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Original Article

Effect of sugar positions in ginsenosides and their inhibitory potency on Na$^+$/K$^+$-ATPase activity

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Aim: To determine whether ginsenosides with various sugar attachments may act as active components responsible for the cardiac therapeutic effects of ginseng and sanqi (the roots of *Panax ginseng* and *Panax notoginseng*) via the same molecular mechanism triggered by cardiac glycosides, such as ouabain and digoxin.
Methods: The structural similarity between ginsenosides and ouabain was analyzed. The inhibitory potency of ginsenosides and ouabain on Na$^+$/K$^+$-ATPase activity was examined and compared. Molecular modeling was exhibited for the docking of ginsenosides to Na$^+$/K$^+$-ATPase.
Results: Ginsenosides with sugar moieties attached only to the C-3 position of the steroid-like structure, equivalent to the sugar position in cardiac glycosides, and possessed inhibitory potency on Na$^+$/K$^+$-ATPase activity. However, their inhibitory potency was significantly reduced or completely abolished when a monosaccharide was linked to the C-6 or C-20 position of the steroid-like structure; replacement of the monosaccharide with a disaccharide molecule at either of these positions caused the disappearance of the inhibitory potency. Molecular modeling and docking confirmed that the difference in Na$^+$/K$^+$-ATPase inhibitory potency among ginsenosides was due to the steric hindrance of sugar attachment at the C-6 and C-20 positions of the steroid-like structure.
Conclusion: The cardiac therapeutic effects of ginseng and sanqi should be at least partly attributed to the effective inhibition of Na$^+$/K$^+$-ATPase by their metabolized ginsenosides with sugar moieties attached only to the C-3 position of the steroid-like structure.

Introduction

Ginseng and sanqi (the roots of *Panax ginseng* and *Panax notoginseng*), 2 well-known traditional Chinese medicinal herbs, have been greatly used in several Asian countries for thousands of years[1]. Despite certain distinct remedial usages, comparable therapeutic effects, such as the promotion of blood circulation, have been reported for these 2 herbs. Both ginseng and sanqi are commonly used for the treatment of coronary heart disease and cerebral vascular disease[2–4]. Belonging to the same genus, ginseng and sanqi possess similar constituents, including their unique active ingredients, ginsenosides[5,6]. Cumulated research outcomes have indicated that identical and variable ginsenosides in these 2 herbs are possibly responsible for the comparable and dis-

tinct therapeutic effects between ginseng and sanqi[7].

Ginsenosides are triterpene saponins that have a common 4 ring hydrophobic steroid-like structure with sugar moieties attached mostly at the C-3, C-6, or C-20 position[8]. So far, more than 80 ginsenosides have been isolated from over 10 *Panax taxa*, and most of these are derived from 4 types of aglycones: protopanaxadiol, protopanaxatriol, oleanolic acid, and ocotillol[9]. Biological activities, such as neuroprotective effects, antitumoral activity, and cardiac therapeutic effects have been documented for these ginsenosides[1,7,10]. The different sugar moieties in ginsenosides are assumed to provide specificity for the diverse therapeutic effects of variable ginsenosides. Although the cardiac therapeutic effects of ginsenosides have been documented, the detailed molecular mechanism triggering this medicinal role is still unknown. It has been speculated that calcium channel blocking or the antifree-radical action of ginsenosides may be responsible for their cardiac therapeutic effect[11].

Cardiac glycosides, such as ouabain and digoxin, also contain a core steroid-like structure and have been utilized for the treatment of congestive heart failure[12]. The cardiac therapeutic effect of cardiac glycosides is a result of their reversible inhibition on the α-subunit of the membrane-bound Na^+/K^+-ATPase, mainly, but not exclusively, located in the human myocardium[13]. This inhibition leads to the accumulation of sodium in cardiac cells, which are enforced to promote the sodium-calcium exchange system in the cell membrane, thus causing a higher level of intracellular and myocardial calcium. The elevated intracellular calcium induces positive inotropy that eventually accentuates the force of myocardial contraction.

In light of the structural similarity between ginsenosides and cardiac glycosides, as well as their consentaneous utilization in cardiac therapy, we determined whether ginsenosides of certain sugar attachments may act as active ingredients responsible for the cardiac therapeutic effects of ginseng and sanqi via the same molecular mechanism triggered by cardiac glycosides. In this study, the inhibitory potency of various ginsenosides and ouabain on Na^+/K^+-ATPase was examined and compared. The molecular modeling and docking of representative ginsenosides to Na^+/K^+-ATPase were exhibited to reveal the observed difference in Na^+/K^+-ATPase inhibition among ginsenosides at the molecular level.

Materials and methods

Chemicals and reagents Ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, PPD, Re, Rf, Rg2, Rg1, Rh1, and PPT, as well as pseudoginsenoside F11, were purchased from Scientific Pharmaceutical Elite Company (Taiwan). Ginsenoside R1 was obtained from ChromaDex (Irvine, CA, USA). Oleanolic acid and ouabain were obtained from Sigma (St Louis, MO, USA). The phosphate assay kit was purchased from Amresco (Solon, Ohio, USA).

Measurement of Na^+/K^+-ATPase activity The Na^+/K^+-ATPase activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP[14]. A commercial Na^+/K^+-ATPase from the porcine cerebral cortex (Sigma, USA; 0.3 units/mg) was incorporated into a 1 mL reaction mixture containing 3 mmol/L ATP, 5 mmol/L $MgCl_2$, 80 mmol/L NaCl, 20 mmol/L KCl, and 40 mmol/L Tris-HCl (pH 7.8). The enzymatic reaction was terminated by adding 250 μL of 30% (*w/v*) trichloroacetic acid after the incubation period. After centrifugation at 10 000×*g* for 10 min, the supernatant was diluted 12.5-fold with deionized water and then 50 μL color development reagent, which was provided by the phosphate assay kit, was added. After 30 min

of incubation at room temperature, the color intensity was measured at 620 nm on a SpectraMax M2 reader (Molecular Devices, Sunnyvale, CA, USA). Sodium pump activity was expressed as μmol Pi liberated from ATP by 1 mg of Na^+/K^+-ATPase in 1 h.

Data analysis Data were expressed as mean±SEM of 5 replicates, and one-way ANOVA was performed on SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $P<0.05$.

Molecular modeling and docking The crystal structure of pig renal Na^+/K^+-ATPase (Protein Data Bank [PDB] code 3B8E) was downloaded from the PDB[15]. In order to facilitate the docking process, we removed the β and γ subunits of the Na^+/K^+-ATPase, as well as the water molecules and counter ions surrounding the remaining α subunit. The modified Na^+/K^+-ATPase after hydrogen saturation was minimized with the CHARMm force field[16] using the Discover Studio 2.0 package (http://accelrys.com/products/discovery-studio). The 2-D structures of ginsenosides used in this study were constructed by using the ChemDraw program, and their corresponding 3-D structures were converted by the Chem3D program (http://www.cambridgesoft.com). All of the ginsenosides were either 20(*S*)-protopanaxadiol or 20(*S*)-protopanaxatriol. The pocket for binding cardiac glycosides in the Na^+/K^+-ATPase α subunit was defined among the extracellular loops linking transmembrane segments as reported by Qiu *et al*[17,18], and the subunit-binding domain was defined as the region of the sphere with a 10 Å radius from the center of the binding pocket, which lies between I315 and L793 of the modified Na^+/K^+-ATPase. Docking of the ginsenosides was performed in silico by employing the LibDock module[19] in the Discover Studio 2.0 package. There were 100 hotspots identified in the binding pocket. The LibDock methodology effectively executed the docking of combinatorial libraries of compounds in a high-throughput manner while keeping the protein structure fixed[20]. With the energy threshold set as 20 kcal/mol, 76 conformations of Rg3, 118 conformations of Rh2, 26 conformations of PPD, and 25 conformations of PPT were generated for docking, respectively. The ginsenoside-Na^+/K^+-ATPase complexes were evaluated by LibDockscore[21]. This scoring resembles piecewise linear potential[22] by summing over interacting atoms in the specific ginsenoside-Na^+/K^+-ATPase complexes.

Results

Structural comparison between ginsenosides and ouabain Similar to cardiac glycosides, such as ouabain,

ginsenosides comprise a core steroid-like structure with sugars and other functional groups attached at variable positions (Figure 1). Protopanaxadiol ginsenosides, 1 of the 2 major ginsenoside groups, possess sugar moieties at the C-3 and C-20 positions, while protopanaxatriol ginsenosides, the other major group, possess a hydroxyl group at the C-3 position and sugar moieties at the C-6 and C-20 positions of the steroid-like structure. Among the 3 sugar attachment positions in ginsenosides, the C-3 position of the steroid-like structure is equivalent to the unique sugar attachment position of cardiac glycosides.

Inhibition of porcine Na⁺/K⁺-ATPase by ginsenosides

To verify whether ginsenosides and their aglycones exhibit a similar therapeutic effect via the same mechanism triggered by ouabain, that is, accentuating the force of myocardial contraction by elevating calcium concentration via the inhibition of Na⁺/K⁺-ATPase, a commercial Na⁺/K⁺-ATPase from the porcine cerebral cortex was used to evaluate the inhibitory potency of 7 protopanaxadiol ginsenosides and their aglycone PPD, 6 protopanaxatriol ginsenosides and their aglycone PPT, pseudoginsenoside F11, oleanolic acid, and ouabain. Among the protopanaxadiol ginsenosides, Rg3, Rh2, and PPD, which contain no sugar moieties at the C-20 position of the steroid-like structure, showed signifi-
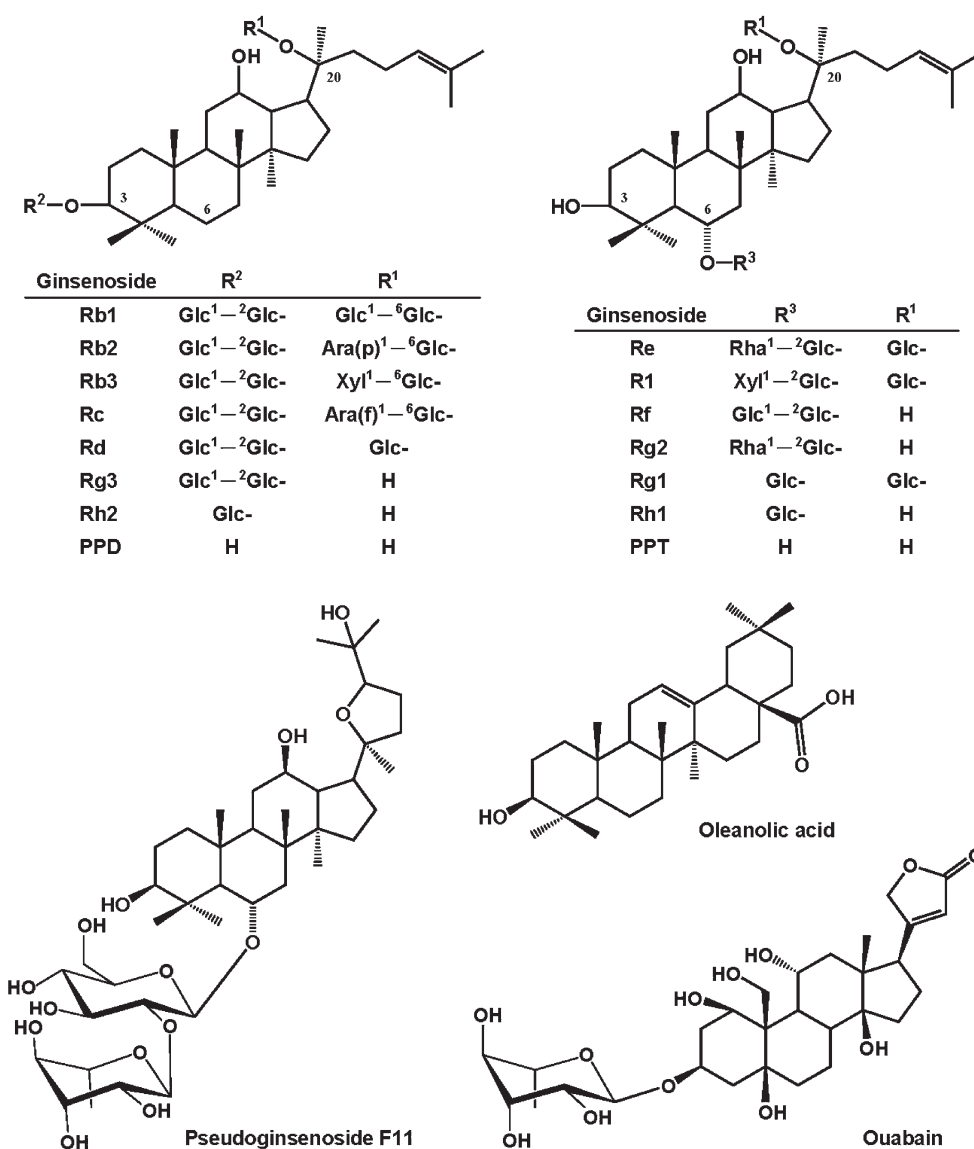

Ginsenoside	R^2	R^1
Rb1	Glc^1—^{2}Glc-	Glc^1—^{6}Glc-
Rb2	Glc^1—^{2}Glc-	Ara(p)^1—^{6}Glc-
Rb3	Glc^1—^{2}Glc-	Xyl^1—^{6}Glc-
Rc	Glc^1—^{2}Glc-	Ara(f)^1—^{6}Glc-
Rd	Glc^1—^{2}Glc-	Glc-
Rg3	Glc^1—^{2}Glc-	H
Rh2	Glc-	H
PPD	H	H

Ginsenoside	R^3	R^1
Re	Rha^1—^{2}Glc-	Glc-
R1	Xyl^1—^{2}Glc-	Glc-
Rf	Glc^1—^{2}Glc-	H
Rg2	Rha^1—^{2}Glc-	H
Rg1	Glc-	Glc-
Rh1	Glc-	H
PPT	H	H

Figure 1. Chemical structures of 7 protopanaxadiol ginsenosides and their aglycone PPD, 6 protopanaxatriol ginsenosides and their aglycone PPT, pseudoginsenoside F11, oleanolic acid, and ouabain.

cant inhibition on Na^+/K^+-ATPase activity, regardless of the sugar attachment at the C-3 position; however, the inhibitory potency was mostly reduced (for ginsenoside Rd) or completely abolished (for ginsenosides, Rb1, Rb2, Rb3, and Rc) when monosaccharide or disaccharide was attached to the C-20 position (Figure 2A). All of the 6 protopanaxatriol ginsenosides (Re, R1, Rf, Rg1, Rg2, and Rh1) containing sugar moieties at the C-6 position of the steroid-like structure did not inhibit Na^+/K^+-ATPase, while PPT, which does not con-

tain sugar moieties at the C-6 and C-20 positions, possessed considerable inhibitory potency (Figure 2B). Similar to protopanaxatriol ginsenosides, pseudoginsenoside F11, which contains a disaccharide attached at the C-6 position, did not inhibit Na^+/K^+-ATPase, while oleanolic acid, which does not contain sugar, possessed significant inhibitory potency (Figure 2C). Under our experimental conditions, the inhibitory potency of all ginsenosides on porcine Na^+/K^+-ATPase was evidently lower than that of ouabain; the IC_{50} of ouabain (0.45 μmol/L) was approximately 120 times lower than that of ginsenoside Rh2 (55 μmol/L), the strongest inhibitor of Na^+/K^+-ATPase among the ginsenosides examined in this study (Figure 3).
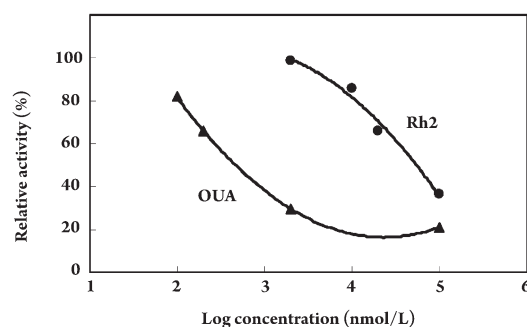

Figure 3. Inhibitory potency of ouabain and ginsenoside Rh2 on porcine Na^+/K^+-ATPase. Inhibitory potency of various concentrations of ouabain and ginsenoside Rh2 was observed as the reduction of Pi liberation released from ATP by a constant amount of commercial porcine Na^+/K^+-ATPase.

Molecular modeling and docking of ginsenosides to Na^+/K^+-ATPase To reveal the observed difference in Na^+/K^+-ATPase inhibition among ginsenosides at the molecular level, representative ginsenosides, Rg2, Rg3, Rd, Rb1, PPD, PPT, and Rh1 were subjected to molecular modeling and docking to the extracellular domain of the α subunit of Na^+/K^+-ATPase. In accordance with the results of the Na^+/K^+-ATPase inhibitory assay for these representative ginsenosides, only PPD, Rh2, and Rg3, which contained 0, 1, and 2 sugar units at the C-3 position, but no sugar attached at the C-6 and C-20 positions, could dock into the extracellular pocket of the Na^+/K^+-ATPase α subunit (Figure 4). The core steroid-like structure of PPD, Rh2, or Rg3 was trapped in a cave (shown in green with dimensional limitations of 10.5 and 9.2 Å for the top and bottom areas, respectively) of the extracellular pocket. The protrusive alkyl side chain attached at the C-20 position of the steroid-like structure was located in a narrow space deeper than the cave. In comparison with PPD, the extra monosaccharide and disaccharide of Rh2 and
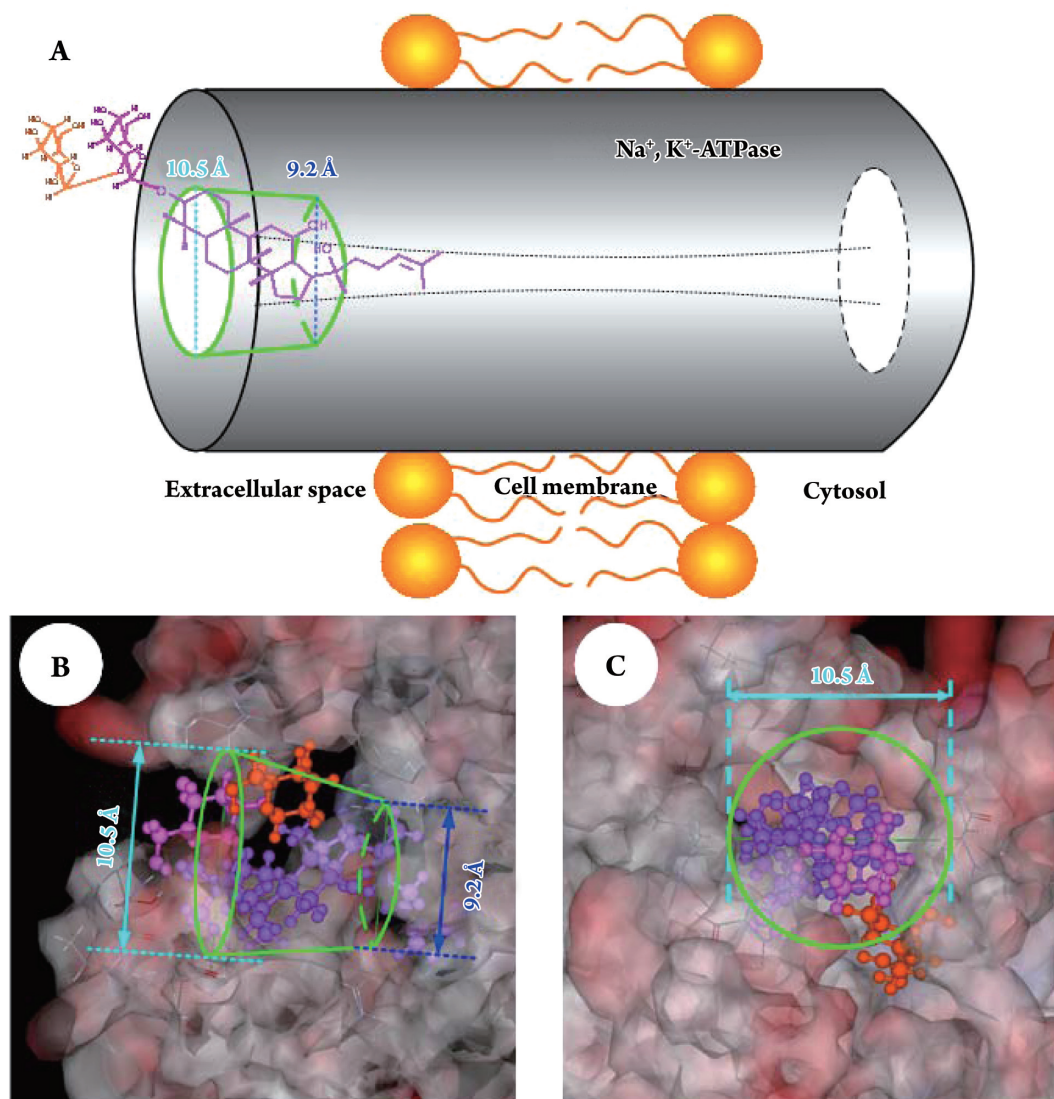

Figure 2. Inhibition of porcine Na^+/K^+-ATPase by ginsenosides. Inhibitory potency of (A) 7 protopanaxadiol ginsenosides and their aglycone PPD, (B) 6 protopanaxatriol ginsenosides and their aglycone PPT, and (C) pseudoginsenoside F11 (PF11), oleanolic acid (OA), and ouabain (OUA) were observed as the reduction of Pi liberation released from ATP by a constant amount of commercial porcine Na^+/K^+-ATPase. Data represent mean±SEM of 5 replicates. $^bP<0.05$, $^cP<0.01$ *vs* control group (CON; deionized water only).

Figure 4. Modeling of ginsenosides binding to the extracellular pocket of the Na^+/K^+-ATPase α subunit. (A) core steroid-like structure of ginsenoside Rg3 was trapped in a cave (shown in green with dimensional limitations of 10.5 and 9.2 Å for the top and bottom areas, respectively) of the extracellular pocket of the Na^+/K^+-ATPase α subunit. Similar structural interaction was observed for ginsenoside Rh2 (Rg3 without the orange glucose molecule) and PPD (Rg3 without the orange and pink glucose molecules). (B) molecular interaction between ginsenoside Rg3 and the extracellular binding pocket of the Na^+/K^+-ATPase α subunit. Ginsenoside Rg3 and the binding pocket of Na^+/K^+-ATPase were displayed in ball-and-stick and space-fill modes, respectively. Left-side view (C) of the extracellular space facing the binding pocket of Na^+/K^+-ATPase (B).

Rg3 at the C-3 position of the steroid-like structure were located outside of the cave and were freely exposed to the extracellular space.

Detailed molecular interactions between Rg3, Rh2, PPD, or PPT and the extracellular pocket of Na^+/K^+-ATPase are shown in Figure 5. Three hydrogen bonds were found between the sugar moieties at the C-3 position of Rg3 or Rh2 and Na^+/K^+-ATPase (Figure 5A, 5B). Among these hydrogen bond interactions, R880 of Na^+/K^+-ATPase was involved

in 2 hydrogen bonds with the sugar moieties at the C-3 position of Rh2. Presumably, the interaction between the sugar moieties at the C-3 position of ginsenosides was beneficial for their binding to the extracellular pocket of Na^+/K^+-ATPase. Only 1 hydrogen bond was observed between the hydroxyl group at the C-20 position of PPD and V798 of Na^+/K^+-ATPase (Figure 5C). Two hydrogen bonds were observed between PPT and Na^+/K^+-ATPase; one was between the hydroxyl group at the C-3 position of PPT and N790 of
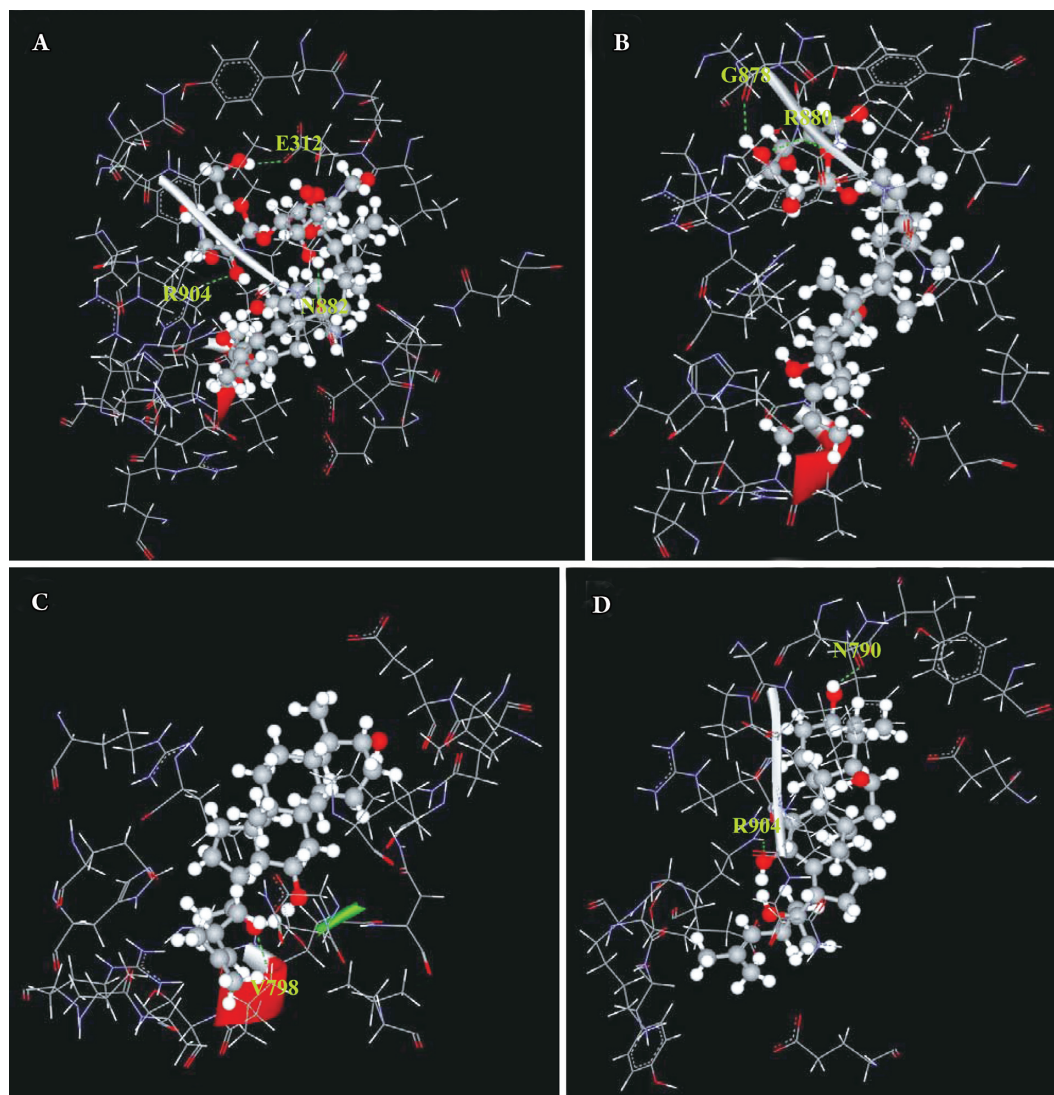
Figure 5. Detailed molecular interactions between the extracellular pocket of Na+/K+-ATPase and (A) Rg3, (B) Rh2, (C) PPD, and (D) PPT. Amino acid residues of Na+/K+-ATPase close to ginsenosides are shown in the wire frame, and the structures of ginsenosides are shown in the scaled ball and stick. Green dashed lines represent the hydrogen bonds between ginsenosides and Na+/K+-ATPase. Amino acids involved in hydrogen bonding to ginsenosides are in green.

Na+/K+-ATPase, and the other between the hydroxyl group at the C-12 position of PPT and R904 of Na+/K+-ATPase (Figure 5D). Our observation of hydrogen bond interactions between Arg and ginsenosides was in accordance with that between Arg in the H7–H8 loop of human Na+/K+-ATPase and cardiac glycosides[23]. The interaction between ginsenosides and the extracellular pocket of Na+/K+-ATPase putatively blocked the entrance of ions rather than impeded the active site of Na+/K+-ATPase located in the intracellular region. LibDockscores of the Rg3, Rh2, PPD, and PPT were 145.069, 148.118, 100.344, and 103.058, respectively. The

docking score results were consistent with the observations in the inhibitory assay.

To rationalize the steric hindrance of sugar attachment at the C-6 or C-20 position of the steroid-like structure, the molecular fitness of PPD, PPT, and Rh1 or Rg3, Rd, and Rb1 in the cave of the extracellular pocket of the Na+/K+-ATPase α subunit was depicted and compared (Figure 6). The altered dimension of ginsenoside increased from 6.1 Å (PPD) to 7.1 Å (PPT) or 11.8 Å (Rh1) when a hydroxyl group or glucose was attached to the C-6 position of the steroid-like structure (Figure 6, left). Since the dimensional
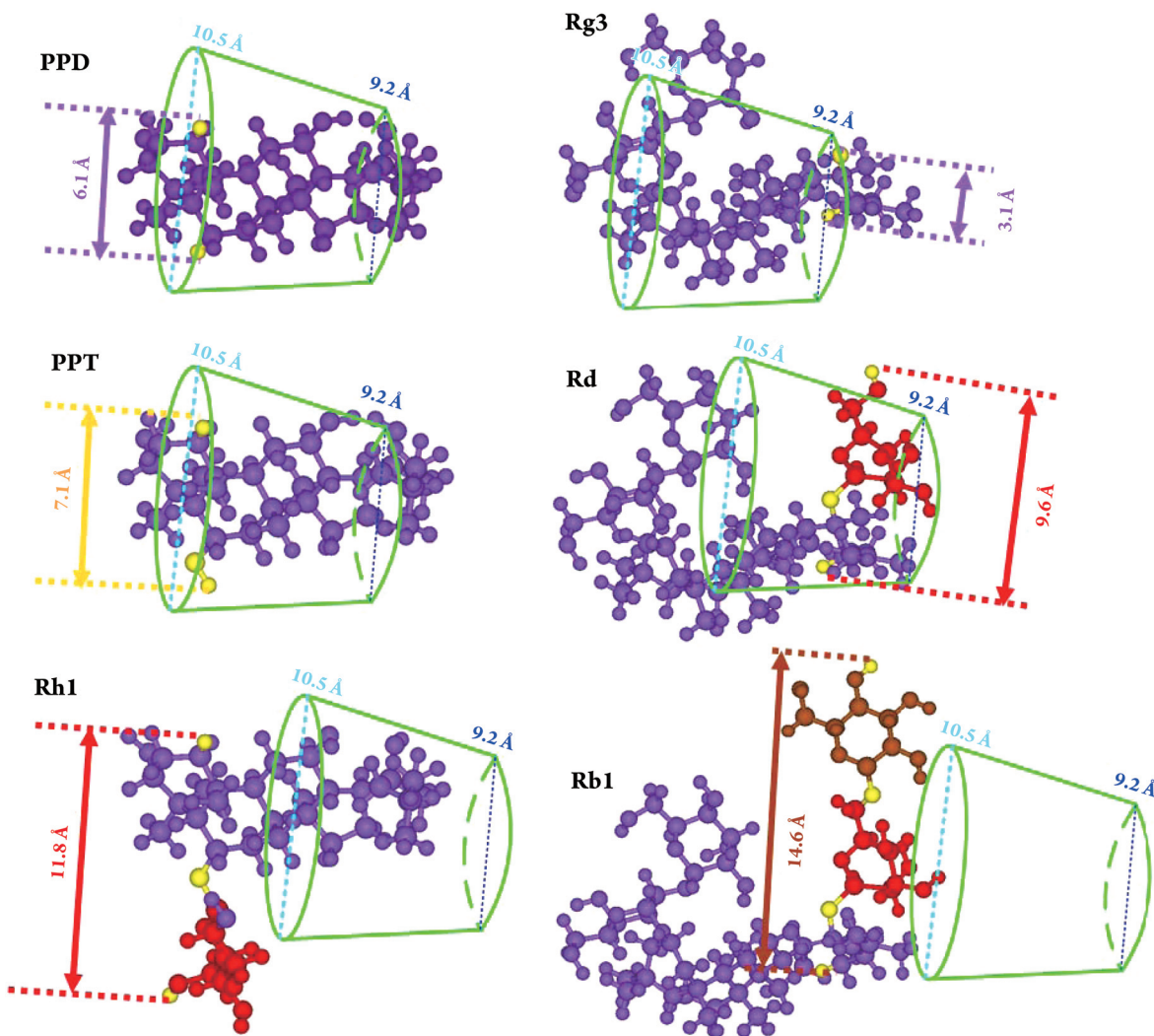
Figure 6. Molecular fitness of representative ginsenosides in the cave of the extracellular binding pocket of the Na$^+$/K$^+$-ATPase α subunit. Green cave of dimensional limitations for ginsenoside fitness was the same as that shown in Figure 4. Red and brown colors show the glucose molecules attached to the C-6 and C-20 positions of the steroid-like structure. Yellow balls indicate the extended dimensions of ginsenosides after hydroxylation (PPT) or glycosylation (Rh1, Rd, and Rb1) at the C-6 or C-20 positions.

limitations of the extracellular cave of the Na$^+$/K$^+$-ATPase α subunit were 10.5 and 9.2 Å, both PPD and PPT, but not Rh1, could fit in the cave. Therefore, PPD and PPT, but not Rh1, possessed inhibitory potency on Na$^+$/K$^+$-ATPase. The altered dimension of ginsenoside increased from 3.1 Å (Rg3) to 9.6 Å (Rd) or 14.6 Å (Rb1) when monosaccharide or disaccharide was attached to the C-20 position of the steroid-like structure (Figure 6, right). Consequently, Rg3, but not Rb1, could fit in the cave and possessed inhibitory potency on Na$^+$/K$^+$-ATPase. Rd (9.6 Å) could pass through the top (10.5 Å), but could not reach the bottom (9.2 Å) of the cave, and thus possessed subtle inhibitory potency on Na$^+$/K$^+$-

ATPase.

Discussion

Based on experimental observation and theoretical modeling, we found that ginsenosides with sugar moieties attached only to the C-3 position of the steroid-like structure, equivalent to the sugar position in cardiac glycosides, and possessed inhibitory potency on Na$^+$/K$^+$-ATPase activity. Sugar attachment to the C-6 or C-20 position of the steroid-like structure presumably caused steric hindrance for the entrance of ginsenosides into the extracellular binding pocket

of the Na⁺/K⁺-ATPase α subunit, and thus significantly reduced or completely abolished their inhibitory potency. Paradoxically, most ginsenosides found in ginseng and sanqi have not appeared to be competent inhibitors for Na⁺/K⁺-ATPase due to their sugar attachment to the C-6 or C-20 position of the steroid-like structure. Nevertheless, ginsenosides might act as prodrugs, as they tend to be metabolized to their active forms by intestinal bacterial deglycosylation after oral administration[8]. Commonly, the metabolites could be easily absorbed by the intestines due to the increase of hydrophobicity after deglycosylation, and might display the same or different pharmacological actions in comparison with their parent compounds[24,25]. While our experimental observation and theoretical modeling were executed using porcine Na⁺/K⁺-ATPase, the observed inhibitory potency of ginsenosides was presumably applicable to human Na⁺/K⁺-ATPase, since isoforms of this enzyme in diverse species were highly conserved evolutionarily. In fact, the amino acid sequence identity between porcine and the human α1 subunit of Na⁺/K⁺-ATPase was found to be up to 98%. In view of the results observed in the current study and those reported in the available literature, we propose that the cardiac therapeutic effects of ginseng and sanqi should be at least partly attributed to the effective inhibition of Na⁺/K⁺-ATPase by their metabolized ginsenosides, with sugar moieties attached only to the C-3 position of the steroid-like structure.

Ginsenosides are also demonstrated to be the pharmacologically-active ingredients responsible for the effects of ginseng on the central and peripheral nervous systems[7]. They have been reported to possess reversible and selective inhibitory effects on voltage-dependent ion channels (such as the Ca²⁺, K⁺, and Na⁺ channels) and ligand-gated ion channels (such as *N*-methyl-*D*-aspartate, some subtypes of nicotinic acetylcholine, and 5-hydroxytryptamine type 3 receptors), although little is known about the exact mechanisms. As the inhibition of Na⁺/K⁺-ATPase also leads to the fluctuation of Ca²⁺, K⁺, and Na⁺ concentrations, it will be interesting to see if any cross-talks are held among these ion channels after being inhibited by ginsenosides, and as a result, exhibit the pharmacological actions of ginseng and sanqi.

Cardiac glycosides have been demonstrated to provide neuroprotection against ischemic stroke in a cortical brain slice-based compound screening platform[26]. Neuroprotective activity and delayed therapeutic potential were observed for cardiac glycosides in this brain slice assay model. Recently, we demonstrated that magnesium lithospermate B, a derivative of caffeic acid tetramer, present as the major soluble ingredient in danshen (the dried roots of medicinal plant *Salvia miltiorrhiza*), possessed a cardiac therapeutic activity by

its effective inhibition on Na⁺/K⁺-ATPase and exhibited the same neuroprotective effect against ischemic stroke in a similar brain slice assay model[27]. Similar to cardiac glycosides and magnesium lithospermate B, we found that ginsenosides possessed inhibitory potency on Na⁺/K⁺-ATPase in this study. In previously published studies, similar neuroprotective effects were reported for ginsenosides against ischemic stroke, and some of the results were observed under the same brain slice assay model[28–32]. It remains to be investigated whether ginsenosides, as well as cardiac glycosides and magnesium lithospermate B, exert neuroprotection against ischemic stroke via the same mechanism triggered by the inhibition of Na⁺/K⁺-ATPase.

Author contribution

Jason TC TZEN designed research; Ronald JY CHEN and Tse-yu CHUNG performed research; Feng-yin LI and Nan-hei LIN contributed new analytical tools and reagents; Jason TC TZEN wrote the paper.

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