Regulation of GLUT Transporters by Flavonoids in Androgen-Sensitive and -Insensitive Prostate Cancer Cells

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Cancer cells show different metabolic requirements from normal cells. In prostate cancer, particularly, glycolytic metabolism differs in androgen-responsive and nonresponsive cells. In addition, some natural compounds with antiproliferative activities are able to modify glucose entry into cells by either modulating glucose transporter (GLUT) expression or by altering glucose binding. The aim of this work was to study the regulation of some GLUTs (GLUT1 and GLUT4) in both androgen-sensitive (LNCaP) and -insensitive (PC-3) prostate cancer cells by 4 structurally different flavonoids (ie, genistein, phloretin, apigenin, and daidzein). Glucose uptake was measured using nonradio-labeled 2-deoxyglucose. The evaluation of protein levels as well as subcellular distribution of GLUT1/4 were analyzed by Western blot and immunocytochemistry, respectively. Androgen-insensitive LNCaP-R and androgen-sensitive PC-3-AR cells were used to study the effect of androgen signaling. Additionally, a docking simulation was employed to compare interactions between flavonoids and Xyle, a bacterial homolog of GLUT1 to -4. Results show for the first time the presence of functionally relevant GLUT4 in prostate cancer cells. Furthermore, differences in GLUT1 and GLUT4 levels and glucose uptake were found, without differences on subcellular distribution, after incubation with flavonoids. Docking simulation showed that all compounds interact with the same location of transporters. More importantly, differences between androgen-sensitive and -insensitive prostate cancer cells were found in both GLUT protein levels and glucose uptake. Thus, phenotypic characteristics of prostate cancer cells are responsible for the different effects of these flavonoids in glucose uptake and in GLUT expression rather than their structural differences, with the most effective in reducing cell growth being the highest in modifying glucose uptake and GLUT levels. (Endocrinology 155: 3238–3250, 2014)
There are 14 different members of GLUT receptors (GLUT1–12, GLUT14, and H/myo-inositol transporter) (8). GLUT1, highly expressed in brain and erythrocytes, is responsible for providing basal glucose levels and is mainly regulated by growth factors (9). GLUT4, an insulin-dependent transporter, is expressed in heart, skeletal muscle, and adipose tissues and is regulated by protein externalization-internalization as a consequence of insulin binding to its receptor (10). In tumor cells, changes in protein levels or membrane translocation of GLUTs are related to glycolytic pathways. Thus, an increase in glucose uptake has been associated mainly with GLUT1 overexpression but may also involve other GLUTs, including GLUT4 (8).

In prostate cancer, as in many other tumors, glucose metabolism is different between early and advanced stages (11), and glycolytic metabolism profile differs in androgen-sensitive and insensitive prostate cancer cells (12). GLUT1 expression in androgen-sensitive LNCaP cells seems to be regulated by androgens (13, 14). An androgenic stimulation increases glucose uptake and lactate production, whereas androgen receptor (AR) signaling pathway stimulates anabolic synthesis (6, 15). Furthermore, a higher glucose uptake is also required for faster proliferation of androgen-insensitive prostate cancer cells (16).

The role of natural compounds as potential antitumor agents is currently under extensive study. In prostate cancer, natural compounds are mainly involved in the inhibition of oxidative stress and inflammation (17). Natural compounds have different molecular targets in biological systems. Additionally, some of them are able to modify the entry of glucose into cells by modulating GLUT1 expression or by altering glucose binding (18). Inhibition of glucose uptake reduces cell growth and induces apoptosis in some tumor cells (19). Consequently, natural compounds that target different tyrosine kinases or ATP binding sites are able to inhibit transporter activity (20).

Phytoestrogens are naturally occurring compounds with similar structure to that of the sexual hormone 17β-estradiol, which displays weak estrogenic activity. In prostate cancer cells, some of them have been associated with apoptosis induction and with angiogenesis inhibition (21, 22). They also modulate sex hormone metabolism often by reducing viability of their active forms (23, 24). Some flavonoids, a type of natural compound with important biological properties, are considered as phytoestrogens (25). However, there is no information on whether these effects might be related to glucose uptake impairment.

The aim of this work was to study the influence of some flavonoids, ie, genistein, phloretin, apigenin, and daidzein, on expression or subcellular location of GLUT1 and GLUT4 as well as their impact in glucose uptake in both androgen-sensitive and -insensitive prostate cancer cells and to study how this influence might correlate with their antiproliferative activity.

### Materials and Methods

#### Chemicals and reagents

Phloretin and daidzein were purchased from Calbiochem. Antibiotic-antimycotic cocktail was obtained from Gibco (Invitrogen). Aprotinin A, Reflotron glucose and Genopure Plasmid Midi Kit were purchased from Roche. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and diaphorase from *Clostridium kluyveri* were purchased from Abnova. 2-Deoxy-d-glucose (2-DG) and resazurin sodium salt were provided by Alfa Aesar. NADP sodium salt and G418 disulfate were obtained from Applichem. Nonfat dry milk and BSA were obtained from Santa Cruz Biotechnology. Fluoromont G fluorescent mounting media was provided by Southern Biotech. Activated carbon was obtained from Merck. FuGENE HD was provided by Promega. All cell culture media employed were provided by Lonza. All other reagents were purchased from Sigma-Aldrich.

#### Cell types and culture conditions

Human androgen-sensitive epithelial prostate cancer cells (LNCaP) and human normal prostate epithelial cells immortalized with simian virus 40 (PNT1A) were purchased from European Collection of Cell Cultures (89110211 and 95012614) and were maintained in RPMI 1640 and DMEM, respectively, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 15mM HEPES, 100 μg/mL ampicillin, and 100 μg/mL kanamycin. PC-3 cells, a human prostate androgen-insensitive adenocarcinoma cell line, were purchased from American Type Culture Collection (CRL-1435) and were grown in DMEM/F12 supplemented with 10% FBS, 2% L-glutamine, and 1% antibiotic-antimycotic cocktail (100 U/mL penicillin, 10 μg/mL streptomycin, and 0.25 μg/mL amphotericin B). All cell lines were grown at 37°C in a humidified 5% CO₂ environment. The medium was changed every 2 days, and cultures were split once a week.

LNCaP-R cells, a clone of androgen-insensitive LNCaP cells, were achieved as previously described (26). Briefly, FBS was incubated with a mixture of 2.5% activated carbon plus 0.025% dextran T70 overnight at 4°C and then centrifuged at 5000 g. Thus, FBS was stripped from low-molecular-weight molecules including steroids. charcoal/dextran-stripped FBS (FBS_{char}) was added to culture medium and filtered. LNCaP cells were grown in RPMI 1640 plus FBS_{char}. Androgen-resistant colonies were isolated and cultured. Clones were then frozen and stored after 6 months growing in the absence of androgens. LNCaP-R 1.3.6 was selected among 12 other clones for its GLUT protein levels. AR was studied, and its expression was 3 times higher than in LNCaP cells (data not shown). Cells were maintained in RPMI 1640 medium with FBS_{char}, but experiments were performed in complete medium with FBS.

PC-3 AR cells were obtained by transfection with FuGENE HD. Plasmid pEGEFP-C1-AR (28235; Addgene database) was given by Michael Mancini (Baylor College of Medicine, Houston, TX) (27). PC-3 cells were transfected when they reached a
density of 80% per well in DMEM/F12 complete medium without antibiotics. Plasmid was diluted in complete medium to a final concentration of 0.020 μg/μL and FuGENE HD solution was added as a reagent to DNA ratio of 3:1. After 48 hours, medium was replaced with complete medium plus 300 μg/mL G418 for selection. Cells were maintained in selective medium for 2 weeks. Resistant colonies were trypsinized and transferred to 6-well plates. AR expression was checked by quantitative PCR showing AR mRNA levels similar to LNCaP cells (data not shown). Each clone was maintained in DMEM/F12 with 200 μg/mL G418 until experiments were performed in complete medium without the selective antibiotic.

**Treatments**

Natural compounds were added directly in complete culture medium from a 100mM stock solution, using dimethylsulfoxide (DMSO) as a vehicle. With the exception of dose-response experiments, the final concentration used for each compound in culture media was the corresponding IC50 values obtained and shown in Results (see Tables 1 and 2). All treatments, including controls, had 0.1% DMSO. All treatments were incubated 48 hours after seeding, and their duration was variable (20 minutes or 1, 6, 24, or 48 hours). Insulin (100nM) was added for 20 minutes to perform 2-DG studies, after which cells were incubated 1 hour with flavonoids.

**Cell proliferation assay**

Per well, 5 × 10⁴ LNCaP cells or 2 × 10³ PC-3 or PNT1A cells were seeded in 96-well plates. After 24 hours, treatments were added at different concentrations (0μM–100μM for genistein and phloretin, 0μM–50μM for apigenin and 0μM–140μM for daidzein). To evaluate the effect of flavonoids in cell proliferation, LNCaP cells were seeded in RPMI 1640 medium with FBS, Schst and 5nM DHT was added 3 hours before starting treatment, LNCaP cells were seeded in RPMI 1640 medium with FB-Schst and 5nM DHT was added 3 hours before starting treatments. The final DMSO concentration was 0.1% in all groups including controls. After 48 hours, media were removed and cells were rapidly frozen at −80°C. After thawing, 100 μL of ddH₂O was added to each well and cells were incubated 1 hour at 37°C. Cells were then frozen once more to ensure cell lysis. After thawing, cells were incubated in buffer containing 10mM Tris-HCl (pH 7.4) and 2M NaCl with 20 μg/mL Hoechst for 5 minutes without light exposure. Finally, fluorescence was measured using 350 nm (excitation)/460 nm (emission) in a Synergy H4 hybrid reader (Bio-Tek) (28). Results shown are the mean (n = 6) ± SEM. Experiments were repeated twice, and a representative one is shown.

**Glucose uptake**

Glucose uptake was assessed as described previously (29) with minor modifications. Cells were seeded in 24-well culture plates. After 48 hours, treatments were added either for a short time (20 minutes or 1 hour) or for a long time (24 hours). After treatments, cells were washed twice with RPMI 1640 complete medium without glucose. Then, medium containing 2mM 2-deoxy-D-glucose (2-DG) was added and cells were further incubated for 20 minutes at 37°C in a 5% CO₂ incubator. After that, cells were washed twice with buffer containing 50mM HEPES, 137mM NaCl, 4.7mM KCl, 1.85mM CaCl₂, and 1.3mM MgSO₄ (pH 7.4) containing 0.1% BSA (Cohn fraction V). Cells were lysed (0.1M NaOH) with graded heating from 60°C to 85°C for 60 minutes. After that, lysates were neutralized with 0.1M HCl, and the same volume of 200mM triethanolamine was added to each well. Twenty five microliters of each well were transferred to a 96-well assay plate, and 200 μL of assay solution (50mM triethanolamine, 50mM KCl, 0.02% BSA, 0.1mM NADP⁺, 0.2 U/mL diaphorase, 6μM resazurin sodium salt, and 20 U/mL glucose-6-phosphate dehydrogenase) was added to each well. Plates were incubated 45 minutes at 37°C. Finally, fluorescence was measured in a Synergy H4 hybrid reader (550 nm excitation and 605 nm emission). For standardization, results are given as glucose uptake per microgram protein. Protein concentrations were determined using a Bradford assay. Results are shown as the mean of 3 samples ± SEM.

**Glucose concentration in cell culture**

Glucose concentration in media was measured with Retrotro Plus System Kit (Roche). Even though the test has been developed for measuring glucose in blood, serum, or plasma, it was previously tested that culture media did not interfere with assay (data not shown). For this purpose, culture media were collected at the end of treatments. The 2-DG was oxidized into D-glucuronolactone by glucose oxidase, and the hydrogen peroxide formed was then oxidized by peroxidase. Color, directly related to glucose concentration, was read at 642 nm at 37°C in a spectrophotometer. Standardization of glucose concentration was accomplished by determining protein concentration with a Bradford assay.

**PAGE and immunoblotting**

After incubation, cells were harvested by soft scraping, washed with ice-cold PBS, pelleted by centrifugation, and lysed in RIPA lysis buffer (50mM Tris-HCl [pH 7.4], 150mM NaCl, 0.1% SDS, 1% IGEPAL C, and 0.5% sodium deoxycholate) supplemented with 1mM DTT and protease inhibitors for 30 minutes at 4°C (10 μg/mL leupeptin, 2 μg/mL aprotinin A, 1 μg/mL pepstatin, 200μg sodium orthovanadate, 1mM sodium fluoride, and 1mM phenylmethylsulfonyl fluoride).

Proteins (20 μg) were separated on a 12% SDS-PAGE gel according to Laemmli’s method (30) using a Mini-Protein III system (Bio-Rad). Proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore) using the Trans-Blot Cell System (Bio-Rad). After blocking, membranes were incubated with 5% nonfat dry milk in Tris-buffered saline (TBS) with Tween 20 (20mM Tris-HCl [pH 7.4], 150mM NaCl, 0.05% Tween 20) for 1 hour at room temperature and then incubated overnight at 4°C and reacted to anti-GLUT1 (Upstate-Millipore), anti-GLUT4 (Upstate-Millipore), or anti–β-actin (Santa Cruz Biotechnology) at a final dilution of 1:2500, 1:2000, and 1:8000, respectively. Primary antibodies were visualized by binding horseradish peroxidase-conjugated antirabbit (Calbiochem) or antimouse (Santa Cruz Biotechnology) secondary antibodies and detected with chemiluminescence substrate (Millipore). Blots were scanned (300 dpi grayscale), and densitometry was quantified using Image J software. Experiments were repeated 3 times, and a representative experiment is shown.

**Immunocytochemistry**

LNCaP and PC-3 cells were seeded on treated plastic coverslips (Thermanox; Nunc-Thermo Fisher Scientific).
After treatment, cells were washed in Dulbecco's PBS and fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4). Then, cells were washed 3 times, permeabilized, and blocked by incubation with TBS plus 0.15% Tween 20 and 0.5% BSA for 30 minutes at room temperature. Cells were incubated overnight in a humid chamber at 4°C with anti-GLUT-1 or anti-GLUT4 antibody at final concentration 1:250 in TBS. After that, coverslips were washed 3 times, and cells were incubated with antirabbit secondary antibody conjugated with phycocerythrin at a final dilution 1:1000 (Jackson ImmunoResearch) for 60 minutes at 37°C. Coverslips were then rinsed thrice with TBS for 5 minutes. Cells were then counterstained with 4′,6-diamino-2-phenylindole (1 μg/mL) for 5 minutes at room temperature. Coverslips were finally mounted using Fluoromount G. A Leica SP2/AOBS microscope was used to study the samples, and Image J software was used for image processing.

Molecular docking simulation

Because GLUTs have not yet been crystallized, a different approach for docking simulation studies was used. For this purpose, the crystal structure of the bacterial protein homolog of GLUT1 to -4 (MFS, major facilitator superfamily, proton:xylose symporter XyIE), which shares more than 45% homology, was used (31). Flavonoid 3-dimensional structures were obtained from PubChem (NCBI). Docking simulations were run under Molegro Virtual Docker version 5.5 software (CLC Bio), using the MolDock score function. Grid resolution was set at 0.30 Å with a radius of 19, and x, y, and z centers were −13.24, 8.44, and 1.30, respectively. The search algorithm used was MolDock optimizer with default settings. The number of runs ranged from 10 to 25, and maximum iterations varied from 2000 to 10 000. The active site region previously described (32) of GLUT1 and GLUT4 proteins (XyIE) was chosen for docking with glucose. It provided 10 conformations for each ligand and returned 5 outputs with MolDock score and other thermodynamically calculated values. The MolDock score is an anonymous value on which we had to suggest the best docked ligand with its conformation. It also showed hydrogen bond information together with other thermodynamic values, which suggest the formation of a stable complex between ligand and receptor molecule (33).

Statistical analysis

Results shown represent mean ± SEM of at least 3 samples per group. Differences among means were calculated using one-way ANOVA, followed by a Student-Newman-Keuls t test. Values were considered statistically significant when \( P < .05 \).

Results

Flavonoids reduce cell growth of prostate cancer cells

The effect of 4 structurally different flavonoids on cell growth was studied. Their molecular structure is shown in Figure 1A. Cell proliferation was determined by Hoechst assay after 48 hours of incubation. Transformed nontumorigenic PNT1A cells were used as normal cells. Results showed that all compounds, i.e., genistein, phloretin, apigenin, or daidzein, significantly inhibited cell growth of tumor cells (Figure 1B). Nonetheless, their influence on cell proliferation was always higher in LNCaP than in androgen-insensitive PC-3 cells. Apigenin was the most effective in reducing cell growth in both cell lines, resulting in a 50% of inhibition at 7 μM and 16μM for LNCaP and PC-3 cells, respectively. Phloretin and daidzein showed a similar effect on LNCaP cells, whereas genistein was clearly less effective in the androgen-sensitive cells. On the other hand, genistein and phloretin were virtually identical in their influence on PC-3 cell growth, whereas daidzein was the least effective in these androgen-insensitive cells, with an IC50 of 100 μM (Table 1). PNT1A cells were more resistant than tumor cells to all compounds studied. None of them reached a 50% inhibition during the time of experiment. To evaluate the influence of androgens in flavonoids’ inhibition of cell growth, LNCaP cells were grown in FBSchst-supplemented media in the presence or absence of 5nM DHT. Thus, after androgen withdrawal in LNCaP cells, an increment in IC50 was observed, particularly when cells were treated with phloretin and apigenin (Supplemental Figure 1), and genistein and daidzein exerted a similar reduction of cell proliferation in the presence or absence of androgens.

Flavonoids change insulin-dependent and -independent glucose uptake

A 2-DG uptake assay was employed to study the effect of flavonoids on glucose uptake in prostate cancer cells (Figure 2, A and B). Twenty minutes after changing media, only phloretin significantly decreased 2-DG uptake in LNCaP cells. However, after 1 hour of incubation, although phloretin still reduced 2-DG uptake, no significant differences were found after genistein or daidzein treatments. Furthermore, even an increase in 2-DG uptake after apigenin treatment was found (Figure 2A). On the contrary, in the case of PC-3 cells, an increase with all treatments but phloretin was found in the first 20 minutes, whereas there was a clear inhibition of 2-DG uptake with phloretin, apigenin, and daidzein after 1 hour of treatment. Differences vanished after 24 hours of culture (Figure 2B). Treatment with apigenin was again the most efficient in reducing glucose uptake in androgen-insensitive prostate cancer cells, because a reduction of more than 90% was found.

A 2-DG uptake assay was again employed to evaluate the effect of flavonoids on insulin-induced glucose uptake in prostate cancer cells. As shown in Figure 2C, differences in insulin sensitivity between cell lines were found. Thus, when cells were incubated with insulin, PC-3 cells showed a higher increase in 2-DG uptake (1.78 ± 0.04) than
In androgen-sensitive cells, treatment with genistein for 1 hour even increased 2-DG uptake in the presence of insulin, whereas phloretin reduced it. On the contrary, in the case of insulin-stimulated PC-3, 2-DG uptake levels significantly decreased when cells were treated with genistein, phloretin, or apigenin. Phloretin and apigenin reduced 2-DG uptake down to the levels found in control cells without insulin.

Figure 1. A and B. Chemical structure of the flavonoids genistein, phloretin, apigenin, and daidzein (A) and proliferation assay after 48 hours of treatment with flavonoids in LNCaP PC-3 and PNT1A cells (B). Results are expressed as mean ± SEM (n = 6); 100% proliferation was assigned to the control group. *, P < .05 vs control; **, P < .01 vs control; ***, P < .001 vs control; ††, P < .01 vs PC-3; †††, P < .001 vs PC-3; +, P < .05 vs PNT1A; ++, P < .01 vs PNT1A; ++++, P < .001 vs PNT1A.

LNCaP cells (1.13 ± 0.01). In androgen-sensitive cells, treatment with genistein for 1 hour even increased 2-DG uptake in the presence of insulin, whereas phloretin reduced it. On the contrary, in the case of insulin-stimulated PC-3, 2-DG uptake levels significantly decreased when cells were treated with genistein, phloretin, or apigenin. Phloretin and apigenin reduced 2-DG uptake down to the levels found in control cells without insulin.
In addition, glucose levels in culture media were measured after 48 hours of treatment (Figure 2D and Supplemental Figure 2). Although no changes were found in net glucose concentration (Supplemental Figure 2), the availability of glucose for each cell was always higher after treatments when concentration was standardized with total protein content because the number of cells was significantly reduced (Figure 2D).

**Flavonoids modulate GLUT1 and GLUT4 expression without changing their subcellular distribution**

Some studies have shown that flavonoids inhibit glucose uptake by blocking GLUTs. However, studies about their effect on GLUT expression are scarce. First, the presence of GLUT1 and GLUT4 was confirmed in LNCaP and PC-3 cells, with GLUT1 and GLUT4 levels being slightly higher in androgen-insensitive PC-3 cells than in androgen-sensitive LNCaP cells at 24 hours after medium re-

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**Table 1.** IC50, Calculated by Hoechst Proliferation Assay, of the Flavonoids Used in LNCaP and PC-3 Cells

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>LNCaP (μM)</th>
<th>PC-3 (μM)</th>
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<tbody>
<tr>
<td>Genistein</td>
<td>15.1</td>
<td>35.3</td>
</tr>
<tr>
<td>Phloretin</td>
<td>25.0</td>
<td>39.4</td>
</tr>
<tr>
<td>Apigenin</td>
<td>7.1</td>
<td>15.7</td>
</tr>
<tr>
<td>Daidzein</td>
<td>35.2</td>
<td>100.0</td>
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*These concentrations were used in all experiments.

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**Figure 2.** A and B, Effect of genistein (Gen), phloretin (Phl), apigenin (Api), and daidzein (Dai) IC50 in 2-DG uptake in LNCaP (A) and PC-3 cells (B) in the first 20 minutes and after 1 and 24 hours of treatment. Flavonoids were employed at the IC50 previously estimated (Table 1). Results are expressed as mean ± SEM (n = 3) and standardized to protein concentration. C, To study effect of insulin (Ins) (100nM) in 2-DG uptake after incubation with flavonoids, LNCaP and PC-3 cells were pretreated for 1 hour with IC50 values of Gen, Phl, Api, or Dai (Table 1) or vehicle (control [Con]) and then were further treated with insulin. A control group without Ins (~Ins) was also incubated. Results are expressed as mean ± SEM (n = 3) and standardized to protein concentration. D, Concentration of glucose at the end of treatments (48 hours) was measured, and the relationship between glucose concentration and protein concentration was obtained. Results are expressed as mean ± SEM (n = 4). An arbitrary value of 1.0 was given to the control group in all experiments. *, P < .05 vs Con; **, P < .01 vs Con; ***, P < .001 vs Con; a, P < .001 vs ~Ins; b, P < .01 vs Con; c, P < .001 vs Con.
newal (Figure 3A). Then, GLUT1 and GLUT4 protein levels were analyzed by Western blot at different incubation times (6, 24, and 48 hours) using IC50 values for cell growth inhibition described above (Table 1).

In LNCaP cells (Figure 3B), GLUT1 decreased after 6 hours of treatment when cells were cultured with genistein and daidzein, but then protein levels increased after 24 and 48 hours. GLUT1 protein levels tended to decrease over time in control groups, and all flavonoids prevented this fall. Protein levels significantly increased after treatment with genistein and phloretin for 24 hours, whereas this increment was observed only after 48 hours of apigenin or daidzein treatment. On the other hand, differences in GLUT4 protein levels were less evident. An important increase was found in LNCaP cells only after 48 hours of culture. Interestingly, the effect of flavonoids on GLUT1 levels was different in PC-3 cells compared with LNCaP cells (Figure 3C). All flavonoids, except daidzein, slightly changed GLUT1 protein levels in PC-3 cells. Only daidzein significantly decreased protein levels after 48 hours of treatment. On the other hand, the most significant changes observed in PC-3 cells were found in GLUT4. After 24 hours of treatment, protein levels were higher with all treatments employed compared with controls (Supplemental Figure 3).

The GLUT1 and GLUT4 subcellular location was analyzed by immunocytochemistry at 24 and 48 hours (Figure 4 and Supplemental Figure 4). As shown, GLUT1 was predominantly located in cell membrane, whereas GLUT4 was found, as expected, in the cytoplasm. We did not observe significant differences in subcellular distribution of GLUTs, contrary to what was found at the protein expression level. Immunocytochemistry confirmed that after incubation with phloretin and apigenin for 48 hours, there was an increase in GLUT1 in LNCaP cells (Figure 4B); however, no differences in subcellular distribution were found.

**Variation in androgen sensitivity of prostate cancer cells changes flavonoids’ effect on cell growth, 2-DG uptake, and GLUT1/4 expression**

Because the effect of flavonoids in glucose uptake and GLUT protein levels were different between LNCaP and PC-3 cells, an androgen-insensitive clone of LNCaP and an androgen-sensitive clone of PC3, namely LNCaP-R and PC-3-AR, respectively, were employed to determine the role of androgen signaling in these differences. First, Hoechst assays after 48 hours showed that the effect of these flavonoids in cell growth were different from their matched cell lines (Figure 5A and Table 2). IC50 values in LNCaP-R was generally higher compared with LNCaP cells, and IC50 values in PC-3-AR were lower compared with PC3 cells, as shown in Table 2. Thus, the genistein, phloretin, and daidzein concentrations required to decrease LNCaP-R proliferation was higher than those necessary to re-

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Figure 3. A, GLUT1 and GLUT4 protein expression in LNCaP and PC-3 cells after 24 hours since medium renewal. Protein expression was determined by Western blot, and β-actin was used as internal standard. An arbitrary value of 1.0 was given to LNCaP cells. B and C, The effect of genistein (Gen), phloretin (Phl), apigenin (Api), and daidzein (Dai) using IC50 values on GLUT1 and GLUT4 protein expression was studied in LNCaP (B) and PC-3 cells (C) after 6, 24, and 48 hours of culture. An arbitrary value of 1.0 was given to control groups. Experiments were repeated at least 3 times, and a representative experiment is shown.
duce LNCaP cell growth. On the contrary, concentrations necessary to inhibit PC-3-AR proliferation were higher than those needed to reduce PC-3 proliferation. Genistein and daidzein showed the highest differences and phloretin and apigenin the lowest.

The effect of flavonoids in 2-DG uptake also changed in these cells (Figure 5B). The 2-DG uptake in LNCaP-R was significantly increased compared with LNCaP cells (1.24 ± 0.02), and the decrease caused by flavonoid treatment was also higher. Phloretin reduced uptake to 5%, being again the most effective compound reducing uptake. On the other hand, PC-3-AR cells showed a 2-DG uptake lower than PC-3 cells (0.74 ± 0.03). The change of 2-DG uptake caused by flavonoids in PC-3-AR cells was similar to that found in LNCaP cells. Phloretin was also the only compound that significantly decreased 2-DG uptake (25% reduction) in PC-3-AR.

Finally, the effect of flavonoids in GLUT1/4 protein levels was also studied at 24 hours (Figure 5C). In LNCaP-R cells, GLUT1 levels were lower than LNCaP cells, and flavonoids changed its expression compared with LNCaP cells. Phloretin was the compound that modified mostly GLUT1 expression, up to 50%. However, the levels of GLUT4 in LNCaP-R cells were higher than in LNCaP cells. GLUT4 was significantly reduced with genistein, phloretin, and daidzein, but it was slightly increased after treatment with apigenin. Also, GLUT1 and GLUT4 levels were lower in PC-3-AR cells than in PC-3 cells. Although genistein and daidzein increased GLUT1 levels in PC-3-AR cells, all compounds recovered GLUT4 expression to PC-3 control levels.

Flavonoids interact with the same site of the GLUT XylE

XylE, an Escherichia coli homolog of GLUT1 to -4, has been recently crystallized. This prompted us to use it as a model to study the interaction of the 4 flavonoids with GLUTs when bound to D-glucose in silico. According to docking study, all compounds seemed to interact with the transporter in the same location and that position was located previous to the active site of the receptor where D-glucose is bound (Figure 6, A–C). According to the docking model, most of the amino acids at which all compounds attach were identical, sharing 50% of the sequence. More interestingly, some of these amino acids are preserved in GLUT1 and GLUT4 (Figure 6D). Only 2 amino acids (Ser/Thr58 and Thr/Ile28) differ between both GLUTs at these positions. One of them was not preserved (Gly in XylE instead of Ser in GLUT1 and Ala in GLUT4) and the other was preserved only in GLUT1 (Thr in XylE and GLUT1, and Ile in GLUT4).
Some natural compounds, including flavonoids, are able to modify glucose uptake by regulating GLUT1 expression and/or altering glucose binding to them (34). In most of the studies published, a single compound is described instead of comparing more than one to relate their properties to their molecular structures. In this study, we chose 4 flavonoids whose effect in glycolytic metabolism has been previously described in other cell types. Genistein and daidzein are isoflavones that differ in a hydroxyl group. Apigenin has a benzene ring in position 2 instead of position 3 of the central ring like genistein. Phloretin is similar to apigenin with an open structure (see Figure 1A). These minor differences have been previously proposed to explain their different effects on cancer cells (35).

GLUT1 expression is increased in cancer cells and is responsible for basal transport of glucose (36). GLUT4 expression in cancer cells has been less studied, but it is well known that its expression is regulated by the IGF-1 pathway in some cancer cells such as MCF-7 (37, 38). In prostate cancer cells, GLUT1 as well as other GLUTs such as GLUT3, GLUT5, GLUT11, and GLUT12 have been previously reported to be overexpressed (12, 39, 40), whereas GLUT4 expression has not been described yet. GLUT1 is inhibited in a competitive way by genistein in human promyelocytic leukemia cells and erythrocytes and in Chinese hamster ovary (CHO) cells overexpressing GLUT1 (41). GLUT1 is also inhibited by phloretin in several cell types (42) and by apigenin in human pancreatic cells (43), unlike daidzein (44). On the other hand, GLUT4 is inhibited competitively by genistein and phloretin (45). However, changes in the expression of GLUTs by these natural compounds are scarcely studied, nor they have been studied in depth in prostate cancer. There are studies showing that apigenin modifies GLUT1 protein levels in human pancreatic cancer cells (46) and daidzein increases GLUT4 mRNA and promotes glucose uptake through GLUT4 translocation in adipocytes (47, 48).

Our data show that basal GLUT protein levels are higher in androgen-insensitive PC-3 cells than in androgen-sensitive LNCaP cells and that flavonoids are usually more potent in reducing cell growth in androgen-sensitive than in androgen-insensitive prostate cancer cells. Not

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**Table 2.** IC\textsubscript{50}, Calculated by Hoechst Proliferation Assay, of the Flavonoids Used in LNCaP-R and PC-3-AR Cells

<table>
<thead>
<tr>
<th>Concentration (µM)a</th>
<th>LNCaP-R</th>
<th>PC-3-AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>45.3</td>
<td>21.5</td>
</tr>
<tr>
<td>Phloretin</td>
<td>29.4</td>
<td>37.1</td>
</tr>
<tr>
<td>Apigenin</td>
<td>19.3</td>
<td>13.7</td>
</tr>
<tr>
<td>Daidzein</td>
<td>99.5</td>
<td>71.0</td>
</tr>
</tbody>
</table>

a These concentrations were used in all experiments.
only is a higher concentration required to reduce PC-3 cell proliferation, but also higher IC50 values were found when comparing LNCaP-R with LNCaP cells and lower when comparing PC-3-AR with PC-3 cells. As mentioned above, isoflavones genistein and daidzein differ only in a hydroxyl group, and both are botanical estrogens that display estrogenic activities. Our results show that particularly genistein and daidzein have different biological activities in prostate cancer cells when they depend on androgen signaling (49). Thus, the effect of these flavonoids in prostate cancer cells might be related to their estrogenic activity (50, 51). Phloretin and apigenin are flavonoids that change their activity to a lesser extent between LNCaP vs LNCaP-R and PC-3 vs PC-3-AR cells.

**Figure 6.** A, Docking model studies of genistein (Gen) (brown), phloretin (Phl) (red), apigenin (Api) (yellow), and daidzein (Dai) (violet) binding to the XylE transporter. This transporter was used for its high homology with GLUT1 and GLUT4. B, Location of flavonoids in this receptor is the same, previous to the active site where glucose (blue) is bound. C, Most of the amino acids of XylE involved in the interaction with these flavonoids are the same, with 50% being common. Amino acids that interacted with each compound are marked with a check and some of these amino acids are preserved both GLUT1 and GLUT4 transporters (D).
and this could be explained by their weaker estrogenic activity (52).

Half-lives of GLUTs are about 6 to 24 hours (53). Here we found an increase in GLUT1 levels after 24 and 48 hours of treatment with each flavonoid, whereas a reduction is observed in control cells with time of culturing in LNCaP cells. As a result of blocking, 2-DG uptake seems to be a concomitant positive feedback leading to an increase in protein levels. A similar rise in GLUT4 after flavonoid incubation is observed in PC-3 cells. In this case, shortly after incubation, 2-DG uptake is reduced in PC-3 cells incubated with phloretin, apigenin, or daidzein, although this effect disappears after 24 hours. This also confirms that blocking the transporter by flavonoids might promote GLUT overexpression, which finally eliminates differences in 2-DG uptake. LNCaP-R and PC-3-AR cells confirm that GLUT4 regulation by flavonoids is increased in androgen-independent prostate cancer cells, whereas GLUT1 expression is less regulated.

The regulation of glucose uptake by flavonoids in LNCaP seems to be more complex than in PC-3 cells. A significant reduction of glucose uptake was found, but this reduction occurs after 24 hours of incubation. In this case, androgenic signaling might play an important role given LNCaP hormone dependence and its particular importance in the glycolytic metabolism in prostate cancer cells (12).

A particular characteristic of 2-DG uptake after treatment is that there is a faster decrease in LNCaP than in PC-3 cells, and then a recovery or even an increase. In LNCaP cells, there is a decrease with all flavonoids in the first 20 minutes, which is not observed in PC-3 cells. It is possible that due to a faster metabolism of PC-3 cells, that first decrease will occur even before. PC-3-AR cells have the same dynamic profile than that observed in LNCaP cells, so androgen receptor pathway seems to be related to glucose uptake.

Surprisingly, the measurement of glucose levels in culture media showed no variation after 48 hours. However, glucose bioavailability considerably increased in flavonoid-treated cells because after 48 hours, each compound reduced over 50% the number of cells in culture plates. Consequently, at this time, there are still differences in the expression of both receptors that might be explained considering the glucose concentration in culture media. More importantly, insulin experiments show that GLUT4, which was not previously described in prostate cancer cells, not only is present but also participates actively in glucose uptake. It also seems that GLUT4 is more important in glucose uptake in androgen-insensitive than in androgen-sensitive prostate cancer cells. LNCaP-R cells show higher levels of GLUT4 than LNCaP cells. On the contrary, PC-3-AR show lower levels than PC-3 cells, which implies that GLUT4 levels are higher when prostate cancer cells are androgen-independent for their growth. GLUT4 protein, in both LNCaP and PC-3 cells, was found to be mainly accumulated in the cytoplasm, but it was not exposed on the cell surface as expected. However, a small amount of receptor still appears in the cell membrane because they can be translocated by IGF-1, and a small amount of insulin is present in FBS. Lack of alterations in subcellular location of GLUTs after 24 and 48 hours could be explained by regulatory mechanisms. Treatments with these natural compounds change only GLUT1 protein levels without altering their location (this being its mechanism of regulation) and GLUT4 remains in the cytoplasm (this being its mechanism of regulation). Differences between treatments in glucose uptake and GLUT expression could be also explained by the concentrations of compounds used and their inhibition constant. Compounds that block glucose transport also decrease glycosylation levels, and as a result, GLUT levels are lower (54). This could be the reason why GLUT1 levels are decreased after treatment with daidzein. Kinetic studies are scarce, but their effect in the inhibition of GLUTs are surely different and might also explain our results.

The blocking effect of GLUTs by these natural compounds is in part validated by docking assays. Until recently, no GLUT homolog structure had been elucidated by x-ray crystallization, which limited considerably the docking studies. Recent crystallization of xylose transporter XylE from E. coli has provided insights into the structure of the closely related GLUT family of transporters. By using this model, we have shown that natural compounds included in our study theoretically bind to this receptor in a region located near the active site center, therefore preventing glucose binding. This site is preserved in both GLUT1 and GLUT4. In addition to drug concentration, androgenic metabolism, or GLUT kinetics, slight binding variations could account for differences in glucose uptake, which should be further addressed.

In conclusion, we show for the first time that the effect of these flavonoids in glucose uptake and GLUT1 and GLUT4 protein expression is dependent on the phenotype of prostate cancer cells in addition to their slight variations in chemical structure. In that sense, isoflavones such as genistein and daidzein are much more efficient in reducing cell proliferation in androgen-sensitive LNCaP and PC-3-AR cells than in androgen-insensitive PC-3 or LNCaP-R cells. Apigenin and phloretin are the most efficient in reducing glucose uptake and in modifying GLUT levels, which (apparently) accounts for the highest antiproliferative effect shown.
Acknowledgments
We thank Marta Alonso-Guervós (Servicios Científicos-Técnicos, Facultad de Medicina, Universidad de Oviedo) for her kind assistance with confocal microscopy and Iván González-Pola (Departamento de Morfología y Biología Celular, Universidad de Oviedo) for his helpful technical assistance.

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P.G.-M. is currently supported by Formacion del Personal Universitario grant from Ministerio de Educacion, Cultura y Deporte, Gobierno de España (AP2012-4924). A.-R.G.-G. is currently supported by a Severo Ochoa Fundacion Para el Fomento a la Excelencia grant from Ministerio de Educacion, Cultura y Deporte, Gobierno de España (AP2012-4924). D.H. acknowledges Instituto Universitario de Oncología del Principado de Asturias sponsorship.

Disclosure Summary: The authors have nothing to disclose.

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