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Ginsenoside Rb1 inhibits fibrillation and toxicity of alpha-synuclein and disaggregates preformed fibrils

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ABSTRACT

Compelling evidence indicates that α-synuclein (α-syn) aggregation plays a central role in the pathogenesis of Parkinson's disease (PD) and other synucleinopathies. Identification of compounds that inhibit or reverse the aggregation process may thus represent a viable therapeutic strategy against PD and related disorders. Ginseng is a well-known medicinal plant that has been used in East Asia for more than two thousand years to treat several conditions. It is now understood that the pharmacological properties of ginseng can be attributed to its biologically active components, the ginsenosides, which in turn have been shown to have neuroprotective properties. We therefore sought to determine for the first time, the potential of the most frequently used and studied ginsenosides, namely Rg1, Rg3 and Rb1, as anti-amyloidogenic agents. The effect of Rg1, Rg3 and Rb1 on α-syn aggregation and toxicity was determined by an array of biophysical, biochemical and cell-culture-based techniques. Among the screened ginsenosides, only Rb1 was shown to be a potent inhibitor of α-syn fibrillation and toxicity. Additionally, Rb1 exhibited a strong ability to disaggregate preformed fibrils and to inhibit the seeded polymerization of α-syn. Interestingly, Rb1 was found to stabilize soluble non-toxic oligomers with no β-sheet content, that were susceptible to proteinase K digestion, and the binding of Rb1 to those oligomers may represent a potential mechanism of action. Thus, Rb1 could represent the starting point for designing new molecules that could be utilized as drugs for the treatment of PD and related disorders.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder caused by the gradual loss of dopaminergic neurons (Obeso et al., 2008). As a consequence, the neurotransmitter dopamine is depleted, resulting in severe debilitation in motor skills (Obeso et al., 2008). Neuropathological studies of PD have revealed the presence of cytoplasmic inclusions, which are abundantly found in the degenerating dopaminergic neurons of the substantia nigra and other cortical and subcortical neurons (Galvin et al., 1999). These inclusions are known as Lewy bodies (LBs) and Lewy neurites (LNs), formed due to α-syn deposition (Spillantini et al., 1998). Intracellular α-syn inclusions are also a prominent feature of other neurodegenerative diseases, including dementia with Lewy bodies and multiple system atrophy (reviewed by Goedert et al., 2013). Genetic, biochemical and animal model studies also provide strong evidence in support of the central role of α-syn aggregation during the pathogenesis of PD and related disorders.

In its native form, α-syn, has little or no ordered structure, existing mostly as an unfolded protein (Weinreb et al., 1996). However, α-syn can undergo conformational changes that promote the self-assembly and aggregation of the protein. α-Syn aggregation proceeds through

Abbreviations: α-syn, α-synuclein; PD, Parkinson's disease; Aβ, beta amyloid; LBs, Lewy bodies; LNs, Lewy neuritis; Gn, ginsenoside; GST, glutathione S-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; Th-T, Thioflavin-T; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEM, transmission electron microscope; WB, Western blot; PK, proteinase K; NMR, nuclear magnetic resonance.

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the formation of oligomers (early aggregates), which ultimately convert into well-ordered fibrils (late aggregates) (Uversky et al., 2001). Recent studies indicate that early aggregates of α-syn, the so-called soluble oligomers, form the pathogenic species that drive neurodegeneration and neuronal cell death rather than mature amyloid fibrils (Conway et al., 2000; El-Agnaf et al., 2003; Winner et al., 2011).

The currently available drugs for the treatment of PD provide symptomatic relief but do not alter the course of the disease (He et al., 2013). However, the effectiveness of these drugs diminishes after several years of treatment. Together with the increasing incidence of PD due to population aging, these facts indicate the compelling need for more effective drugs and treatments for PD. Although modern therapeutic options that target disease modifications are on the rise, the multiple mechanisms involved in the pathogenesis of PD create considerable difficulty for producing effective treatments. Hence, inhibition of α-syn aggregation may represent a viable strategy for therapeutic intervention in PD and related disorders. It is therefore essential to identify compounds that can serve as potent inhibitors and interrupt the early stages of aggregation.

Ginseng is a well-known medicinal herb that has been used for more than two thousand years in China, Korea and Japan to promote well-being and alleviate fatigue. Although there are eleven different species of ginseng that belong to the genus *Panax* of the *Aralliaceae* family, the most commonly used species are *Panax ginseng* (Asian or Korean ginseng), *Panax quinquefolius* (American ginseng), *Panax japonicus* (Japanese ginseng) and *Panax notoginseng* (Chinese notoginseng or Sanchi) (Chen et al., 2006). Named after its ability to treat several conditions – *Panax* means panacea in Greek language – ginseng is now a well-documented anti-carcinogenic, anti-diabetic, anti-oxidant and vasorelaxing agent that exhibits immunomodulatory properties and improves the function of central nervous system (Lü et al., 2009). The numerous pharmacological properties of ginseng are attributed to its biologically active ingredients, the ginsenosides (reviewed by Im and Nah, 2013), which can be extracted from many parts of the ginseng plant, including the root, the leaves and the ginseng berries (Attele et al., 1999). Ginsenosides, which are also referred to as ginseng saponins, are derivatives of triterpenoid dammarane with a four-ring, steroidal structure bearing sugar moieties and an aliphatic side chain (Wee et al., 2011). The variations in the structure of ginsenosides, namely the type of aglycone (triterpene), the type of sugar moieties (glucose, maltose, fructose, saccharose etc.), their number and their site of attachment (Wee et al., 2011), give rise to three categories of ginseng saponins, the panaxadiol group, the panaxatriol group and the oleanolic acid group (Kim et al., 2013). More than 100 ginsenosides have been identified so far (Nag et al., 2012), but the most frequently studied ones are Rb1, Rg1, Rg3, Rd, Re, Rh1 and Rh2.

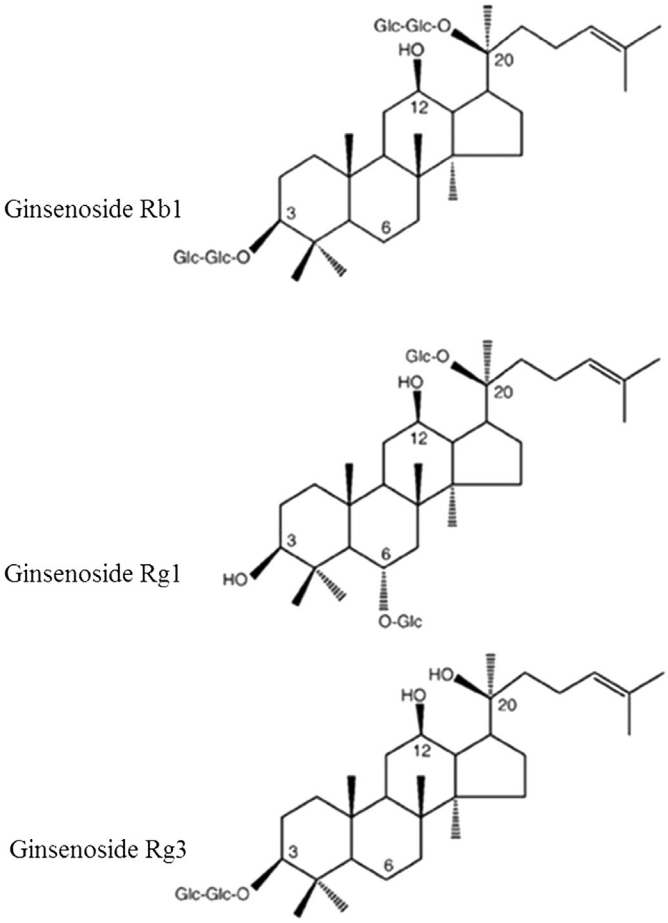

Fig. 1. Chemical structure of ginsenoside Rb1, ginsenoside Rg1, and ginsenoside Rg3.

Ginsenosides have been shown to affect voltage-gated ion channels, such as the Ca^{2+}, Na^{+}, and K^{+} channels, as well as the ligand-gated ion channels, such as the 5-HT_3-, the α7 nicotinic acetylcholine and the N-methyl-D-aspartate (NMDA)-gated channels (reviewed by Nag et al., 2012; Radad et al., 2011). This property of ginsenosides appears to underlie many pharmacological effects of ginseng, including neuroprotection (Kim et al., 2007), since it has a beneficial effect on many neurological conditions, including neurodegenerative diseases such as PD (reviewed by Cho, 2012; Kim et al., 2013). However, despite the numerous studies exploring the effect of various ginsenosides on the nervous system, there are no reports on the effect of ginsenosides on the aggregation propensity of amyloidogenic proteins such as α-syn. As a consequence, we sought to determine the potential of the most frequently used and studied ginsenosides, namely Rb1, Rg1 and (20S)-Rg3, i.e. the stereoisomer of Rg3 with its C-20 OH being spatially close to the C-12 OH group (Fig. 1).

Materials and methods

Expression and purification of recombinant human α-syn

A GST-α-syn fusion construct in the pGEX-4T1 vector (kindly provided by Dr. Hyangshuk Rhim of the Catholic University College of Medicine, Seoul, Korea). The expression and purification was carried out as described elsewhere (Ardah et al., 2014). Briefly, the construct was inserted into BL21 *Escherichia coli* bacteria by heat shock. The transformed bacteria were grown in LB medium supplemented with 0.1 mg/ml ampicillin at 37 °C in an orbital shaker to an OD_{600} of 0.5. Expression was then induced by adding 0.5 mM IPTG (Sigma-Aldrich Chemie GmbH, Germany), and the culture was incubated for 2 h at 37 °C. The cells were harvested by a 15 minute centrifugation at 9000 ×*g*, and the resulting pellet was then resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% DTT) and shaken for 10 min at room temperature. To improve the efficiency of cell lysis, the resuspended pellet was subjected to 6 freeze–thaw cycles in liquid nitrogen and a 37 °C water bath. The lysate was then centrifuged at 27,000 ×*g* for 15 min, and the resulting supernatant was retained for purification by affinity chromatography using sepharose beads conjugated to glutathione, which has a high affinity for the GST tag. The cell lysate was mixed with glutathione sepharose beads and incubated for 1 h at room temperature, followed by centrifugation at 500 ×*g* at 4 °C for 8 min. The beads were then washed twice with wash buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, pH 8.0); twice with 50 mM Tris–HCl, pH 8.0; and once with 1× PBS. The washed beads were resuspended in 5 ml of 1× PBS, and the GST tag was cleaved by human plasma thrombin (1 unit/μl) (Sigma-Aldrich, USA). The thrombin-catalyzed cleavage reaction was incubated overnight at room temperature with continuous mixing followed by 5 min of incubation at 37 °C. The reaction mixture was then centrifuged for 8 min at 500 ×*g* at 4 °C, and benzamidine sepharose beads (Amersham, Sweden) were used to 'fish out' thrombin. Pure α-syn