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Spectroscopic and molecular dynamics characterization of glycyrrhizin membrane-modifying activity

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ABSTRACT

Glycyrrhizic acid (GA) is a triterpene glycoside extracted from licorice root. Due to its amphiphilicity GA is capable of forming complexes with a variety of hydrophobic molecules, substantially increasing their solubility. GA can enhance the therapeutic effects of various drugs. It was hypothesized that the increased bioavailability of the drug by GA is not only due to increased solubility, but also to enhancement of drug permeability through cell membranes. In this study the interaction of GA with POPC liposomes and model DOPC, POPC and DPPC bilayers was investigated by NMR with addition of shift reagents and MD simulations. This work helps to better understand the mechanism of enhanced drug bioavailability in the presence of GA. NMR and MD reveal that GA does penetrate into the lipid bilayer. NMR shows that GA changes the mobility of lipids. GA is predominantly located in the outer "half-layer" of the liposome and that the middle of the hydrophobic tails is the preferred location. GA freely passes through the bilayer surface to the inner part bringing a few water molecules. Also both approaches indicate pore formation in the presence of GA. The GA interaction with membranes is an additional aspect of the biological activity of GA-based drug delivery systems.

1. Introduction

A significant increase has occurred over the past few years in the number of publications on physicochemical studies of supramolecular complexes of drugs [1–6]. The main purpose of such supramolecular systems is to increase the solubility and bioavailability of poorly soluble drugs. A hydrophobic drug molecule is able to pass through the hydrophobic interior of a lipid bilayer by passive transport. This requires a relatively high extracellular concentration of the drug to overcome any concentration gradient across the cell membrane, which is often problematic due to low drug solubility (about 40% of drug compounds are classified as practically insoluble). The fact that about 85% of the most popular drugs are taken orally makes supramolecular drug delivery highly significant.

An example of such a carrier (or drug delivery system) is glycyrrhizic acid (glycyrrhizin, GA, Fig. 1). GA is a triterpenoid saponin from an extract of licorice root. It has long been used to treat and prevent various diseases from the common cold to stomach and duodenal ulcers. It has pronounced anti-inflammatory activity and according to recent studies it is able to induce apoptosis in cancer cells [7–12]. Inaddition, GA affects the biosynthesis, properties and blood level of cholesterol [13,14].

Apart from its intrinsic biological activity GA forms inclusion complexes with a variety of drugs due to its amphiphilicity (see Fig. 1). Such supramolecular complexes could increase the solubility of hydrophobic compounds up to dozens of times [15–21]. It was suggested that the enhancement of drug bioavailability and other effects in the presence of GA is related to complex formation. Physicochemical and pharmaceutical studies of such complexes examined the contribution of complexation with GA to the enhancement of therapeutic effects. The formation of inclusion "host-guest" complexes with GA was confirmed by various physicochemical methods (in particular, optical and magnetic resonance spectroscopy) and the stability of such complexes in most cases turned out to be two orders of magnitude higher than the corresponding values for inclusion complexes of cyclodextrins [7,22]. This allows substantially lower concentrations of complexants for drug delivery. A number of animal studies have shown

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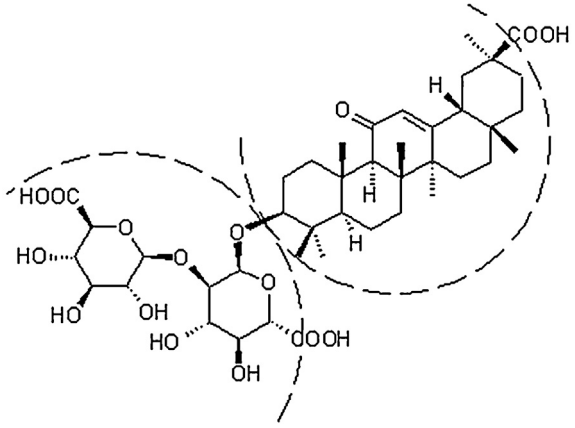

Fig. 1. The structure of glycyrrhizic acid.

that phenibut (nootropic and anxiolytic drug) complexes with GA have a similar effect at a 16-fold lower dose [15]. Furthermore, glycyrrhizin increases animals mnemonic ability and reduces phenibut side effects such as drowsiness and allergic reactions. Toxicity is reduced 1.7-fold, and the therapeutic index is increased 17-fold [15]. Similar effects were observed for GA complexed with other classes of drugs. For example, the nifedipine complex with GA has antihypertensive activity at a 10-fold lower dose [7,15,20]. In vivo and ex vivo studies show that glycyrrhizin inhibits angiogenesis in vascular endothelial cells [23]. Glycyrrhizin delivery of doxorubicin (an anthracycline antibiotic having antitumor activity) to liver cells shows some evidence of efficacy [24]. GA is capable of self-association in aqueous and water-alcohol solutions due to its amphiphilic properties. The structure of such aggregates depends on the GA concentration and the solution pH [25–30]. GA exists mainly as a dimer at low concentrations (10^{-5}–10^{-3} M), while at high concentrations (>10^{-3} M) it forms large micelle-like aggregates [25,31].

These examples show that GA may have promise for drug delivery. A safe GA dose of 2 mg/kg for daily use was proposed from clinical studies, suggesting an acceptable daily intake of 0.2 mg/kg body weight with a safety factor of 10. This means consumption of 12 mg glycyrrhizic acid/day for a person with a body weight of 60 kg [32]. Increased drug solubility in the presence of GA accompanies a significant decrease in the therapeutic dose of the drug; decreased toxicity and side effects; and in some cases, changes in the mechanism of action of the drug in the supramolecular complex. So we can anticipate safe use of GA to deliver low-dose drugs and in some cases, even high-dose drugs. However, the exact mechanism of action for GA as a drug delivery system is still unknown at the molecular and cellular level.

A number of physicochemical studies were carried out to better understand the effects of GA in living systems [33–35]. GA was found to affect functional membrane properties such as permeability and elasticity [33,34]. We suggested that increased drug bioavailability in supramolecular complexes of GA occurs not only from enhancement of the solubility but also from modification of the cell membrane properties. Further study of glycyrrhizin-membrane interaction is needed to elucidate the mechanism of glycyrrhizin action on cell membranes.

In the present study, the glycyrrhizin-membrane interaction was investigated using nuclear magnetic resonance (NMR) and molecular dynamics (MD) techniques. These two methods, although covering different motional time scales (the micro- to milliseconds range for NMR, the nano- to microseconds range for MD), provide largely complementary views of membrane structure and dynamics. NMR obtained structural and dynamic information on the different regions of the phospholipid bilayer by the use of shift reagents. On the other hand, MD simulations reveal other aspects of structure and dynamics that probe the effects of membrane-interacting molecules, such as GA, on the bilayer properties.

To elucidate the mechanism of GA action on cell membranes in this study, the mobility of the various functional groups of the lipid in bilayer was studied by dynamic NMR. Unilamellar liposomes were chosen as a model bilayer membrane. Liposomes are widely used in biophysics as a model of the cell membrane lipid core. Molecular dynamics simulation of the GA interaction with lipid bilayers provide additional evidence of the mechanism of GA action.

2. Materials and methods

2.1. Nuclear magnetic resonance

Liposomes were formed from 1-palmitoyl-2-oleoyl-sn-*glycero*-3-phosphocholine (POPC, Avanti Polar Lipids, purity >99%) and cholesterol (MP Biomedicals). Powder components were predissolved in chloroform and cholesterol (0.05 mM, 0.25 mM and 0.5 mM) was added, as appropriate, at this stage. After removing the solvent, the dry lipid film was hydrated with D_2O. The final concentration of lipid was 10 mM and the maximum cholesterol concentration was 5 mol% with respect to lipid. The suspension was then sonicated (about 37 kHz, 1 h) to obtain unilamellar liposomes. NMR spectra were recorded in 5-mm NMR tubes for 0.6 ml samples of vesicle suspension supplemented with 4 mM $PrCl_3$. GA was added to the liposome/$PrCl_3$ suspension to GA concentrations of 0.05 mM, 0.25 mM or 0.5 mM. The maximum GA concentration corresponds to 5 mol% relative to the lipid. ^{1}H NMR spectra were recorded on Bruker Avance III 500 MHz spectrometer, and the spin-spin relaxation time, T_2, was measured using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence.

Spin-spin relaxation times (T_2) are sensitive to molecular motions. The T_2 is sensitive to low frequency (e.g., large amplitude chain wagging) lipid motions [36,37]. The T_2 of different lipid functional groups (-$N^+(CH_3)_3$-group in the lipid polar head and terminal CH_3-groups in the hydrophobic tails) were measured to characterize the influence of GA. The T_2 is closely related to the mobility of a molecule and is inversely proportional to the rotational correlation time. Thus, using T_2 data, one can probe changes in the environment or state (free/bound) of molecules. The use of shift reagents to resolve lipid resonances from the inner and outer headgroup region and from terminal methyl groups allows us to study the effect of GA on lipid motions in the different parts of the membrane. The NMR measurements were made at 300 K and repeated three times. The arithmetic means of the NMR spectral parameters are reported.

2.2. Molecular dynamics

All simulations were performed using the GROMACS 5.0 molecular dynamics package [38]. Parameters sets and equilibrated fragments of lipid bilayers were taken from the database lipidbook.bioch.ox.ac.uk. We employed Berger's lipids model [39] for all the lipids used: DOPC, POPC and DPPC bilayers. Each model contain 128 molecules of lipid, oriented in the x-y plane, and about 5000–7000 SPC-water molecules [42] as well as one or more glycyrrhizin molecules. A time step of 2 fs and the leap-frog integration algorithm were used. All bonds were constrained with the LINCS algorithm [43]. A Verlet cut-off Scheme [37] was used for non-bonded interactions with rlist and rvdw parameters all equal to 1.2 nm. Long-range corrections for energy and pressure were applied. Electrostatic interactions were calculated using the particle-mesh Ewald method [44] with a real space cutoff of 1.2,