ORIGINAL PAPER

Endocrine Disrupting Activities of the Flavonoid Nutraceuticals Luteolin and Quercetin

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Abstract Dietary plant flavonoids have been proposed to contribute to cancer prevention, neuroprotection, and cardiovascular health through their anti-oxidant, anti-inflammatory, pro-apoptotic, and antiproliferative activities. As a consequence, flavonoid supplements are aggressively marketed by the nutraceutical industry for many purposes, including pediatric applications, despite inadequate understanding of their value and drawbacks. We show that two flavonoids, luteolin and quercetin, are promiscuous endocrine disruptors. These flavonoids display progesterone antagonist activity beneficial in a breast cancer model but deleterious in an endometrial cancer model. Concurrently, luteolin possesses potent estrogen agonist activity while quercetin is considerably less effective. These results highlight the promise and peril of flavonoid nutraceuticals and suggest caution in supplementation beyond levels attained in a healthy, plant-rich diet.

Introduction

Diets rich in fruits and vegetables are associated with lower incidence of disease including cancer and cardiovascular disease. Flavonoids are major components of plant-rich diets and active ingredients of Chinese herbal medicines. Myriad beneficial activities have been ascribed to flavonoids. These activities may contribute to a reduced risk of cancer, the benefits of a Mediterranean diet, and may explain the "French paradox", the low incidence of cardiovascular mortality despite ingestion of a high fat diet associated with consumption of red wine [1–4]. As natural products, flavonoids are marketed as supplements for relief of menopausal symptoms, chronic fatigue syndrome, autism, inflammatory syndromes, cancer prevention, and more. Consumption of flavonoids via supplements may far exceed the amount ingested via a normal diet.

The flavone luteolin and related flavonoids such as quercetin have anti-oxidant, anti-inflammatory, and antiproliferative activities and are found widely in fruits and vegetables [3–5] (http://www.nal.usda.gov/fnic/foodcomp/Data/Other/AICR03_VegFlav.pdf). The hydrophobic ring structure of these flavonoids and the presence of hydroxyls as potential hydrogen bond donors prompted us to assess their activity on steroid signaling. The studies reported here demonstrate that luteolin and quercetin display multifunctional endocrine-disrupting activities at levels achievable by oral consumption. The implication of these results toward the use of flavonoids as supplements is discussed.

Materials and Methods

Reagents Flavonoids were obtained from R&D Systems and suspended in DMSO at 20 mM. The antibodies used are: rabbit anti-cytokeratin 5, Epitomics, 2290–1, used at 1:250; fluorescent goat anti-rabbit, InVitrogen, A-11037, used at 1:250; and mouse anti-progesterone receptor antibody [6], Dako, PgR 1294 (1:1,000), fluorescent goat anti-mouse, LiCor 926–32210 (1:5,000).

Cell Lines All T47D cells lines used express progesterone and estrogen receptors. T47D (A1-2) cells (Fig 1b) also express glucocorticoid receptors [7]. Experiments shown in

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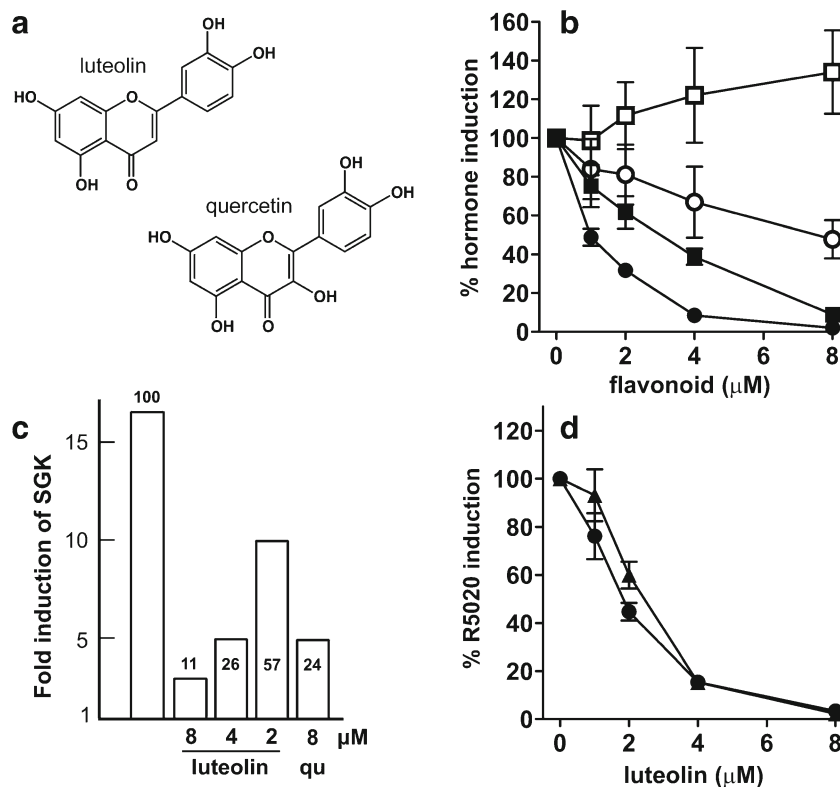
Fig. 1 **a** Structures of the flavone, luteolin, and the flavonol, quercetin. **b** The effect of the flavonoids luteolin and quercetin on steroid signaling. T47D (A1-2) cells were treated with 1 nM R5020 (*filled symbols*) or 1 nM dexamethasone (*open symbols*) and the indicated concentration of luteolin (*circles*) or quercetin (*squares*) or vehicle for 20 h. Induction of endogenous alkaline phosphatase in the absence of flavonoid is defined as 100 % (see "Materials and Methods"). **c** Flavonoid inhibition of progestin-mediated induction of a classical progestin target gene, serum- and glucocorticoid-regulated kinase (*SGK*). CK5Pro-Fluc-

T47D cells were treated with 1 nM R5020 or vehicle with the indicated concentration of luteolin or quercetin (*qu*). Induction of SGK RNA was assessed by qRT-PCR. The *number on each bar* is the induction relative to cells treated with hormone alone which is set at 100. **d** Luteolin inhibition of the progestin-mediated induction of the cytokeratin 5 promoter. Cytokeratin 5-luciferase (*triangles*) or endogenous alkaline phosphatase (*circles*) induction by 1 nM R5020 in CK5Pro-Fluc-T47D cells with the indicated concentration of luteolin

Figs. 1c, d, 2, and 5 employed CK5Pro-Fluc-T47D cells that contain a stably integrated firefly luciferase reporter gene driven by the cytokeratin 5 promoter [8]. T47DKBluc cells (Fig. 3) contain a synthetic estrogen-responsive promoter-luciferase reporter [9]. The Ishikawa cell line [10] used in experiments shown in Fig. 4 expresses both estrogen and progesterone receptors.

Alkaline Phosphatase and Luciferase Gene Activity T47D cell lines were plated into opaque 96 well dishes (Nunc 136101) at 50,000 cells per well for 24 h before treatment with 1 nM steroid hormones, R5020, dexamethasone, or 17β-estradiol for 20 h. For T47D-KBluc cells cultures were transferred to phenol red-free, estrogen-depleted medium before plating. For assay, cells were washed twice then harvested in 40 μl lysis buffer (1 % Triton X-100, 10 % glycerol, 20 mM K_2HPO_4 pH 7.8). Half of the lysate was transferred to another 96-well plate (Greiner 655075) for assay of alkaline phosphatase. Assay reagent, 60 μl of 2× buffer (0.2 M diethanolamine pH 9.5, 2 mM $MgCl_2$), 26 μl H_2O, 12 μl Emerald II, and 2 μl

CSPD (Life Technologies) were added per well. After 30 min, luminescence was assessed using a BioTek plate reader and Gen5 Software. For luciferase assay, 50 μl luciferin reconstituted in buffer (Promega) is injected into each well of the original plate and light signal captured for 10 s following a 2-s delay. Four to six independent wells were assessed for each condition. To quantify antagonism, induction percentage was calculated by dividing net hormone-induced activity in the flavonoid-treated conditions by the net hormone-induced activity without flavonoid multiplied by 100. Net activity is the activity in the presence of hormone minus the activity in vehicle-treated controls. Neither quercetin nor luteolin induced alkaline phosphatase nor cytokeratin 5-luciferase activity on their own at the concentrations used down to a sensitivity of less than 0.5 % that of hormone at concentrations up to 10 μM. For estrogen agonist activity, induction percentage was calculated by dividing net activity in flavonoid-treated cells by net activity in cells treated with 1 nM 17β-estradiol multiplied by 100. Standard deviations are shown where larger than the symbol. Induction of alkaline phosphatase
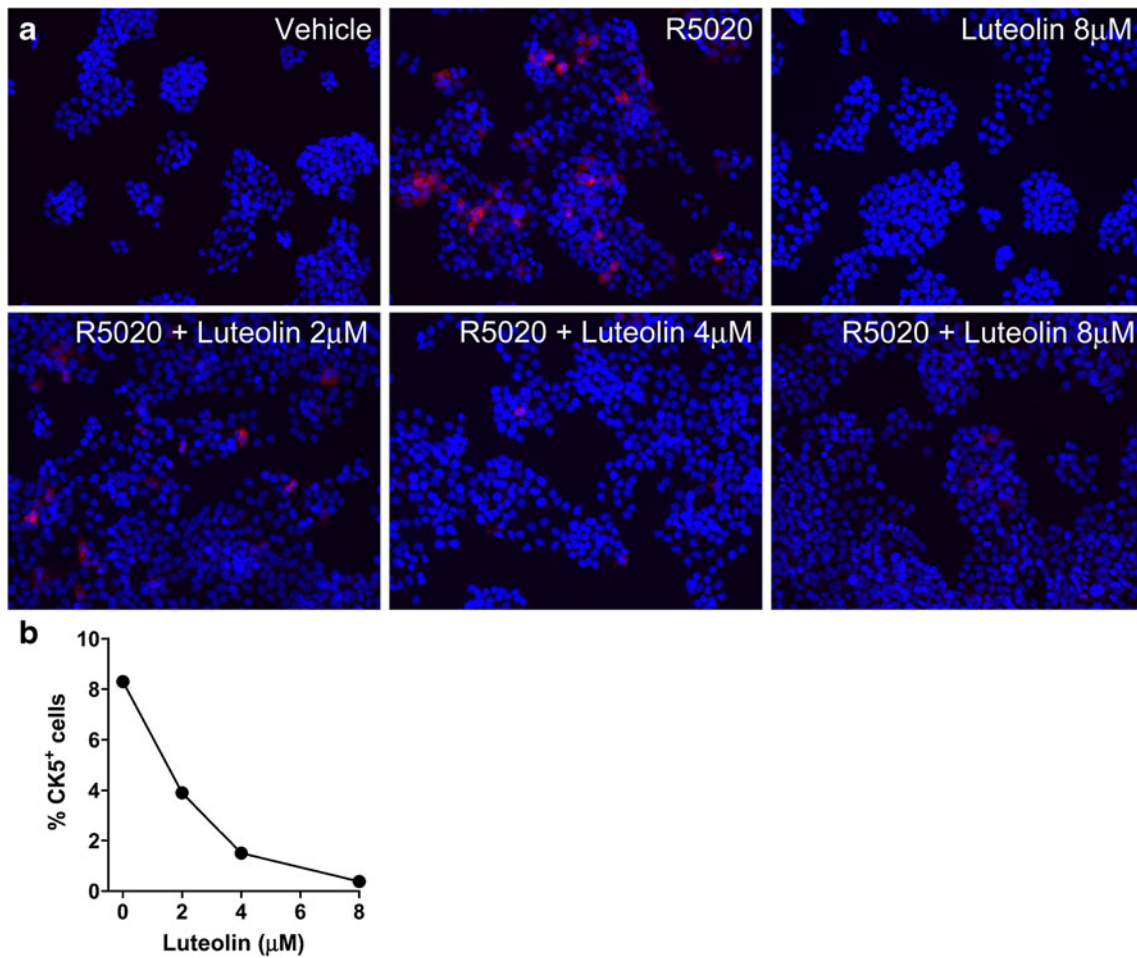
Fig. 2 a Dose-dependent inhibition of progestin induced cytokeratin 5 expression by luteolin in CK5Pro-Fluc-T47D cells treated with 1 nM R5020 or vehicle and the indicated concentration of luteolin for 24 h

before fixation. Cytokeratin 5-expressing cells are stained with Texas Red and nuclei are counter stained with DAPI. **b** Quantitation of dose-dependent inhibition by luteolin of R5020-induced cytokeratin 5 expression

was 40–60-fold over untreated cells for R5020 and 10-fold for dexamethasone. Induction of cytokeratin 5-luciferase was over 200-fold.

Immunofluorescence Two hundred thousand CK5Pro-Fluc-T47D cells were plated in six-well dishes containing sterile coverslips for 48 h. Cells were treated with hormone and/or
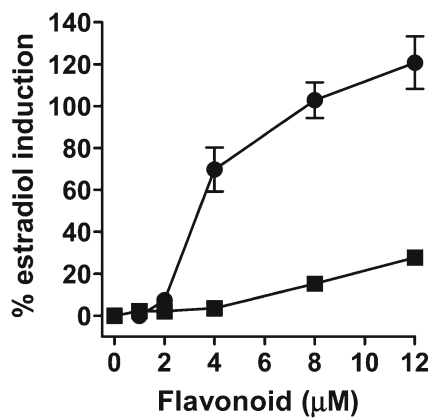
Fig. 3 Estrogen agonist activity of luteolin and quercetin. T47D KBluc cells were treated with luteolin (*circles*), quercetin (*squares*), or vehicle for 20 h. Induction of luciferase activity by 1 nM 17β-estradiol is defined as 100 %
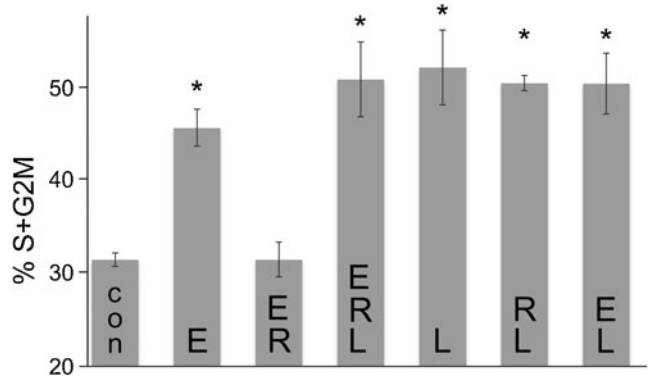
Fig. 4 Luteolin abolishes the progestin-mediated block of estrogen-stimulated cell growth in Ishikawa endometrial cancer cells. Cells were treated for 24 h with the indicated combinations of 1 nM 17β estradiol (*E*); 1 nM R5020, (*R*); 8 µM luteolin (*L*); or vehicle. *$p \leq 0.001$ compared to vehicle-treated control (*con*)

luteolin for 24 h before fixing. For quantitation, a total of 1,000–3,000 cells in two to six fields per condition were scored for detectable cytokeratin 5 expression .

Western Blot 300,000 CK5Pro-Fluc-T47D cells were plated in six-well dishes for 48 h then treated with 1 nM R5020 and/or luteolin (8 μM) for 24 h before harvest. Western blots were imaged on a LiCor Odyssey Infrared imager.

Statistical Analysis Hormone induction is defined as the luciferase or alkaline phosphatase activity in the presence of hormone minus the activity in the absence of hormone. Percent induction is hormone induction in the presence of flavonoid divided by the hormone induction of vehicle-treated controls times 100. The calculation of standard deviation of percent hormone induction values accounts for the error propagation arising from the multiple independent variables that comprise percent induction.

RNA Isolation and qRT-PCR Analysis Total RNA was isolated using the RNeasy Plus kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed in a total volume of 20 μL using MMLV reverse transcriptase (Promega, Madison, WI). Real-time PCR was performed on 1/10 of the synthesized cDNA using the oligonucleotide primers SGK-F (5′-TGCAGAAGGACAGGACAAAG-3′) and SGK-R (5′-GACAGGCTCTTCGGTAAACTC-3′) for SGK and Bact1219U (5′-GTTGCGTTACACCCTTTCTTGA-3′) and Bact1552L (5′-AATGCTATCACCTCCCCTGTG-3′) for β-actin and SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Amplification signals were detected with an ABI 7500 Real-Time PCR system (Perkin Elmer, Waltham, MA). Fold change in expression was calculated using the comparative Ct method [11]. Values were calculated according to the following equation: Fold change=$2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct=\Delta Ct1-\Delta Ct2$. $\Delta Ct1=(Ct_{\text{SGK, treated}}-Ct_{\text{β-actin, treated}})$ and $\Delta Ct2=(\Delta Ct_{\text{SGK, control}}-\Delta Ct_{\text{β-actin, control}})$.

Flow Cytometry After 36 h of serum and steroid starvation, Ishikawa cells were treated with flavonoids and/or steroid hormones for 24 h. Cells were then harvested, washed with cold PBS, and incubated with Krishan stain for 12–16 h [12]. Flow cytometry was performed using a Beckman Coulter FC500 at the University of Colorado Cancer Center Flow Cytometry Shared Resource. The percentage of cells in each cell cycle phase was determined using ModFit LT (Verity Software House) software.

Modeling Docking of luteolin and other related ligands to PR was performed using Autodock Vina [13]. The protein model for docking was based on the structure of progesterone receptor bound to the antagonist asoprisnil (PDB IDs 2OVM and 2OVH) [14]. Residues within 4 Å of asoprisnil were compared to 11 other PDB entries for PR solved in the agonist induced state, and those residues that showed significant deviation in their conformations between structures were allowed to adapt their conformation during docking calculations. The structure of the protein and ligands were prepared for docking calculations using AutodockTools [15]. Docking calculations were repeated four times starting with different random conformations of the luteolin ligand. In each calculation, the conformation with the lowest predicted binding energy was essentially identical.

Results

We have previously employed the T47D (A1-2) cell line to screen for novel effectors of progestin and glucocorticoid signaling [16]. This line has been engineered to express glucocorticoid receptors at levels comparable to the levels of endogenous progesterone receptors [7]. Activity of receptors is easily screened by assessing hormone induction of the endogenous tissue non-specific alkaline phosphatase whose enzyme activity can be readily assessed by a lumimetric assay. This gene is strongly induced by progestins (40–60-fold) and glucocorticoids (10-fold). Cells were treated with the flavones luteolin or quercetin (Fig. 1a). Luteolin strongly inhibited induction of endogenous alkaline phosphatase by the strong, synthetic progestin, R5020 (EC$_{50}$ 1–2 μM). Luteolin also inhibited glucocorticoid signaling, albeit less potently. Quercetin, with its additional hydroxyl, displayed less potent anti-progestin activity than luteolin and failed to inhibit glucocorticoid-dependent alkaline phosphatase expression (Fig. 1b). Thus, antagonism of steroid signaling is receptor and structure specific. No agonist activity was detected at doses up to 10 μM with a sensitivity down to 0.5 % that of 1 nM R5020.

To ensure that luteolin and quercetin were acting at the level of RNA, we assessed R5020-mediated induction of a classical progesterone target, the gene for the serum- and glucocorticoid-regulated kinase (SGK). R5020 induced SGK over 16-fold and luteolin inhibited this induction in a dose-dependent fashion. Quercetin inhibited SGK expression also but less potently than luteolin (Fig. 1c). These data exhibit a very similar pattern to that shown for the flavonoid-mediated inhibition of endogenous alkaline phosphatase enzyme activity.

Results of the Women's Health Initiative and the Million Women studies indicate that in the context of menopausal women receiving long-term hormone replacement therapy, progestins increase breast cancer incidence and mortality [17, 18]. Progestins have been shown to induce a population of drug-resistant, basal-like, tumor-initiating cells [8, 19]. Thus, the progestin *antagonist* activity of luteolin may be

beneficial in this context. These tumor-initiating cells are characterized by hormone-induced expression of cytokeratin 5. We utilized T47D cells engineered to contain a cytokeratin 5 promoter driving a luciferase reporter [8] to determine the effect of luteolin on this population. As shown in Fig. 1d, luteolin inhibits progestin-dependent induction of luciferase with a dose response similar to that of the inhibition of endogenous alkaline phosphatase.

Direct immunofluorescence analysis of cytokeratin 5 expression showed that R5020 increased the number of cytokeratin 5-positive cells from 0.2 to 8 % (Fig. 2). This increase was inhibited by luteolin in a dose-dependent fashion. Co-treatment with 8 μM luteolin almost entirely abrogated the R5020-induced increase in the fraction of cytokeratin 5-positive cells but inhibition was substantial at even lower doses (Fig. 2). Notably, these functional levels of luteolin in the low micromolar range are below that needed for many of its reported effects in vitro. Most of these reported activities, which are used to justify usage as supplements, are significant only at levels of 10 μM or higher. Levels in the range of several micromolar are achievable by dietary supplementation [20–22] but effects imposed only at 10 μM or above may not be physiologically meaningful in vivo.

Together these data suggest a potential role for luteolin to suppress progestin-mediated induction of tumor cells with enhanced progenitor properties; however, this beneficial activity is countered by the fact that luteolin also acts as an estrogen agonist in a similar dose range (Fig. 3). Thus, luteolin acts as a multi-functional endocrine disruptor, imposing estrogenic activity while concurrently antagonizing progesterone and glucocorticoid signaling.

Estrogens promote growth in both the uterine and breast epithelium. Treatment of estrogen receptor-positive Ishikawa endometrial cancer cells with 17β-estradiol drives them into cell cycle as evidenced by an increase in the fraction of cells in S+G2M (Fig. 4). Unlike breast, where progestins may promote cancer, progesterone provides a critical brake on endometrial growth driven by estrogens [23–25]. In normal tissue, progestins inhibit estrogen-driven growth of the uterine epithelium through an interplay with stromal components [26]. However, in Ishikawa cells, progestins can inhibit estrogen-stimulated growth directly. The 17β-estradiol-mediated increase in cells in S+G2M is blocked by concurrent treatment with R5020 (Fig. 4). When 17β-estradiol, R5020, and luteolin are added concurrently, the growth inhibitory effect of R5020 is abrogated by the progestin-antagonist activity of luteolin. In Ishikawa cells, as in the breast model, luteolin alone has estrogen agonist activity. This activity drives an increase of Ishikawa cells into S+G2M as effectively as 17β-estradiol (Fig. 4). R5020 is again unable to inhibit this increase due to the coincident progestin–antagonist activity of luteolin. Thus, luteolin has two deleterious activities in this endometrial cancer model. It stimulates growth via estrogen agonist activity and abrogates the protective effect

of progestins via progestin antagonist activity. This dual activity suggests supplementation with luteolin be contraindicated for women at risk for endometrial cancer.

A potential mechanism by which luteolin may antagonize progesterone receptor signaling is simply to reduce progesterone receptor expression. At high levels (10–100 μM), the flavonoid quercetin has been reported to reduce expression of the androgen receptor in LNCaP prostate cancer cells [27]. In contrast, we found that 8 μM luteolin, a dose that almost completely abrogates the activity of 1 nM R5020, has no effect on levels of progesterone receptor in the absence of R5020 and inhibits the modest agonist-mediated downregulation observed (Fig. 5). The antagonism of hormone-dependent gene induction by luteolin is diminished by increasing doses of agonists, R5020 or progesterone, consistent with luteolin acting via a competitive binding mechanism. For this reason and because plant-derived isoflavones like genistein are known to bind to estrogen receptors, we have performed molecular modeling of luteolin interaction with the ligand binding domain of the progesterone receptor.

Modeling luteolin binding using the crystal structure of an antagonist-progesterone receptor complex [14] yields a series of high affinity poses, the most avid of which has a predicted K_D of 80–90 nM in multiple calculations. Luteolin binding to the antagonist conformation of the receptor is stabilized by an interaction with Glu723 (Fig. 6a). This interaction is unavailable in the agonist-bound conformation of receptor because Glu723 is positioned to stabilize helix 12 by capping the helix dipole [28]. The agonist conformation of helix 12 is further disfavored in this predicted conformation of luteolin due to steric interference with methionine 909 of helix 12 (Fig. 6b). Thus, the modeling predicts that luteolin precludes helix 12 from assuming a position that permits coactivator binding much like steroidal antagonists with bulky substitutions at the 11 position of the C ring [14, 29].

Discussion

Isoflavones, most notably genistein, present in certain forage crops have long been recognized to interfere with the reproductive capacity of livestock [30, 31] and to display estrogenic
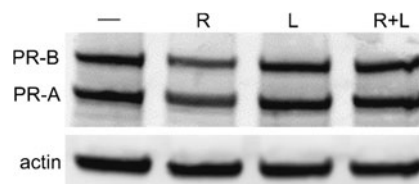
Fig. 5 Luteolin does not inhibit progesterone receptor expression itself and blocks agonist-mediated downregulation. Western blot of progesterone receptor levels at 24 h following treatment of CK5Pro-Fluc-T47D cells with vehicle or hormone and/or luteolin. *PR*, progesterone receptor; *R*, 1 nM R5020, *L*; 8 μM luteolin
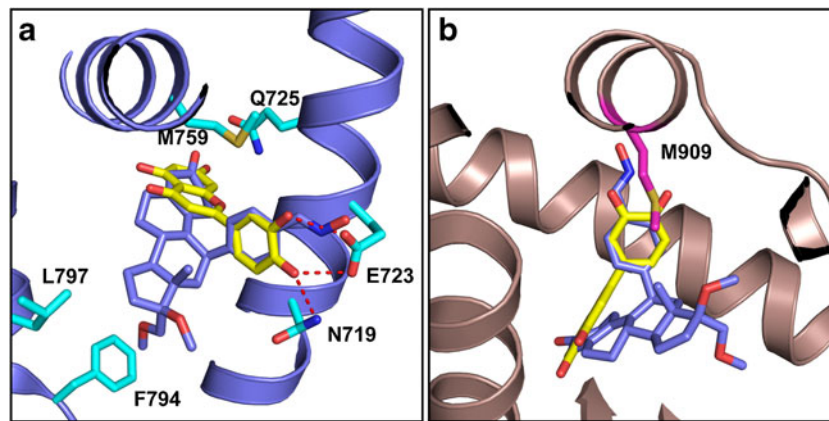
Fig. 6 Predicted binding mode of luteolin to progesterone receptor. In panel **a**, the predicted lowest energy binding mode of luteolin (*yellow*) is shown in comparison to the crystal structure of asoprisnil (*blue*) [14]. Residues in *cyan* were allowed to adapt their conformation during docking. Potential hydrogen bond interactions with Glu273 that caps helix12 in the activated state and with Asn719 are shown as *dashed red lines*. Panel **b** illustrates the clash between luteolin (*yellow*) and methionine 909 when helix 12 is in an agonist conformation. Asoprisnil (*blue*) is shown for comparison

activity [32–35]. This has led to many studies assessing the estrogenicity of plant-derived polyphenolic compounds, luteolin and quercetin among them [36–40]. Screening for other hormonal activities has received less attention. Luteolin exhibits weak androgen antagonist activity [41] as does apigenin, a flavone closely related to luteolin [41, 42]. Apigenin binds to progesterone receptors though, curiously, binding by luteolin was not detected [43]. Apigenin has been reported to possess progestational activity [44, 45] or both progestin agonist and antagonist activity [42]. Apigenin inhibits the growth of progestin-dependent tumor models in vivo consistent with progesterone antagonist activity [46, 47]. These tumor studies attest to the biological activity of orally administered flavones in vivo.

The present studies highlight the potential of flavonoids, especially luteolin, to act as multi-functional endocrine disruptors. Luteolin displays potent progesterone antagonist and estrogen agonist activities and weaker glucocorticoid antagonist activity. Quercetin exhibited weaker estrogen agonist activity and progestin antagonist activity than luteolin but little anti-glucocorticoid activity. Notably, these activities of quercetin and particularly luteolin are significant at low micromolar levels, levels achievable by supplementation in vivo [20–22]. Many of the myriad activities attributed to flavones that are significant at levels of 10 μM and above may have little physiologic relevance in vivo. These results underscore the need for further studies in vivo to assess the complexities of flavonoid pharmacokinetics and the potential of flavonoids and their metabolites to impose or disrupt hormonal activities.

Nutraceuticals are the basis of a multi-billion dollar business but, as natural compounds universally present in plant matter and consumed in food, they are subject to limited oversight. Despite a wealth of data supporting the benefits of a plant-rich diet and many studies showing beneficial effects in pre-clinical systems, evidence supporting the value of supplementation with purified flavonoids is lacking. Here our studies reveal that luteolin, a flavone marketed for a variety of therapeutic applications, including pediatric applications, has potent multi-functional endocrine-disrupting activity. Luteolin displays estrogen agonist activity that can drive cell growth in estrogen-dependent tissues. Additionally, luteolin can simultaneously act as a progesterone antagonist at physiologically attainable levels. This progestin antagonist activity is beneficial in a breast cancer model, inhibiting the progestin-stimulated increase in a population of cells with stem-like or tumor-initiating properties, but deleterious in an endometrial cancer model, blocking the progestin-mediated brake on estrogen-driven growth. These studies highlight the promise and peril of supplementation with nutraceuticals and suggest caution in supplementing well beyond the intake of a normal, healthy diet.

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