Red wine and green tea flavonoids are cis-allosteric activators and competitive inhibitors of glucose transporter 1 (GLUT1)-mediated sugar uptake

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The antioxidant- and flavonoid-rich contents of red wine and green tea are reported to offer protection against cancer, cardiovascular disease, and diabetes. Some studies, however, show that flavonoids inhibit GLUT1-mediated, facilitative glucose transport, raising the possibility that their interaction with GLUT1 and subsequent downstream effects on carbohydrate metabolism may also impact health. The present study explores the structure–function relationships of flavonoid–GLUT1 interactions. We find that low concentrations of flavonoids act as cis-allosteric activators of sugar uptake, whereas higher concentrations competitively inhibit sugar uptake and noncompetitively inhibit sugar exit. Studies with heterologously expressed human GLUT1, -3, or -4 reveal that quercetin–GLUT1 and -GLUT4 interactions are stronger than quercetin–GLUT3 interactions, that epicatechin gallate (ECG) is more selective for GLUT1, and that epigallocatechin gallate (EGCG) is less GLUT isoform–selective. Docking studies suggest that only one flavonoid can bind to GLUT1 at any instant, but sugar transport and ligand-binding studies indicate that human erythrocyte GLUT1 can bind at least two flavonoid molecules simultaneously. Quercetin and EGCG are each characterized by positive, cooperative binding, whereas ECG shows negative cooperative binding. These findings support recent studies suggesting that GLUT1 forms an oligomeric complex of interacting, allosteric, alternating access transporters. We discuss how modulation of facilitative glucose transporters could contribute to the protective actions of the flavonoids against diabetes and Alzheimer’s disease.

Moderate consumption of red wine or green tea is associated with protection against cancer, cardiovascular disease, and diabetes (1–4). These benefits are hypothesized to result from the flavonoid-rich, antioxidant capacity of the beverages (5). The present study investigates an alternative pathway by which the flavonoids may impact organismal health: direct interaction with the facilitative glucose transporters GLUT1, GLUT3, and GLUT4, leading to downstream effects on carbohydrate metabolism.

The flavonoids are a large group of polyphenolic secondary metabolites with over 4,000 types identified in fruits, flowers, vegetables, and leaves (5, 6). The general structure of flavonoids is a flavan backbone (Fig. 1A) consisting of two benzene rings linked together by a heterocyclic pyran ring (7). Substitutions in the flavan structure by hydroxyls, methyl groups, and sugars create the wide range of flavonoid derivatives (5). Quercetin, epigallocatechin gallate (EGCG), and epicatechin gallate (ECG) are present in red wine and green tea and are among the most extensively characterized flavonoids. Each cup of green tea is estimated to contain up to 300 mg of EGCG, 49 mg of ECG, and 14 mg of quercetin (8–10). Red wine contains 4–16 mg/liter quercetin (11, 12), but whereas EGCG and ECG are known to be present in red wine (8), their levels are not well-defined. The health benefits of the flavonoids have been attributed to their strong antioxidant capacity. The flavonoids chelate metal ions, such as Fe³⁺, and trap reactive species, including singlet oxygen, superoxide radicals, nitric oxide, and peroxynitrite (5, 13, 14).

Some studies, however, show that quercetin, EGCG, and ECG inhibit GLUT1-mediated facilitative glucose transport (15, 16), raising the possibility that it is their interaction with GLUT1 and their downstream effects on carbohydrate metabolism that impact health. Whereas this may explain their actions in cancer, where GLUT1 expression and nonoxidative glucose metabolism are up-regulated (17), it is harder to understand how sugar transport inhibition would ameliorate diabetes and cardiovascular disease.

Recent studies have suggested that GLUT1 forms an oligomeric complex of interacting, allosteric, alternating access transporters (18, 19) and that low concentrations of GLUT1 inhibitors acting at exofacial or endofacial sugar binding sites stimulate sugar transport (18, 20). The present study explores the detailed structure–function relationships of flavonoid–GLUT1 interactions to elucidate flavonoid action on cellular function. We find that the flavonoids act as heterotropic, cis-allosteric activators of sugar uptake at low concentrations and as competitive inhibitors of sugar uptake at higher concentrations. Whereas some health ben-
Flavonoids are exofacial GLUT1 ligands

A

B

C

D

E

Figure 1. Chemical structures and dose-dependent inhibition of human erythrocyte zero-trans 3MG (0.1 mM) uptake by quercetin, EGCG, and ECG. A, the flavan skeleton for over 4,000 identified flavonoids, including quercetin (B), EGCG (C), and ECG (D). E, ordinate: 3MG uptake in mmol/liter cell water/min. Abscissa, [inhibitor] in μM. Results are shown for quercetin (●), EGCG (○), and ECG (▲). Each point represents the mean ± S.E. (error bars) of at least three duplicate measurements. The curves were computed by nonlinear regression, assuming that uptake inhibition is described by Equation 1 with the following results: quercetin-treated cells (●): $K_{i(app)} = 1.88 ± 0.33 \mu M, R^2 = 0.92$, S.E. of regression = 0.01 mmol/liter cell water/min; EGCG-treated cells (○): $K_{i(app)} = 9.63 ± 1.95 \mu M, R^2 = 0.87$, S.E. of regression = 0.01 mmol/liter cell water/min; ECG-treated cells (▲): $K_{i(app)} = 1.90 ± 0.32 \mu M, R^2 = 0.93$, S.E. of regression = 0.01 mmol/liter cell water/min.

Results

Dietary flavonoids are reported to impair cellular sugar transport to varying degrees in different cell types (16, 21–25). We therefore examined the effects of quercetin, EGCG, and ECG (Fig. 1, B–D) on GLUT1-mediated, zero-trans uptake of 3-O-methylglucose (3MG; a transported but nonmetabolized sugar) in human red blood cells. Quercetin, EGCG, and ECG inhibit uptake of 0.1 mM 3MG in a dose-dependent manner, with $K_{i(app)} = 1.88 ± 0.33, 9.63 ± 1.95$, and 1.90 ± 0.32 μM respectively (Fig. 1E).

We determined the sidedness of the action of flavonoids on GLUT1 by examining their effects on two modes of red cell sugar transport: 1) zero-trans 3MG uptake (influx into sugar-free cells) and 2) zero-trans 3MG exit (efflux from sugar-loaded cells into medium lacking sugar). Transport theory informs us (26, 27) that a ligand competing with sugar for binding at the exofacial sugar binding site serves as a competitive inhibitor of sugar uptake and as a noncompetitive inhibitor of sugar exit. Conversely, a ligand competing with sugar for binding at the endofacial sugar binding site serves as a noncompetitive inhibitor of sugar uptake and as a competitive inhibitor of exit.

The effects of the flavonoids on the concentration dependence of initial rates of 3MG uptake are shown in Fig. 2A. Using...
The data of Fig. 2A were analyzed by nonlinear regression analysis assuming Michaelis–Menten kinetics (Equation 2) or by Lineweaver–Burk analysis, which also assumes Michaelis–Menten kinetics (Fig. 2A, inset). The resulting parameters ($V_{\text{max}}$ and $K_{m(\text{app})}$ for 3MG uptake) are shown as mean ± S.D. of the analysis. The fit statistics ($S_yx$ and $R^2$) are also shown.

| Table 1 Analysis of flavonoid inhibition of 3MG uptake |
|----------------|----------------|----------------|----------------|
| Nonlinear regression analysis using Michaelis–Menten equation | $V_{\text{max}}$ (mM/min) | $K_{m(\text{app})}$ (mM) | $S_yx$ (mM/min) | $R^2$ |
| Control | 1.20 ± 0.08 | 2.39 ± 0.36 | 0.07 | 0.93 |
| Quercetin | 1.98 ± 0.66 | 11.07 ± 5.03 | 0.07 | 0.89 |
| EGC | 2.14 ± 0.39 | 10.64 ± 2.63 | 0.05 | 0.97 |
| ECG | 1.72 ± 0.29 | 7.14 ± 1.85 | 0.07 | 0.94 |

| Lineweaver–Burk analysis | $V_{\text{max}}$ (mM/min) | $K_{m(\text{app})}$ (mM) | $S_yx$ (mM/min) | $R^2$ |
| Control | 0.95 ± 0.10 | 1.49 ± 0.24 | 0.06 | 1.00 |
| Quercetin | 0.68 ± 0.13 | 2.50 ± 0.04 | 0.46 | 0.99 |
| EGC | 1.49 ± 0.17 | 3.59 ± 0.06 | 0.32 | 1.00 |
| ECG | 0.83 ± 0.13 | 2.51 ± 0.04 | 0.21 | 1.00 |

The extra sum of squares F-test (28) to test the null hypothesis that 3MG uptake is described equally well by nonsaturable sugar uptake (uptake = $k \times [S]$), by Michaelis–Menten uptake (Equation 2), and by Michaelis–Menten uptake plus nonsaturable uptake fails for all conditions. All uptakes are best described by simple Michaelis–Menten kinetics. Analysis of 3MG uptakes by nonlinear regression analysis using Equation 2 reveals that the flavonoids increase $K_{m(\text{app})}$ for sugar uptake from 2.39 ± 0.36 to 11.07 ± 5.03 mM (quercetin), 10.64 ± 2.63 mM (EGCG), and 7.14 ± 2.63 mM (ECG), without significantly affecting $V_{\text{max}}$ for uptake (1.202 ± 0.082 mmol/liter cell water/min; Fig. 2A; Table 1). Lineweaver–Burk analysis results in lower estimates of $V_{\text{max}}$ and $K_{m(\text{app})}$ (Fig. 2A (inset) and Table 1), but the same conclusion ($V_{\text{max}}$ is unchanged but $K_{m(\text{app})}$ is increased). This suggests that quercetin, EGCG, and ECG inhibit GLUT1-mediated sugar uptake by binding at the exofacial 3MG binding site or at a site whose occupancy is mutually exclusive with 3MG occupancy of the exofacial sugar binding site. Consistent with this idea, the flavonoids act as noncompetitive inhibitors of net 3MG exit; they are without effect on $K_{m(\text{app})}$ (12.9 mM) for exit, but they decrease $V_{\text{max}}$ for exit by more than 2-fold from 2.03 mmol/liter cell water/min to 0.89 (quercetin), 0.72 (EGCG), and 0.60 mmol/liter cell water/min (ECG; Fig. 2B).

We have previously shown that low concentrations of exofacial inhibitors (e.g. WZB117 and maltose) or endofacial inhibitors (e.g. cytochalasin B) of GLUT1-mediated sugar transport modestly stimulate red cell sugar uptake (20, 29, 30). Consistent with these reports, quercetin ($\leq 0.5 \mu M$), EGCG ($\leq 2.5 \mu M$), and ECG ($\leq 0.5 \mu M$) reproducibly stimulate erythrocyte zero-trans 3MG uptake by up to 35% ($p < 0.05$; Fig. 3 and Table 2). These results suggest that GLUT1 presents at least two exofacial flavonoid binding sites: one that stimulates sugar uptake and a second site that inhibits uptake.

CB is a membrane-permeant GLUT1 inhibitor that binds at or close to the endofacial glucose-binding site (26, 27). Extracellular maltose, but not glucose, inhibits equilibrium binding of the CB to the endofacial glucose-binding site of GLUT1 (29, 31, 32). We therefore asked whether the flavonoids, which appear to act as exofacial ligands, also interfere with GLUT1 equilibrium [3H]CB binding. Quercetin, EGCG, ECG, and non-radioactive CB inhibit [3H]CB binding to GLUT1 with $K_{i(\text{app})}$ (assuming simple inhibition (33)) of 0.637 ± 0.071, 6.097 ± 0.726, 0.871 ± 0.103, and 0.055 ± 0.003 μM, respectively (Fig. 4A). Whereas these curve fits produce good correlation coefficients ($R^2 > 0.91$ in all cases), the S.D. of the residuals of each fit ($p > 0.09$; excluding CB) is greater than 20% of the S.D. of the y values, suggesting that the fits are poor.

Closer examination reveals that inhibition by quercetin and EGCG increases more steeply, whereas inhibition produced by ECG increases less steeply than is expected for simple Michaelis–Menten inhibition. Inhibition of radiolabeled CB binding by unlabeled CB is well-described by simple Michaelis–Menten inhibition. We therefore asked if quercetin, EGCG, and ECG inhibitions of CB binding are better approximated by inhibition involving multiple cooperative ligand-binding sites and applied a simple Hill-type model (33) to analyze these results (Fig. 4B). This analysis produces fits with residuals that do not deviate significantly from zero, and the S.D. of the resid-
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Table 2

| Inhibitor | UTU | GLUT1 | GLUT3 | GLUT4 | RBC-Glut1 |
|-----------|-----|-------|-------|-------|-----------|
| Quercetin | IC₅₀ (µM) | 9.59 ± 1.42 | 2.00 ± 0.99 | 17.68 ± 1.71 | 1.70 ± 0.25 | 1.88 ± 0.33 |
| Const₁ | (m⁻¹) | NA | (4.69 ± 1.92) x 10⁻⁵ | 0.47 ± 0.02 | (7.59 ± 3.75) x 10⁻⁸ | (4.69 ± 1.92) x 10⁻⁵ |
| Const₂ | (m⁻¹) | NA | (2.59 ± 0.05) x 10⁸ | (5.96 ± 0.55) x 10⁸ | (5.95 ± 6.12) x 10⁻⁸ | (2.59 ± 0.06) x 10⁸ |
| Const₃ | (m⁻¹) | NA | (1.79 ± 0.04) x 10⁹ | (2.77 ± 0.16) x 10⁹ | (2.37 ± 0.61) x 10⁻¹⁴ | (1.79 ± 0.04) x 10⁴ |
| Const₄ | (m⁻¹) | NA | (1.17 ± 0.10) x 10ⁿ | (3.05 ± 0.55) x 10⁹ | 507 ± 266 | (1.17 ± 0.10) x 10⁻¹⁰ |
| EGCG | Kᵣ₆ (µM) | 5.49 ± 1.86 | 9.09 ± 4.99 | 14.44 ± 8.19 | 8.45 ± 5.47 | 9.63 ± 1.95 |
| Const₁ | (m⁻¹) | NA | 0.12 ± 0.03 | 0.28 ± 0.14 | 0.59 ± 0.48 | 0.12 ± 0.03 |
| Const₂ | (m⁻¹) | NA | (5.02 ± 0.33) x 10⁸ | (9.33 ± 0.53) x 10⁴ | (3.99 ± 2.44) x 10⁴ | (5.02 ± 0.33) x 10⁸ |
| Const₃ | (m⁻¹) | NA | (1.03 ± 0.33) x 10⁸ | 6.2 ± 3.8 x 10⁻⁷ | (2.37 ± 0.76) x 10⁸ | (1.03 ± 0.33) x 10⁸ |
| Const₄ | (m⁻¹) | NA | (8.68 ± 0.78) x 10⁸ | 4.76 ± 1.30 x 10⁹ | (1.98 ± 0.86) x 10⁴ | (8.68 ± 0.78) x 10⁹ |
| ECG | Kᵣ₆ (µM) | 5.54 ± 1.84 | 22.7 ± 17.3 | 199.4 ± 333.4 | 126.2 ± 106.3 | 1.90 ± 0.32 |
| Const₁ | (m⁻¹) | NA | (5.24 ± 2.18) x 10⁻⁵ | (8.37 ± 2.33) x 10⁻² | (4.39 ± 0.87) x 10⁻² | (5.24 ± 2.18) x 10⁻⁵ |
| Const₂ | (m⁻¹) | NA | (4.61 ± 0.17) x 10⁸ | (9.38 ± 0.18) x 10⁴ | (7.94 ± 0.85) x 10⁴ | (4.61 ± 0.17) x 10⁸ |
| Const₃ | (m⁻¹) | NA | (4.14 ± 0.17) x 10⁹ | (4.63 ± 0.12) x 10⁵ | (6.14 ± 0.89) x 10⁵ | (4.14 ± 0.17) x 10⁹ |
| Const₄ | (m⁻¹) | NA | (2.48 ± 0.29) x 10² | (9.54 ± 0.65) x 10⁵ | (1.06 ± 0.38) x 10⁸ | (1.84 ± 0.29) x 10⁹ |

Flavonoids are significantly reduced. The analysis indicates that quercetin, EGCG, ECG, and CB interact with Hill coefficients (m) (34) of 1.49 ± 0.13, 1.41 ± 0.16, 0.75 ± 0.08, and 0.99 ± 0.05 sites per CB-binding site, respectively, with inhibitory constants (Kᵢ) of 1.02 ± 0.10, 23.75 ± 9.07, 1.24 ± 0.12, and 0.10 ± 0.01 µM, respectively. Hill coefficients greater than 1 indicate multiple ligand-binding sites interacting with positive cooperativity, whereas Hill coefficients significantly less than 1 indicate multiple ligand-binding sites (34) but could also indicate multiple sites that interact with negative cooperativity (33, 34). Application of the extra sum of squares F-test (28) indicates, for the range of inhibitor concentrations used, that the Hill model provides a significantly better fit for quercetin (p = 0.0002), EGCG (p = 0.0033), and ECG (p = 0.0084) data sets than the simple inhibition model but that both models adequately describe inhibition of [³H]CB binding by unlabeled CB.

To further test the effects of flavonoids on CB binding to erythrocyte GLUT1, we measured the concentration dependence of CB inhibition of 3MG (0.1 mM) uptake with and without flavonoids. The presence of quercetin (2 µM), EGCG (20 µM), or ECG (5 µM) inhibits basal sugar uptake (uptake in the absence of CB) and increases Kᵣ₆ for CB inhibition of 3MG uptake by at least 2.5-fold (Fig. 4C). Assuming simple competition between the flavonoids and CB for binding to GLUT1 (but see Fig. 4B), the computed Kᵣ₆ for quercetin (2 µM), EGCG (20 µM), and ECG (5 µM) inhibition of CB binding (Kᵣ₆ = 0.24 ± 0.03 µM) of transport are 1.88 ± 0.33, 9.63 ± 1.95, and 1.90 ± 0.32 µM, respectively. These results indicate that exofacial inhibitors impaire CB binding to the GLUT1 endofacial sugar-binding site and thereby reduce the potency of CB inhibition of sugar transport.

To act as cytoplasmic antioxidants, the flavonoids must cross the cell membrane. Previous studies have suggested that dietary flavonoids enter cells both by protein-independent trans-bilayer diffusion (35, 36) and via carrier proteins, including GLUT1, GLUT4, SGLT1, and MCT (15, 37–40).

Incubation of RBCs with 1 µM [³H]quercetin at 4 °C for 0.25–30 min indicates that the flavonoid achieves equilibrium association with RBCs within 30 s of exposure to the cells. The [³H]quercetin equilibrium space of the cells is 70% of the equilibrium [³H]3MG space of RBCs (Fig. 5A). If quercetin enters cells via GLUT1, as suggested by Cunningham et al. (15), [³H]quercetin uptake in human erythrocytes should be inhibited by inhibitors of sugar transport. CB (20 µM) almost completely inhibits [³H]3MG uptake but is without effect on [³H]quercetin association with human RBCs (Fig. 5B).

We investigated potential interactions of β-D-glucose, quercetin, EGCG, and ECG with the exofacial sugar-binding site by molecular docking using the homology-modeled GLUT1 outward-open structure (GLUT1-e2 ((18)). The exofacial, interstitial-exposed cavity of GLUT1-e2 presents three potential β-D-glucose docking sites: peripheral, intermediate, and core (18, 20). Benzene ring A (Fig. 1, A–D) in quercetin, EGCG, and ECG overlaps with the proposed core β-D-glucose docking site (20) (Fig. 6, A–D), whereas benzene ring B in quercetin interacts with intermediate and peripheral sites via hydrophobic and/or hydrophobic interactions (Fig. 6B). In EGCG and ECG, benzene ring B is inverted 45° and makes additional interactions with the core β-D-glucose docking site, whereas their galactose group overlaps with the intermediate β-D-glucose docking site (Fig. 6, C and D).

The residues contributing to β-D-glucose docking at core, intermediate, and peripheral β-D-glucose sites have been described previously (20). Fig. 6 (E–H) illustrates the putative hydrogen bond and hydrophobic contacts of core β-D-glucose, quercetin, EGCG, and ECG with GLUT1-e2. All ligands form five common hydrophobic interactions (Ile-164, Val-165, Ile-168, Phe-291, and Phe-379) and one hydrogen bond interaction at Glu-380 (Fig. 6, E–H). Additionally, each inhibitor forms hydrogen bonds with Asn-34 and Gln-283 (Fig. 6, F–H), but quercetin forms three additional hydrogen bonds (Gln-283, Glu-380, and Asn-415; Fig. 6F). EGCG and ECG form more hydrophobic contacts with GLUT1-e2 than quercetin (Fig. 6, G and H).

Neuronal GLUT3 and insulin-sensitive GLUT4 share 93% and 85% sequence similarity with GLUT1, respectively (41). We
Flavonoids are exofacial GLUT1 ligands

Figure 5. Quercetin uptake by RBCs. A, relative [3H]3MG and [3H]quercetin spaces of RBCs. Ordinate, equilibration relative to the equilibrated 3MG space of the cell. Equilibrium [3H]quercetin (1 μM) uptake is observed within 5 min of exposure to cells. [3H]3MG (100 μM) equilibration is achieved after 30-min exposure. Each bar shows the mean ± S.E. (error bars) of three separate duplicate experiments. Unpaired t test analysis indicates significant difference between the 3M and quercetin cell volume; **, p = 0.0044. B, effect of CB on [3H]quercetin and [3H]3MG uptake by human erythrocytes. Ordinate, substrate uptake relative to control. Abscissa, radiolabeled substrate. Results are shown for control (■) and 20 μM CB-treated cells (▲). Each bar shows the mean ± S.E. of three separate experiments measured in duplicate. Unpaired t test analysis indicates the following: ***, significant [3H]3MG uptake inhibition by CB (p < 0.0005); n.s., no significant difference in [3H]quercetin uptake with or without CB treatment (p > 0.05).

Figure 4. Flavonoids inhibit CB binding to human RBCs. A, inhibition of [3H]CB binding to human RBCs. Ordinate, normalized ratio of bound [3H]CB to free [3H]CB. Abscissa, [inhibitor] in μM (log scale). Results are shown for cells treated with quercetin (●), EGCG (○), ECG (▲), and nonradioactive CB (■). Each data point represents the mean ± S.E. (error bars) of three separate duplicate experiments. Curves were computed by nonlinear regression using Equation 4, assuming [3H]CB = 0.05 μM and K_CB = 0.055 μM, with the following results: quercetin treatment (●), K_app = 1.02 ± 0.09 μM, n = 1.49 ± 0.13, R^2 = 0.97, Sy.x = 0.064; EGCG treatment (○): K_app = 23.75 ± 9.07 μM, n = 1.41 ± 0.16, R^2 = 0.97, Sy.x = 0.074; ECG treatment (▲): K_app = 1.24 ± 0.12 μM, n = 0.75 ± 0.08, R^2 = 0.94, Sy.x = 0.082; nonradioactive CB treatment (■): K_app = 0.10 ± 0.01 μM, n = 0.99 ± 0.05, R^2 = 0.99, Sy.x = 0.036. The residuals of the quercetin, EGCG, and ECG fits are plotted below the inhibition plot. B, reanalysis of the same data set assuming multiple binding sites for competing ligand using the Hill equation (Equation 5). The results are as follows: quercetin treatment (●): K_app = 1.02 ± 0.09 μM, n = 1.49 ± 0.13, R^2 = 0.97, Sy.x = 0.064; EGCG treatment (○): K_app = 23.75 ± 9.07 μM, n = 1.41 ± 0.16, R^2 = 0.97, Sy.x = 0.074; ECG treatment (▲): K_app = 1.24 ± 0.12 μM, n = 0.75 ± 0.08, R^2 = 0.94, Sy.x = 0.082; nonradioactive CB treatment (■): K_app = 0.10 ± 0.01 μM, n = 0.99 ± 0.05, R^2 = 0.99, Sy.x = 0.036. The residuals of the quercetin, EGCG, and ECG fits are plotted below the inhibition plot. C, ordinate, 3MG uptake in mmol/liter cell water/min. Abscissa, concentration of inhibitors in μM (log scale). Results are shown for CB-treated cells (○) and CB-treated cells plus 2 μM quercetin (●), 20 μM EGCG (△), or 5 μM ECG (▲). Each data point represents the mean ± S.E. of at least three duplicate measurements. Curves were computed by nonlinear regression using Equation 1 and have the following results: CB treatment (○): K_app = 0.24 ± 0.03 μM, R^2 = 0.94, Sy.x = 0.007; CB + 2 μM quercetin (●): K_app = 0.62 ± 0.17 μM, R^2 = 0.83, Sy.x = 0.004; CB + 20 μM EGCG (△): K_app = 0.619 ± 0.123 μM, R^2 = 0.897, Sy.x = 0.004; CB + 5 μM ECG (▲): K_app = 0.71 ± 0.12 μM, R^2 = 0.94, Sy.x = 0.002.

Figure 3. Quercetin uptake by RBCs. A, relative [3H]3MG and [3H]quercetin uptake at 37 °C. Each bar shows the mean ± S.E. (error bars) of three separate duplicate experiments. Unpaired t test analysis indicates significant difference between the 3M and quercetin cell volume; **, p = 0.0044. B, effect of CB on [3H]quercetin and [3H]3MG uptake by human erythrocytes. Ordinate, substrate uptake relative to control. Abscissa, radiolabeled substrate. Results are shown for control (■) and 20 μM CB-treated cells (▲). Each bar shows the mean ± S.E. of three separate experiments measured in duplicate. Unpaired t test analysis indicates the following: ***, significant [3H]3MG uptake inhibition by CB (p < 0.0005); n.s., no significant difference in [3H]quercetin uptake with or without CB treatment (p > 0.05).

Discussion

Red wine and green tea flavonoids inhibit the facilitative glucose transporter, GLUT1, by interacting at its exofacial sugar-binding site. Quercetin, EGCG, and ECG competitively inhibit
Flavonoids are exofacial GLUT1 ligands

Figure 6. Molecular docking of β-D-glucose, quercetin, EGGC, and ECG to homology-modeled exofacial GLUT1 conformation. A, homology-modeled exofacial GLUT1 (GLUT1-e2) is shown in a cartoon representation, and membrane-spanning helices 1, 3–6, and 8–10 are indicated. Ligands (peripheral, intermediate, and core β-D-glucose (red; A and E); quercetin (cyan; B and F); EGGC (sky blue; C and G); and ECG (light blue; D and H)) are shown as spheres complexed with GLUT1-e2. The insets zoom in to illustrate the spatial arrangement of ligand (shown in a stick representation) interaction sites within GLUT1-e2 but with the GLUT1-e2 cartoon eliminated. Boxes E and F illustrate core β-D-glucose (E), quercetin (F), EGGC (G), and ECG (H) coordination in the exofacial cavity showing residues that form hydrogen bonds and hydrophobic interactions. The analysis suggests that EGGC and ECG binding to GLUT1-e2 promotes a 45° flip of benzene ring B.

net sugar uptake by human erythrocytes but are noncompetitive inhibitors of red cell sugar exit. Molecular docking studies using homology-modeled GLUT1 reveal that quercetin, EGGC, ECG, and β-D-glucose share overlapping interaction sites in the exofacial ligand-binding cavity of GLUT1. Whereas docking studies suggest that only one flavonoid can bind to GLUT1 at any instant, sugar transport and ligand binding studies indicate that the erythrocyte sugar transporter can bind at least two flavonoid molecules simultaneously.

The sidedness of flavonoid action (exofacial) is compatible with previous reports suggesting that quercetin (21, 24) and EGGC (16) act as competitive inhibitors of GLUT1-mediated sugar uptake. Consistent with this conclusion, molecular docking analysis suggests that quercetin, EGGC, and ECG share overlapping interaction envelopes in the exofacial ligand-binding cavity of GLUT1-e2, including the previously defined core β-D-glucose interaction site (20) (Fig. 6). Docking analysis also suggests that flavonoid binding is coordinated by the side chains of amino acids that form the previously defined exofacial intermediate and peripheral β-D-glucose interaction sites. The validity of our docking analysis is bolstered by the finding that the coordination of β-D-glucose at the core site in homology-modeled GLUT1-e2 involves the same amino acid residues coordinating β-D-glucose binding in the β-D-glucose–human...
GLUT3-e2 crystal complex (42). Core β-d-glucose, quercetin, EGCG, and ECG all form hydrophobic interactions with GLUT1 Ile-164, Val-165, Ile-168, Phe-291, and Phe-379 and form a hydrogen bond with Glu-380, suggesting that these residues are integral to ligand binding in the exofacial cavity. The chemical structures of EGCG and ECG are almost identical (the one difference being the addition of a hydroxyl group at C3’ in EGCG), yet our docking analysis reveals no striking differences in EGCG and ECG coordination to GLUT1-e2. Notwithstanding, EGCG inhibits GLUT1 with 5-fold lower affinity than ECG, illustrating the limitations of this docking analysis.

Prior equilibrium ligand-binding studies demonstrate that extracellular maltose inhibits CB binding at the GLUT1 endofacial sugar binding site (27, 29). Here, we show that exofacial quercetin, EGCG, and ECG have the same effect. $K_{i(app)}$ for inhibition of CB binding is 1.6–3-fold lower than $K_{i(app)}$ for transport inhibition. This is not explained by competition between inhibitor and transported sugar for binding at the exofacial site because GLUT1 saturation by 0.1 mM 3MG is less than 5% ($K_{i(app)}$ for 3MG = 2.4 mM; see Fig. 2A). Closer review of the curve fit statistics for flavonoid inhibition of CB binding indicates that the fits to the simple inhibition model are poor. Fitting the data to a Hill model comprising multiple cooperative binding sites (33) (Equation 5) produces significantly better fits for quercetin, EGCG, or ECG but not for CB. Moreover, using the extra sum of squares F-test (28) to test the null hypothesis that the simple inhibition and Hill models provide equally good fits of the binding data fails for quercetin, EGCG, or ECG. These results suggest that GLUT1 CB binding is affected by at least two positively cooperative binding sites each for quercetin and EGCG and two or more (possibly negatively cooperative) binding sites for ECG (32, 33). If multiple exofacial flavonoid-binding sites exist, docking analysis suggests that they are unlikely to co-exist within the same GLUT1 molecule. Rather, they must be present in adjacent molecules that interact with each cytochalasin B–binding GLUT1 protein.

We also considered that flavonoids compete directly with CB for binding at the endofacial sugar-binding site. This would explain their displacement of CB from GLUT1 and would also be consistent with demonstrations of cooperativity between endofacial ligand-binding sites present in adjacent GLUT1 subunits of the GLUT1 tetramer (29, 30, 44, 45). Molecular docking of CB and quercetin to the endofacial orientation of GLUT1 (GLUT1-e1) (46) supports the hypothesis that CB and quercetin could compete for high-affinity binding to GLUT1 (not shown). However, inhibition of transport by a molecule that can bind with equal avidity to both exo- and endofacial sugar binding sites should noncompetitively inhibit both net uptake and net exit (26, 27). Because flavonoid inhibition of net uptake is competitive, this possibility is refuted. If an inhibitor were to bind with significantly lower affinity to the endofacial binding site versus the exofacial site, it would not only increase $K_{m(app)}$ for net sugar uptake but also reduce $V_{max}$ for uptake by >65% (26, 27), and this is not observed.

The actions of the flavonoids on sugar transport demonstrate an additional form of cooperativity, which we term cis-allostery (18). Quercetin, EGCG, and ECG, like other exofacial GLUT1 inhibitors (e.g. WZB117 (20) and maltose (29)), stimulate

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**Figure 7. Isoform specificity of sugar transport inhibition by flavonoids.** Results are shown for transport inhibition by quercetin (A), EGCG (B), and ECG (C) in transfected HEK293 cells transiently expressing hGLUT1 (●), hGLUT3 (▲), or hGLUT4 (▲). Ordinate, relative 2-deoxyglucose uptake (v/v); abscissa, [inhibitor] in μM (log scale). The dashed curves are the curves describing quercetin, EGCG, and ECG modulations of hGLUT1-mediated 3MG uptake in RBCs at 4 °C (Fig. 3) computed using Equation 3. The parameters used were those used in Fig. 3 with one exception. Const, for ECG modulation of GLUT1 was reduced 7-fold. The solid curves describe flavonoid modulations of hGLUT3 and -4 and were computed by nonlinear regression using Equation 3. The parameters describing the best fits are summarized in Table 2. The following summarizes the quality of these fits: for quercetin treatment: hGLUT3 (▲): $R^2 = 0.998$, $Sy.X = 0.017$; hGLUT4 (▲): $R^2 = 0.999$, $Sy.X = 0.008$; for EGCG treatment: hGLUT3 (▲): $R^2 = 0.998$, $Sy.X = 0.017$; hGLUT4 (▲): $R^2 = 0.999$, $Sy.X = 0.008$; for ECG treatment: hGLUT3 (▲): $R^2 = 0.976$, $Sy.X = 0.048$; hGLUT4 (▲): $R^2 = 0.482$, $Sy.X = 0.144$. 2DG uptake in untreated, untransfected cells averaged 2.7 ± 0.6 pmol/μg of protein/min with a range of 1.6–3.8 pmol/μg of protein/min. hGLUT3, hGLUT4, and hGLUT4 heterologous expression increased basal 2DG uptake by 6.3 ± 3.3, 3.4 ± 0.8, and 4.5 ± 1.5-fold, respectively. Error bars, S.E.
GLUT1-mediated sugar uptake at low inhibitor concentrations and then inhibit transport as their concentration is raised. This reinforces the idea that at least two flavonoid-binding sites modulate GLUT1 function. Two possibilities exist: 1) one of these sites is presented by GLUT1 and the second by a non-GLUT1 but nevertheless GLUT1-interacting protein, or 2) the...
flavonoid-binding sites exist on individual GLUT1 molecules whose adjacency in the GLUT1 homotetramer (20, 29, 30) results in cooperative interactions. In both instances, flavonoid binding at the first, high-affinity site stimulates sugar uptake by flavonoid-free GLUT1 molecules. As flavonoid concentration is raised, ligand and sugar now compete for binding at the GLUT1 exofacial sugar-binding site, and transport is inhibited. Whereas the former hypothesis cannot be eliminated by the current study, our previous ligand-binding (18, 20, 27, 29–32, 43), hydrodynamic analysis (44, 47), biochemical cross-linking (48), freeze-fracture EM (47), and co-immunoprecipitation (49) studies of membrane-resident and purified GLUT1 support the latter hypothesis.

Studies with heterologously expressed GLUT1, GLUT3, and GLUT4 indicate that quercetin inhibits GLUT1 and GLUT4 with comparable avidity but is up to 9-fold less potent against GLUT3. EGCG shows similar inhibitory potency toward GLUT1, GLUT3, and GLUT4. ECG appears to be only a very poor inhibitor of sugar uptake in GLUT1-, GLUT3-, or GLUT4-expressing HEK293 cells, but this conclusion should be tempered by the ligand’s reported instability at 37 °C (42).

GLUT1 inhibition at high flavonoid concentrations could explain the anti-cancer action of flavonoids. Small molecule inhibition of cellular sugar transport results in a cascade of downstream events, including down-regulation of glycolytic enzymes, cell cycle arrest, and, ultimately, cell death (49). GLUT1 stimulation by low concentrations of flavonoids could explain their protective actions against diseases such as diabetes (50–53) and neurodegenerative diseases (54–57), where enhanced cellular glucose uptake could be ameliorative. However, flavonoids also directly or indirectly modulate other cellular targets, including mitogen-activated protein kinase, cyclin-dependent kinases, HIF-1α, and vimentin (14). Whereas some studies suggest that flavonoids may not readily cross the plasma membrane (58), others suggest that flavonoids enter cells via carrier proteins, including SGLT1 (37, 38, 59, 60), MCTs (39), GLUT1 (15), and GLUT4 (40), or via transbilayer diffusion (35, 36). The current study indicates that, whereas quercetin may cross the plasma membrane, it does so via a CB-insensitive, GLUT1-independent pathway.

Whereas some health benefits of the flavonoids may derive from their antioxidant capacities, the dual actions of flavonoids on glucose transport (interaction with the exofacial sugar-binding site and at regulatory sites that modulate sugar transport) present new tools to explore glucose transporter function and downstream glucose metabolism in tumors, in insulin-secreted and -responsive tissues, and in the central nervous system.

**Experimental procedures**

**Reagents**

Tritium-labeled 3-O-methylglucose ([3H]3MG), 2-deoxy-D-glucose ([3H]2DG), cytochalasin B ([3H]CB), and quercetin ([3H]quercetin) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled 3MG, CB, quercetin, EGCG, ECG, and phloretin were purchased from Sigma-Aldrich. Phosphate-buffered saline was purchased from Thermo Fisher Scientific (Waltham, MA). WZB117 was purchased from EMD Millipore (Billerica, MA).

**Solutions**

KCl medium comprised 150 mM KCl, 5 mM HEPES, 0.5 mM EDTA, pH 7.4. Stop solution comprised ice-cold KCl medium plus 50 μM WZB117 and 100 μM phloretin. Sugar uptake/exit medium was made up in KCl medium containing 0–20 mM 3MG with or without inhibitors and contained [3H]3MG or [3H]quercetin, as indicated. HEK293 solubilization buffer comprised KCl medium with 1% Triton X-100.

**Cells**

De-identified whole human blood was purchased from Biological Specialty Corp. (Colmar, PA). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 37 °C humidified 5% CO2 incubator.

**Heterologous expression of GLUTs**

Heterologous expression of hGLUT1, hGLUT3, and hGLUT4 in HEK293 cells was as described previously (49, 61). Both hGLUT1 and hGLUT4 contain a Myc epitope in exofacial loop 1 (61), whereas the 13 C-terminal amino acids in hGLUT3 are replaced by the corresponding residues of hGLUT4 (49) to facilitate detection of heterologously expressed transporter.

**Red blood cell sugar transport measurements**

All human erythrocyte sugar transport experiments were performed at 4 °C as described previously (20, 49). Red blood cells were isolated from whole blood and glucose-depleted as described previously (62). Sugar transport and ligand-binding experiments reported below typically involve the addition of 2–10 volumes of uptake or ligand-binding medium with or without inhibitor to 1 volume of a 50% suspension of red cells. Our measurements show that preincubating RBCs with increasing volumes of medium containing the test inhibitor progressively reduces $K_{i(app)}$ for inhibition of sugar transport or cytochalasin B binding. The explanation (44) is that the very high GLUT1 content of RBCs depletes [inhibitor]free as it interacts with GLUT1, resulting in [inhibitor]free $\leq$ [inhibitor]total at the time of transport or ligand-binding assay. The most practical solution to this problem is to preincubate RBCs with an excess volume of uptake or ligand binding medium lacking sugar or CB but containing the inhibitor at the requisite concentration. We observe that $K_{i(app)}$ for test compound inhibition of sugar transport or CB binding approaches its minimum asymptote when preincubation conditions are 1 volume of RBCs to 50–400 volumes of preincubation assay medium (the necessary dilution falls with increasing [inhibitor]total). We therefore preincubated RBCs with 50–400 volumes of assay medium prior to centrifugation and resuspension in uptake or ligand binding medium to ensure optimal equilibration of inhibitor with GLUT1 before performing transport or ligand-binding measurements.

**Zero-trans uptake**

Zero-trans [3H]3MG or [3H]quercetin uptake (uptake into cells lacking intracellular sugar or quercetin) was initiated by...
**Flavonoids are exofacial GLUT1 ligands**

adding 10 volumes (100 μl) of uptake medium with or without inhibitor to 1 volume (10 μl) of sugar-depleted, 50% hematocrit red cells, and sugar uptake was allowed to proceed for 30–60 s at 4 °C. Uptake was stopped by adding 50 volumes (1 ml) of ice-cold stop solution containing 50 μM WZB117 and 100 μM phloretin. Cells were washed one more time in stop solution and lysed in 3% perchloric acid, and radioactivity was assayed in clarified lysates using liquid scintillation counting. Radioactivity measurements were done in duplicates.

**Zero-trans exit**

Glucose-depleted, packed RBCs were loaded with 10 mM 3MG by incubating 1 volume of cells with 20 volumes of 20 mM 3MG (containing 1 μCi of [3H]3MG/ml of cold 3MG) for 1 h at 37 °C. Immediately following 3MG loading, cells were transferred to 4 °C and preincubated with or without inhibitors for 10–15 min. Cell suspension were spun at 10,000 g for 1 min, and supernatant was discarded. One volume (0.5 ml) of sugar-loaded RBCs were added to 50 volumes of KCl medium with or without inhibitor on a shaker with magnetic stirrer. Aliquots (0.5 ml) of the suspension were withdrawn at the indicated time intervals and immediately added to 1 ml of ice-cold stop solution. Cells were washed again in stop solution, lysed in 3% perchloric acid, and assayed in duplicate for radioactivity.

**HEK293 cell sugar uptake**

All HEK293 cell sugar uptake measurements were performed at 37 °C, using 100 μM 2-deoxyglucose (2DG plus [3H]2DG) as described previously (49, 63).

**Equilibrium CB binding**

CB binding to human red cells was performed as described previously (20, 31). Briefly, 50 μl of sugar-depleted RBC (50% hematocrit) with or without inhibitors were mixed with 50 μl of ice-cold KCl medium containing 40 mM [3H]CB and 10 μM cytochalasin D for 15 min at 4 °C, with constant end-over-end rotation. Total [CB] was obtained from 2 × 10 μl of the cell suspension lysed in 100 μl of 3% perchloric acid, and radioactivity was assayed by liquid scintillation counting. To obtain free [CB], cell suspension was centrifuged at 10,000 × g for 30 s, and 2 × 10 μl of clarified supernatant were assayed for radioactivity. Bound [CB] was calculated as total [CB] – free [CB].

**Homology modeling**

The homology models of the outward-open (e2) conformations of GLUT1 and GLUT4 were generated using the maltose-bound human GLUT3 structure (Protein Data Bank code 4ZWC) (42). Maltose was removed from the GLUT3 structure, and chain A was used as the template for modeled structures. Sequence alignments were generated using ClustalX (64). Homology models were built using Modeler version 9.9 (65) and analyzed using PROCHECK (66).

**Stochastic docking**

The crystal structure of outward-open hGLUT3-e2 (4ZWC) (42) was obtained from the Protein Data Bank. The structures for β-D-glucose, quercetin, EGCG, and ECG were obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov). Docking was performed using the Schrödinger software suite. The protein structure was preprocessed with the Protein Preparation Wizard, bond orders were assigned, hydrogens were added, and the hydrogen bond network was optimized. The system was energy-minimized using the OPLS2005 force field. Ligand structures were prepared with the LigPrep module, and the pKₐ of the ligands was calculated using the Epik module. Molecular docking was performed by the GLIDE module in standard precision (SP) mode using default values for grid generation. Cavities for docking were calculated using the CastP server (http://sts.bioe.uic.edu/castp/) (67), and the grid was centered on the residues forming the cavity. No restraints were used during the docking.

**Data analysis**

Linear and nonlinear regression analysis of data sets and statistical tests were performed using GraphPad Prism version 7.0a (GraphPad Software, Inc., La Jolla, CA).

Michaelis–Menten inhibition of sugar transport is assumed to be described by Equation 1,

\[
v = v_c - \frac{v_c[I]}{K_{i(app)} + [I]} \tag{Eq. 1}\]

where \(v_c\) is measured in the absence of inhibitor I, \([I]\) is the concentration of inhibitor, and \(K_{i(app)}\) is that [I] producing 50% inhibition of uptake.

Michaelis–Menten transport is assumed to be described by Equation 2,

\[
v = \frac{v_{max}[3MG]}{K_{m(app)} + [3MG]} \tag{Eq. 2}\]

where \(v_{max}\) is the maximum rate of 3MG transport, \([3MG]\) is the concentration of 3MG, and \(K_{m(app)}\) is the [3MG] where the rate of uptake is \(v_{max}/2\). Sugar exit was analyzed by nonlinear regression analysis using Mathematica version 10.4.1.0 (Wolfram Research), assuming that exit follows Michaelis–Menten kinetics and that the first derivative of the exit progress curve represents \(d[S]/dt\) at any given [3MG] (20).

Transport stimulation followed by inhibition by inhibitors was approximated first by normalizing all uptake to \(v_c\) and then using the following model,

\[
v_i = \frac{\text{Const}_1 + [I](\text{Const}_2 + [I])}{\text{Const}_1 + [I](\text{Const}_2 + [I](\text{Const}_3))} \tag{Eq. 3}\]

where \(v_c\) is uptake measured in the absence of inhibitor I, \(v_i\) is uptake measured in the presence of inhibitor, \([I]\) is the concentration of inhibitor, and \(\text{Const}_1\) through \(\text{Const}_4\) are model-dependent (19).

Inhibition of [3H]CB binding to GLUT1 by ligands was analyzed by simple competitive inhibition using the model,

\[
\frac{[\text{CB}]_b}{[\text{CB}]_b} = \frac{K_{i(app)}([\text{CB}] + [\text{CB}])}{K_{[\text{CB}]} + K_{i(app)}([\text{CB}] + [\text{CB}])} \tag{Eq. 4}\]

3 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
where $[\text{CB}]_{bi}$ is bound [CB] measured in the presence of inhibitor I, $[\text{CB}]_b$ is bound [CB] measured in the absence of inhibitor, $K_{(app)}$ is the apparent inhibitory constant for inhibition of CB binding by inhibitor I, and $K_{CB}$ is the dissociation constant for CB binding to GLUT1.

CB binding was also analyzed using the Hill equation for inhibition of equilibrium binding in which the transporter is allowed to bind more than one molecule of competing ligand, I,

$$\frac{[\text{CB}]_{bi}^f}{[\text{CB}]_{bi}} = \frac{K'}{K' + [I]^n} \quad \text{(Eq. 5)}$$

where $[\text{CB}]_{bi}^f$ is the ratio of bound to free [CB] measured in the presence of inhibitor I, $[\text{CB}]_{bi}$ is the ratio of bound to free [CB] measured in the absence of inhibitor, and $K'$ is $K_{(app)}$ for I binding to GLUT1 to the power of $n$, where $n$ is the number of inhibitor-binding sites. Comparisons of quality of model fits were made using the extra sum of squares F-test (28) using GraphPad Prism.

**Author contributions**—O. A. O. conducted most of the experiments, analyzed the results, and wrote most of the paper. K. P. L. constructed the GLUT1 homology models; J. K. D. Z. performed the heterologous expression and transport studies. A. C. and O. A. O. conceived the idea for the project, analyzed the results, and wrote most of the paper. K. P. L. conceived the idea for the project, analyzed the results, and wrote the manuscript.

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