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In-vitro anti-proliferative and anti-oxidant activity of galangin, fisetin and quercetin: Role of localization and intermolecular interaction in model membrane

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ABSTRACT

Flavonols are an important class of naturally occurring molecules and are known for their pharmacological activity. The activity is associated with the ability of flavonols to influence membrane-dependent processes. We have investigated the *in-vitro* anti-proliferative and anti-oxidant activity of galangin (GLN), fisetin (FTN) and quercetin (QTN), which possess variable number of phenolic hydroxyl groups. An attempt has been made to correlate the biological activity of these molecules with their interaction and localization in dipalmitoyl phosphatidyl choline (DPPC) bilayers, using differential dcanning calorimetry (DSC) and nuclear magnetic resonance (NMR) methods. Results indicate that GLN interacts to the alkyl chains of the lipid bilayer involving hydrophobic interactions. FTN and QTN interact with head region and *sn-1*-glycero region involving hydrogen bonding. Ring current induced chemical shifts of lipid protons, due to intermolecular interaction indicate that GLN acquires a parallel orientation with respect to the bilayer normal whereas FTN and QTN resume a mixed orientation. The membrane binding constants of these molecules are in the order GLN > QTN > FTN. It has been shown that the number and position of hydroxyl groups in these molecules play an important role in membrane binding and thereby in biological activity.

1. Introduction

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is the most abundant bioflavonol found in common dietary sources and has been widely investigated for its pharmacological activities [1,2]. Lately, attention has been focused on other hydroxy analogues of quercetin (QTN), namely fisetin (3,7,3′,4′-tetrahydroxyflavone) and galangin (3,5,7-trihydroxyflavone). Fisetin (FTN) is abundantly found in fruits and vegetables [3]. It is emerging as a multifunctional drug with a broad spectrum of biological activities for its antioxidant, anti–inflammatory [4,5], *in-vitro* cytotoxic and apoptotic properties [6,7]. Fisetin has recently shown *in-vivo* anticancer activity in mouse lung carcinoma [8], human melanoma [9] and human prostrate tumors [10]. On the other hand, galangin (GTN) is known for its inhibitory action to iNOS mRNA expression and COX-2 transcription during the inflammatory response [11], suppression of bacterial cell growth [12] and inhibition of tumor growth and the cell cycle [13].

The effect of flavonols as bioactive compounds has been exclusively explained by their binding to and interference with enzymes, receptors, transporters and signal transduction systems [14]. Such events are known to occur in membrane environments and therefore, the bioactivity of flavonols may be attributed to their mode of interaction with cell membrane. Thus, their mode of binding and possible location in membrane is essential for a clear understanding of structure–activity relationship [15,16]. Moreover, membrane partitioning experiments on flavonols have shown that the antioxidant activity depends on their capacity to interact/permeate into the lipid bilayer [17]. To establish these aspects of structure–activity relationships, we have evaluated the mode of interaction of GLN, FTN and QTN (Fig. 1) with DPPC model membrane using DSC and multinuclear NMR techniques and correlated functional activity with respect to their anti-oxidant and anti-proliferative properties.

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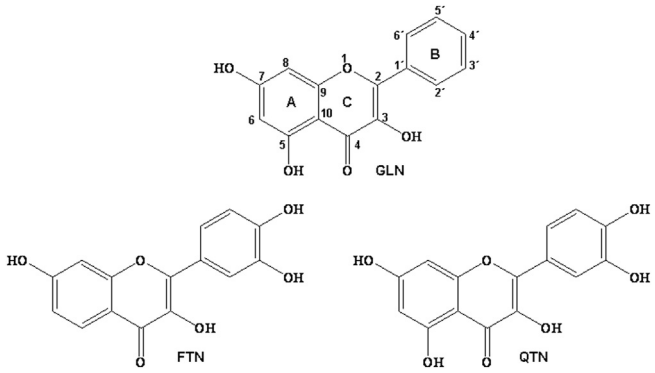

Fig. 1. Molecular structure of galangin (GLN), fisetin (FTN) and quercetin (QTN).

2. Materials and methods

2.1. Materials

Galangin (3,5,7-trihydroxyflavone), fisetin (3,7,3′,4′-tetrahydroxyflavone), quercetin (3,5,7,3′,4′-pentahydroxyflavone), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 2,2-diphenyl-1-picrylhydrozyl (DPPH) were purchased from Sigma chemicals Co. USA.

2.2. NMR and DSC experiments

NMR experiments were recorded on a BRUKER AVANCE 500 MHz NMR spectrometer. 2D-COSY and 2D-NOESY spectra were recorded using standard pulse programs [18,19], with a mixing time of 400 ms. ^{31}P and ^{13}C NMR experiments were carried out with a relaxation delay of 1 s using broadband proton decoupling. NMR software Topspin 2.0 was used for data processing. DSC measurements were performed on differential scanning calorimeter VP-DSC (Microcal, Northampton, MA, USA) using procedure used earlier [20]. The buffer and lipid samples were degassed under vacuum before being loaded into the reference and sample cells. A scan rate of 90 °C/hr was generally employed. Data were analyzed using the software ORIGIN provided by MicroCal. All the experiments were carried out in the temperature range 20 °C–60 °C.

Repeated scans for the same samples were generally superimposable. Data were analyzed with the software ORIGIN provided by Microcal.

2.3. Sample preparation

Multilamellar vesicles (MLV's) from DPPC were prepared using standard procedure [21]. The desired quantity of DPPC and flavonol was dissolved in chloroform. The solvent was evaporated with a stream of nitrogen so as to deposit a lipid film on the walls of the container. The last traces of the solvent were removed under vacuum. MLV's sample thus prepared was hydrated with the required amount of D_2O at pH 7.2, followed by incubation in water bath at 50 °C with repeated vortexing. The lipid concentration for NMR samples was maintained at 100 mM while the concentrations of the flavonols were varied from 10 to 50 mM. For DSC experiments, samples were prepared by mixing the lipid and flavonol solutions to obtain flavonol/lipid molar ratios from 1:20 to 1:2 by maintaining the lipid concentration to 50 mM. Unilamellar vesicles (ULVs) for NMR experiments were prepared by sonicating the lipid dispersions using a Branson Sonicator-450 at 50% duty cycles till optical clarity was obtained.

2.4. Determination of flavonol-MLV binding constants

Flavonol-MLV binding constants were determined by the centrifugation method [22]. MLVs were prepared by varying lipid concentration from 0.25 mg/ml to 2 mg/ml with fixed flavonol concentration of 100 μM. This corresponds to a flavonol:lipid molar ratios in the range 1:2.5 to 1:20. The resulting solutions were incubated for 2 h and subsequently transferred into ultracentrifuge tubes. Separation of liposomes from the aqueous phase was achieved by centrifugation at 40,000 rpm for 2 h. Optical density of 100 μM solution of the pure flavonol was measured at a wavelength range of 220–400 λ. The amount of flavonol bound to liposomes was determined from the difference in optical density measured for the pure flavonol and that of the supernatant. The flavonol-liposome apparent binding constant (k) has been analyzed using the double reciprocal plot of 1/(fraction bound) versus 1/[lipid concentration] which yields a straight line with a slope 1/k.

2.5. Determination of antioxidant activity by DPPH assay

Varying concentrations of each flavonol (0–200 μg/ml, 0.5 ml in methanol) was mixed with methanolic solution of DPPH (0.1 mM, 1.0 mL) and Tris-HCl buffer (0.1 M, pH 5.5, 1.0 mL) to make a total volume 2.5 mL. The absorbance of the sample was measured at 517 nm after 0.5 h of incubation [23]. The reaction solution without DPPH was used as a blank test. Measurements were performed in triplicate. Free radical scavenging activity of the flavonol was measured as the difference in absorbance between the test sample and the control (sample without flavonol). Concentration required for a 50% reduction (IC_{50} in μg/ml) of DPPH radical solution was determined graphically.

2.6. Determination of anti-proliferative activity

Anti–proliferative activity was evaluated by the Sulforhodamine B assay method [24]. Three different cell lines, viz. K562, HepG2 and MCF-7 were used. Cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM of L-glutamine. The test compounds were dissolved in dimethyl sulfoxide and diluted suitably before adding them to the culture medium. After incubation at standard conditions for 48 h, percent growth inhibition of cells has been calculated.

3. Results and discussion

3.1. Binding studies

Fig. 2 shows a plot of fraction of flavonols bound to MLVs with increasing concentration of lipid. In all cases, an increase in binding affinity is observed with increasing concentration of lipid. All the three flavonols show nearly 60%–70% binding even at a relatively low (0.5 mg/ml) concentration of lipid, and thereafter the binding curves acquire a plateau. Double inverse plot of fraction of flavonol bound vs. inverse of lipid concentration (inset Fig. 2) has been used to calculate the binding constants. The results indicate that these molecules bind to the MLVs with a variable degree of affinity, in the order GLN > QTN > FTN. The apparent binding constants measured are: GLN: 16,701 M^{-1}, FTN: 10,438 M^{-1} and QTN: 10,710 M^{-1}. It may be noted that the hydroxyl groups present on the ring A are responsible for overall enhanced binding. For example, FTN which is devoid of one hydroxyl group in ring A, shows least binding. Moreover, absence of two hydroxyl groups in ring B is likely to promote the binding as in the case of GLN which shows highest binding. Thus, the presence of hydroxyl groups in ring A in these molecules point to a possibility of interaction with the polar head