vivo is reduced significantly only when either the trypsin-like or caspase-like sites are inhibited, together with the chymotrypsin-like sites (*25*).

Furthermore, ginsenoside Rb1 has been shown to act as a phytoestrogen in MCF-7 human breast cancer cells via activation of the estrogen receptors, whereas it increases SW480 human colorectal cancer cell apoptosis significantly (*26*). Reported data show that ginsenoside Rb2 inhibits tumor angiogenesis and metastasis (*27*) and that ginsenosides Rc and Re stimulate c-fos expression in MCF-7 human breast carcinoma cells, whereas ginsenoside Rg1 served as a functional ligand of the glucocortico receptor and as an estrogen-like activity (*28, 29*). Ginsenoside Rg1 also can be used as a novel therapeutic modality for inducing angiogenesis (*30*). However, our data show that ginsenosides Rb1, Rb2, Rc, Re, and Rg1 did not significantly inhibit the chymotryptic activity of 26S proteasome.

Michaelis−Menten Kinetics of Ginsenoside Rd. To further investigate the inhibition kinetics, ginsenoside Rd was analyzed by the Lineweaver−Burk method. The experiment used the

same protocol as described above. The concentration of Suc-Leu-Val-Tyr-AMC varied from 1 to 5 μM. The velocities of reactions were measured without and with inhibitor (50 and 100 μM). The Lineweaver−Burk plot is shown in **Figure 2**. The Michaelis−Menten kinetics had a V_{max} = 0.95 pmol of AMC·s^{-1}·mg of proteins^{-1} and a K_m (Suc-LLVY-AMC) = 11.7 μM. 26S activity in the presence of ginsenoside Rd at 50 and 100 μM exhibited V_{max} values of 0.53 and 0.32 pmol of AMC·s^{-1}·mg of proteins^{-1} and K_m values of 8.9 and 7.5 μM, respectively. Ginsenoside Rd was a mixed type inhibitor of the chymotrypsin-like activity of 26S proteasome with a K_i value of 109.9−135.1 μM and a K_i' value of 50.8−63.3 μM.

Ginsenoside Rd was used at a concentration of 100 μM ($\cong$ 9.47 μg/mL), which is below its K_i value for the chymotryptic activity (109.9−135.1 μM). Our data show that the inhibitory kinetic of chymotrypsin-like activity on its substrate, Suc-LLVY-AMC, follows a mixed type kinetics with $K_i' < K_i$. The efficiency of ginsenoside Rd in inhibiting chymotrypsin-like activity depends on its chemical structure—3-*O*-Glc$_2$-Glc and 20-*O*-β-Glc positions. We have found that the kinetic rate of enzyme−ginsenoside Rd association is faster than that of enzyme−substrate−ginsenoside Rd association. However, although those fragments are affected by the association of chymotrypsin-like enzyme with ginsenoside Rd, the trypsin-like and caspase-like activities of 26S proteasome are still able to hydrolyze proteins.

Combined Ginsenoside Rd and Other Ginsenosides Trigger Decreased 26S Proteasome Activity. Chinese herbal medicine is usually made of two or more ingredients. Here we try to address whether combinations of ginsenosides could be more efficient in inhibiting the chymotrypsin-like activity of 26S proteasome. Because many of the previously reported research efforts were focused on crude ginseng extracts, it is thought studies on combinations of purified ginsenosides may provide a better understanding of the diverse pharmacological properties of ginseng. For these reasons, we used ginsenoside Rd at a concentration of 100 μM, which is lower than its respective IC_{50}. During the experiment the 26S preparations were treated for 5 min with ginsenoside Rd or a combination of other ginsenodies and then analyzed for the inhibitory effect by peptide assay. A higher inhibitory effect (64%) was noted when combined ginsenosides Rd and Rb1 were combined than with either ginsenoside alone (**Figure 3**). Data obtained from the proteolysis assay showed that ginsenoside Rd and ginsenoside Rb1 inhibit 64% of the chymotrypsin-like activity of 26S proteasome. Combination of four ginsenosides (Rd, Rb1, Rf, and Rg1) showed only a 62% inhibitory effect of the chymotrypsin-like activity of 26S proteasome. Other combinations of ginsenosides showed no significant inhibition.

Our data showed a higher inhibitory effect when the enzymatic activity of 26 proteasome preparation was treated with combined ginsenosides Rd and Rb1 than with either alone. However, for the difference of the biological SAR, ginsenoside Rd and ginsenoside Rb1 are similar (**Figure 1A**). Nevertheless, there are more sugar moieties (glycons) in ginsenoside Rb1 than in ginsenoside Rd. These results suggest that the 3-*O*-Glc$_2$-Glc position of the ginsenoside might play an important role in the inhibitory effect on the chymotrypsin-like activity of 26S proteasome. That is why combined ginsenoside Rd and ginsenoside Rb1 inhibit 64% of the chymotrypsin-like activity of 26S proteasome, whereas each alone inhibited about 26.2% for Rb1 and 52.9% for Rd. Because ginsenosides Rf and Rg1 did not have the 3-*O*-Glc$_2$-Glc position, other combinations with these two ginsenosides showed no significant inhibition.

In conclusion, our results may be useful for evaluating the structure−activity relationship of other gensenoides and for developing novel anticancer drugs. Our findings also suggest that ginsenoside Rd could function as a potential compound for cancer treatment and prevention.

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