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Urolithins, Ellagic Acid-Derived Metabolites Produced by Human Colonic Microflora, Exhibit Estrogenic and Antiestrogenic Activities

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Urolithins A and B (hydroxy-6*H*-dibenzo[*b,d*]pyran-6-one derivatives) are colonic microflora metabolites recently proposed as biomarkers of human exposure to dietary ellagic acid derivatives. Molecular models suggest that urolithins could display estrogenic and/or antiestrogenic activity. To this purpose, both urolithins and other known phytoestrogens (genistein, daidzein, resveratrol, and enterolactone) were assayed to evaluate the capacity to induce cell proliferation on the estrogen-sensitive human breast cancer MCF-7 cells as well as the ability to bind to α- and β-estrogen receptors. Both urolithins A and B showed estrogenic activity in a dose-dependent manner even at high concentrations (40 μM), without antiproliferative or toxic effects, whereas the other phytoestrogens inhibited cell proliferation at high concentrations. Overall, urolithins showed weaker estrogenic activity than the other phytoestrogens. However, both urolithins displayed slightly higher antiestrogenic activity (antagonized the growth promotion effect of 17-β-estradiol in a dose-dependent manner) than the other phytoestrogens. The IC_{50} values for the ERα and ERβ binding assays were 0.4 and 0.75 μM for urolithin A; 20 and 11 μM for urolithin B; 3 and 0.02 for genistein; and 2.3 and 1 for daidzein, respectively; no binding was detected for resveratrol and enterolactone. Urolithins A and B entered into MCF-7 cells and were metabolized to yield mainly urolithin-sulfate derivatives. These results, together with previous studies regarding absorption and metabolism of dietary ellagitannins and ellagic acid in humans, suggest that the gut microflora metabolites urolithins are potential endocrine-disrupting molecules, which could resemble other described "enterophytoestrogens" (microflora-derived metabolites with estrogenic/antiestrogenic activity). Further research is warranted to evaluate the possible role of ellagitannins and ellagic acid as dietary "pro-phytoestrogens".

KEYWORDS: Breast cancer; phytoestrogen; hydroxy-6*H*-dibenzo[*b,d*]pyran-6-one derivative; endocrine-disrupting; estrogen receptor

INTRODUCTION

There is a great concern about the potential of the so-called "endocrine-disrupting chemicals" (EDCs) to either mimic or inhibit endogenous hormones such as estrogens and androgens. Kavlock et al. defined an EDC as an "exogenous agent that interferes with the production, release, transport, metabolism, and binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (*1*). These compounds are present in the environment and range from natural plant-derived molecules to man-made substances, such as pharmacological agents and organochlorine pesticides. EDCs may behave physiologically as agonists like endogenous estrogen, oppose estrogen action as an antagonist, or even have mixed agonist properties, behaving as agonists in some tissues and as antagonists in others. This mixed agonist/antagonist effect is characteristic of a new and potentially therapeutically useful class of EDCs, termed "selective estrogen receptor modulators" (SERMs) (*2*). These compounds are under intense research for their potential to treat estrogen-related conditions such as malignancies and osteoporosis and to alleviate the symptoms associated with menopause.

From a dietary point of view, the plant-derived EDCs ("phytoestrogens") have attracted the attention as "natural" SERMs. Currently, four different classes of phytoestrogens have been dientified: the stilbenes (mainly resveratrol), lignans (converted to enterolactone and enterodiol by gut microflora), coumestans (predominantly coumestrol), and isoflavonoids, which are by far the most studied phytoestrogens (among others, genistein and daidzein, as well as the microflora-derived metabolites such as equol).

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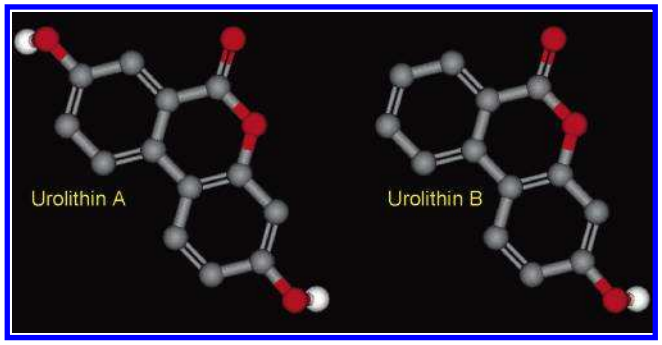

Figure 1. Structures of urolithins A and B.

About 105 clinical trials (61% of them yielded "positive results") have been carried out so far to evaluate the effects associated with the intake of phytoestrogens (*3*) focusing on the effects on bone density (*4*), cancer prevention (*5*), cognitive ability (*6*), cardiovascular health (*7*), and menopausal symptoms (*8*, *9*). Hormone replacement therapy (HRT) has proved to be less safe or effective than initially thought (*10*), and phytoestrogens, especially isoflavones, have been proposed as a potential alternative to diminish estrogen-related disorders in menopause (*11*).

It is known that the biological activity of dietary molecules, including phytoestrogens, is strongly affected by their uptake and metabolism because molecules that appear in blood or are excreted in urine can be very different from those ingested (*12*). For instance, the isoflavones genistein and daidzein, the most studied phytoestrogens, are metabolized by the gut microflora to yield dihydrodaidzein, *O*-demethylangolensin, and equol from daidzein and dihydrogenistein from genistein (*13*, *14*).

Ellagic acid is a polyphenol that has been reported to show a plethora of biological properties including antioxidant and anticancer activities (*15*−*17*). Very recently, estrogenic/antiestrogenic activity via the estrogen receptor (ER) subtypes ERα and ERβ has been also proposed for ellagic acid (*18*). From a dietary point of view, the occurrence of free ellagic acid in foodstuffs is rather uncommon. Ellagic acid is found as part of polymeric molecules called ellagitannins, which are abundant in strawberries, raspberries, walnuts, oak-aged wines, pomegranates, etc. (*19*). We have demonstrated that complex dietary ellagitannins from different sources are not absorbed in humans but hydrolyzed to yield ellagic acid, which is further metabolized by the human colonic microflora to yield bioavailable hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one derivatives (mainly urolithins A and B) (*20*, *21*) (**Figure 1**). These metabolites reach concentrations of micromolar levels in the blood and are excreted in the urine and feces of humans. Taking into account the above, a more plausible context to fully assess potential biological effects of ellagic acid should include the assay of ellagitannins (as the main dietary ellagic acid source) and ellagic acid (as the in vivo hydrolysis ellagitannins product) in the gastrointestinal tract (stomach, colon) because these molecules do reach these organs and their complete metabolism can take several days (*21*). In addition, as urolithin metabolites reach systemic circulation, their bioactivity should be also explored. In this context, urolithins have been reported to show very poor antioxidant capacity (*20*), and nothing is yet known about other (if any) potential biological activities of these bioavailable colonic metabolites in the human body. The presence of urolithins A and B in urinary sediments from sheep feeding on estrogenic clover pasture (*Trifolium subterraneum*) was first described by Nottle in 1976 (*22*). However, the author did not describe the origin of urolithins or the possible involvement of these metabolites in the estrogenic capacity of clover.

A number of scientists have approached structure−activity relationship studies to predict estrogenic activity of chemicals (*23*, *24*). One of the most recent studies on this topic was carried out by Klopman and Chakravarti using the MultiCASE expert system (*25*). This study identified substructural features associated with ER binding activity such as the presence of specific molecular moieties (the so-called "biophores") as well as the intramolecular distance between two of these biophores (*25*).

Taking into account all of the above, we hypothesized that urolithins could exert estrogenic and/or antiestrogenic activities, because they have some key structural features related to the estrogenic activity. To this purpose, the estrogenic and antiestrogenic activities of both urolithins A and B as well as other known phytoestrogen (genistein, daidzein, resveratrol, and enterolactone) compounds were assayed on MCF-7 human breast cancer cells. We also assayed the ability of these compounds to bind both ERα and ERβ. In addition, the uptake and metabolism of both urolithins by MCF-7 cells were also explored.

MATERIALS AND METHODS

Chemicals. Genistein, enterolactone, resveratrol, daidzein, 17-β-estradiol (estradiol), and crystal violet were purchased from Sigma (St. Louis, MO). [2,4,6,7,16,17-^{3}H]Estradiol was obtained from Amersham International (Bucks, U.K.). Estrogen receptors ERα and ERβ were purchased from Panvera (Madison, WI). Urolithin A (95% purity) (3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one) and urolithin B (98% purity) (3-hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one) were chemically synthesized by Kylolab S.A. (Murcia, Spain). Dextran-coated charcoal (DCC)−fetal bovine serum was obtained from Hyclone (Erembodegem, Aalst, Belgium). Eagle's minimal essential medium (EMEM) without phenol red and insulin were purchased from Gibco BRL (Grand Island, NY). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Cell Culture. MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in EMEM containing 10% fetal calf serum (FCS) and 1.5 g/L sodium bicarbonate and supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.01 mg/mL bovine insulin. For the determination of estrogenic and antiestrogenic activities, the medium consisted of a phenol red-free EMEM with the same supplements and containing 5% DCC−FCS (FCS treated with DCC to remove steroids).

Measurement of Estrogenic and Antiestrogenic Activities. Assay methods to test estrogenic/antiestrogenic activities were based on those recommendations raised by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (*26*). Estrogenic activity was defined as the capacity to induce proliferation of MCF-7 breast cancer cells. "Control" estrogenic activity was that measured in the presence of estradiol without other compounds. The E-SCREEN assay (based on the estrogen receptor binding induced proliferation of MCF-7 cells) was performed according to the method of Payne et al. (*27*) with some modifications. Cells were seeded at 500 cells/well in 96-well plates. After 24 h, cells were washed with PBS and replaced with the estrogen-free medium and preincubated for 3 days prior to treatment. Afterward, different concentrations of the assayed compounds were added to the cells, that is, estradiol (1 pM and 1 nM); genistein, daidzein, enterolactone, or resveratrol (10 nM, 50 nM, and 1 μM); and urolithins A and B (0.1, 1, 10, 20, and 40 μM). DMSO concentration did not exceed 0.5%. Cell proliferation was measured after 7 days of incubation with the different compounds using the crystal violet assay (*28*).

Antiestrogenic activity was defined as the capacity to prevent or diminish the proliferation of MCF-7 cells in the presence of 1 pM estradiol. Urolithin concentrations were those assayed for the estrogenic activity. The rest of the phytoestrogens were assayed at the concentrations of 0.1 and 1 μM. The E-SCREEN assay was repeated five times to measure both estrogenic and antiestrogenic responses.

ER Competitive Binding Assay. The ability of resveratrol, daidzein, enterolactone, genistein, and urolithins A and B to bind to the estrogen receptors α and β was carried out according to the method of Arcaro et al. (*29*) with some modifications. The assay was based on the ability of the different compounds to compete with ^{3}H-labeled estradiol for the estrogen receptor. ERα binding assay included 2.4 nM pure estrogen receptor and 5 nM tritium-labeled estradiol ([2,4,6,7,16,17-^{3}H]estradiol). The ERβ binding assay included 1.2 nM pure estrogen receptor and 2.4 nM [^{3}H]estradiol. Unlabeled estradiol, urolithins A and B, genistein, daidzein, enterolactone, and resveratrol were prepared in DMSO at a concentration of 10 mM. All of these compounds (including receptors and [^{3}H]estradiol) were diluted in Tween/PBS (0.15:99.85, w/v). The assay mixture consisted of ER (either ERα or ERβ), [^{3}H]estradiol, and the different compounds at concentrations ranging from 1 pM to 100 μM in a final volume of 150 μL. The mixture was incubated for 2 h at 25 °C to allow the binding with the receptor. To remove the nonbound [^{3}H]estradiol, the reactions were incubated with 50 μL of Tween/PBS (0.15:99.85, w/v) containing 10% of DCC (Sigma) and 2% of bovine serum albumin (Roche, Indianapolis, IN) for 15 min at 4 °C, followed by centrifugation at 6000*g* for 5 min at 4 °C. An aliquot of this supernantant (150 μL) was added to 4 mL of scintillation counting liquid (Optifase HiSafe3, Perkin-Elmer, Salem, MA). The bound [^{3}H]-estradiol was measured in a WinSpectral 1414 liquid scintillation counter (Perkin-Elmer). Three independent experiments containing three replicates were performed for each compound tested. Results are expressed as the percentage of specific binding of [^{3}H]estradiol to ER versus log of competitor concentration. IC_{50} values represent the concentration of test compound required to displace 50% [^{3}H]estradiol from the receptor. IC_{50} values were determined by nonlinear regression fitting of experimental data to a sigmoid equation.

Cellular Uptake. MCF-7 cells (10^6) were incubated during 8, 24, and 48 h with a 40 μM concentration of either urolithin A or B in EMEM media without phenol red and with the supplements specified above in the cell culture section. The presence of metabolites excreted to the medium as well as those located inside the cells was explored. After treatments, media were recovered and acidified with formic acid (1.5 % in media) and filtered through a Sep-Pak cartridge (a reverse phase C-18 cartridge; Millipore Corp.), which retains phenolic compounds. The cartridges were previously activated with 10 mL of MeOH and 10 mL of water. Every 10 mL of medium was eluted with 2 mL of MeOH, and all methanolic fractions were collected and concentrated at 40 °C under reduced pressure, filtered through a 0.45 μm membrane Millex-HV_{13} filter, and then analyzed by LC-MS/MS.

Cells were washed twice with PBS and then lysed with 2 mL of MeOH, sonicated on ice during 5 min, and centrifuged; the supernatant was concentrated under reduced pressure, and an aliquot (100 μL) was analyzed by LC-MS/MS.

In addition, both media and cell extracts were treated with 40 units/mL β-glucuronidase from bovine liver (G-0251, EC 3.2.1.31; 1000 units/g of solid, Sigma) and 0.3 unit/mL sulfatase from *Helix pomatia* (S-9626, EC 3.1.6.1; 10000 units/g of solid, Sigma) to determine the possible conjugation to sulfate and/or glucuronide moieties upon metabolism of urolithins by MCF-7 cells. Experiments to assess cellular uptake and metabolism of urolithins by MCF-7 cells were performed in triplicate.

LC-MS/MS Analyses. The HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) was equipped with a mass detector in series (Agilent). The mass detector was an ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system (capillary voltage, 4 kV; dry temperature, 350 °C). Mass scan (MS) and MS/MS daughter spectra were measured from *m*/*z* 150 to *m*/*z* 600. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode.

Chromatographic separations of samples were carried out on a reverse phase C_{18} LiChroCART column (25 cm × 0.4 cm, particle size = 5 μm, Merck, Darmstadt, Germany) using water/acetic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The gradient started with 5% B in A to reach 40% B in A at 30 min and 95% B in A at 31 min. UV chromatograms of extracts were recorded at 280, 305, and 360 nm.

Urolithins A and B were identified in cell media and cell extracts according to their UV and MS spectra as well as MS/MS fragments using the corresponding pure urolithins as standards. Both urolithins as well as the corresponding cell-derived metabolites were quantified as urolithins at 305 nm (*14*).

Modeling Methodology. Structure−activity relationships (SAR) were determined following the procedure of Klopman and Chakravarti (*25*). The intramolecular distance between groups was measured using the DS ViewerPro 6.0 software (Accelrys Inc., San Diego, CA).

Statistics and Graphs. All of the experiments were carried out more than three times. Data were analyzed using one-way ANOVA followed by Dunnett's multiple-comparisons post test. Data were deemed to be significant at $p < 0.01$ (***), $p < 0.05$ (**), and $p < 0.1$ (*). Graphs of the experimental data and nonlinear regression fittings were executed by using the Sigma Plot 6.0 program for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Phytoestrogens have attracted attention as potential SERMs, which could be beneficial in countering menopausal symptoms and in lowering the incidence of hormone-dependent diseases including breast cancer (*2*, *3*). Many polyphenols (isoflavones, flavanones, stilbenes, etc.) have been described as SERMs with health-promoting properties (*3*). Besides dietary polyphenols, there are other described "enterophytoestrogens", that is, molecules such as equol or enterolactone, derived from dietary phytoestrogens and produced by gut microflora (*13*, *30*) which can exhibit even higher estrogenic/antiestrogenic properties than their corresponding precursors (daidzein, lignans, etc.).

The gut microflora molecules 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]-pyran-6-one (urolithin A) and 3-hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one (urolithin B) (**Figure 1**) are metabolites derived from ellagic acid and the related molecules ellagitannins (*14*). Urolithins are excreted in urine and feces and reach concentrations of micromolar level in human plasma after the ingestion of ellagitannin-rich sources such as strawberries, raspberries, walnuts, and pomegranates (*20*, *21*). We have recently described the molecular regulation of the apoptotic pathway of colon cancer cells by the pomegranate ellagitannin punicalagin and ellagic acid (*17*). However, unlike ellagic acid and ellagitannins with reported chemopreventive properties, no biological activity has yet been described for the microbial metabolites urolithins. In this context, the present paper investigates the in vitro estrogenic and antiestrogenic activities of urolithins A and B, which suggest a potential role for these metabolites as in vivo microflora produced EDCs (enterophytoestrogens).

SAR studies indicate potential binding of urolithins to estrogen receptors. Klopman and Chakravarti analyzed the binding potential of 313 chemicals to estrogen receptors by using the MultiCASE expert system (*25*). This analysis identified substructural features associated with ER binding activity (the so-called "biophores") (**Figure 2**). Among these biophores, the hydroxyl group (biophore type 1, **Figure 2**) was the most important biophore responsible for the estrogenic activity of most of the chemicals analyzed. However, other biophores such as types 2 and 3 (**Figure 2**) together with other structural features (the so-called "modulators") such as the intramolecular distance between biophores (12.8 Å between two biophores type 1; 6 Å between biophores types 1 and 2) were also of great importance. The analysis of both urolithins A and B as well as the other "control" phytoestrogens assayed (genistein, resveratrol, daidzein, and enterolactone) using the above criterion revealed the existence of biophores and modulators in these molecules (**Figure 3**). The presence of biophores and modulators is related to the binding potential to estrogen receptors (*25*). However,

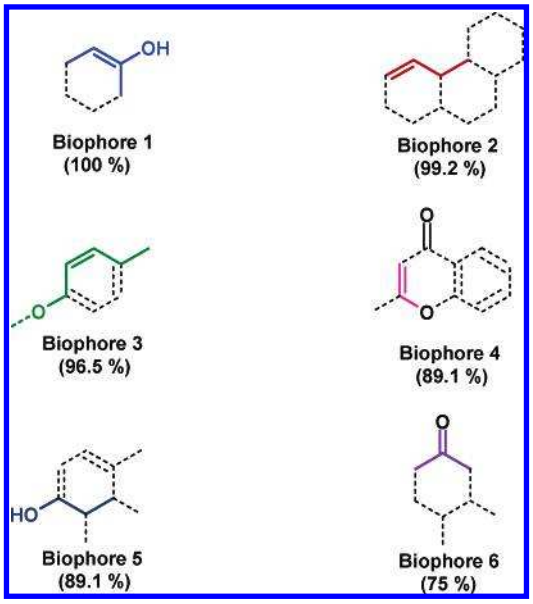

Figure 2. Most statistically significant biophores [adapted from Klopman and Chakravarti (*25*)]. Probability of relevance in the binding to the estrogen receptor is indicated in parentheses.

the exact binding affinity cannot be easily predicted when molecules with different structure are compared due to the presence of a mixture of biophores and modulators. This comparison can be made when two molecules show only slight differences. This is the case of genistein and daidzein as well as urolithins A and B (**Figure 3**). According to **Figure 3**, genistein should bind with higher affinity than daidzein to estrogen receptors due to the presence of an additional biophore type 1. In the case of urolithin A, the presence is noted of two biophores type 1 and two biophores type 2 as well as the modulator 6 Å. In addition, there is a portion of the molecule in which biophore types 1 and 2 are overlapped, which has been correlated with a higher binding potential to ER (**Figure 3**) (*25*). In the case of urolithin B, there is only one biophore type 1 but three biophores type 2. The modulator 6 Å also occurred, but the overlapped portion between biophore types 1 and 2 is missing. Therefore, according to the biophores and modulators detected in the molecules assayed, both urolithins A and B may be able to bind to estrogen receptors and display estrogenic/antiestrogenic activities. In addition, according to the type and number of biophores, urolithin A may bind to ER with higher affinity than urolithin B. The next step in our study was to assay the binding to both estrogen receptors ERα and ER*β* to validate the above theoretical SAR analysis.

Relative Affinity of Urolithins for ERα and ER*β*. The IC_{50} values for urolithins are in the average for other known phytoestrogens. The first step in the action of any estrogenic molecule involves the binding of ligand to an estrogenic receptor, which can be located in different sites within the cell. The main pools of ERs are located in the nucleus and cytosol, although a small pool of ERs is also located in the cell membrane (*31*). This involves a complex relationship between the membrane and nucleus effects of estrogens. For instance, distinctive actions of membrane-targeted and nuclear-localized ERs in breast cancer cells have been recently proposed (*32*).

The exact binding affinity values of phytoestrogens for both ERα and ER*β* can be different depending on the assay method. However, the relative affinity order for different phytoestrogens is often constant, independent of the binding method used (*33*, *34*). In our study, IC_{50} values (i.e., micromolar concentration required to achieve 50% inhibition of the binding of [^{3}H]-estradiol to the corresponding ER) and the molar excess to achieve this 50% inhibition with respect to the initial [^{3}H]-estradiol assayed are shown in **Table 1**. As predicted by the SAR analysis (**Figure 3**), genistein showed higher affinity values than daidzein to bind ER*β*, although the values were approximately the same to bind ERα (**Table 1**). Urolithin A showed a relatively high affinity for both receptors, especially for ERα, in comparison with the rest of the compounds assayed (**Table 1**). Urolithin B showed the lowest affinity for both receptors ERα and ER*β* among the compounds tested (**Table 1**). Urolithin A showed better affinity values than other acknowledged phytoestrogens such as genistein and daidzein (with the exception of genistein for ER*β*, which showed a lower IC_{50} value) (**Table 1**). No binding to either of the two receptors was detected for resveratrol or enterolactone in accordance with previous findings (*34*). Focusing on urolithins and taking into account both SAR and ER binding assays, both urolithins, especially urolithin A, may exert some estrogenic/antiestrogenic activities. Therefore, our next step was to assay these compounds on the estrogen-sensitive MCF-7 breast cancer cells to determine whether these metabolites can induce hormone-dependent cell proliferation.

Effect on the Estrogen-Sensitive MCF-7 Cell Proliferation: Estrogenic and Antiestrogenic Activities of Urolithins. The adenocarcinoma MCF-7 breast cancer cell line expresses high levels of ERα as well as lower but significant levels of ER*β* (*35*). In addition, an important ERα pool is extranuclear, located in the cytosol (*34*, *36*). The use of MCF-7 cells as a tool to check the estrogenic/antiestrogenic activities of different compounds (E-Screen assay) has been widely approached (*34*, *37*, *38*). Estrogenic assays such as the E-Screen are necessary to validate the binding affinity assays with pure estrogen receptors according to the ICCVAM endocrine-disruptor report (*26*).

Table 1. Relative Affinity of Urolithins and Other Phytoestrogens for ERα and ER*β*[a]

compound	ERα IC_{50} (μM)	molar excess to achieve 50% inhibition (ERα)	ER*β* IC_{50} (μM)	molar excess to achieve 50% inhibition (ER*β*)
estradiol	0.0064 ± 0.0005	1.3×	0.0048 ± 0.0004	2×
genistein	3.4 ± 0.5	530×	0.023 ± 0.002	9×
daidzein	2.3 ± 0.2	460×	1.1 ± 0.1	440×
resveratrol	NA	NA	NA	NA
enterolactone	NA	NA	NA	NA
urolithin A	0.44 ± 0.01	88×	0.75 ± 0.04	300×
urolithin B	20.1 ± 3	4000×	11.2 ± 2	4500×

[a] IC_{50} is defined as the concentration required to achieve 50% inhibition in the binding of [^{3}H]estradiol to the corresponding estrogen receptor (ER). NA, not achieved (no binding). IC_{50} values are shown as mean ± SD ($n = 9$). Molar excess is referred to the initial [^{3}H]estradiol assayed in the medium (5 and 2.4 nM for ERα and ER*β* assays, respectively). Assay conditions are specified under Materials and Methods.

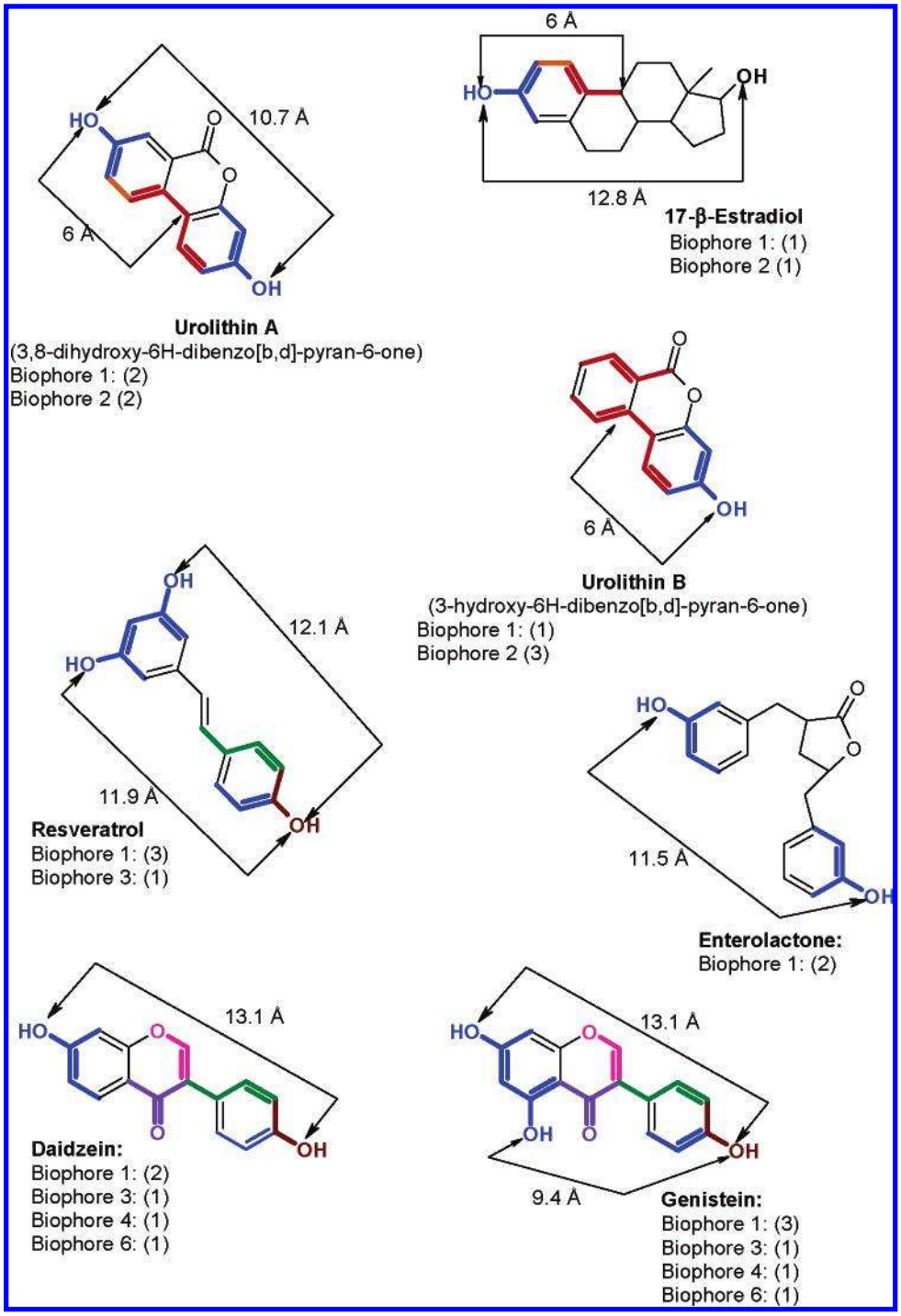

Figure 3. Presence of biophores and major modulators in the compounds assayed. Color code: blue, biophore 1; red, biophore 2; green, biophore 3; pink, biophore 4; purple, biophore 6; orange, overlapping biophores 1 and 2; brown, overlapping biophores 1 and 6. Biophore 5 (**Figure 2**) is not present in the compounds assayed. The number of each biophore present in the molecule is indicated in parentheses.

In the present study, the estrogenic activity measured as the induction-fold of MCF-7 cells proliferation was not strictly coincident with the binding affinity values reported above (**Figure 4**). The lowest urolithin concentration that provoked significant induction of cell proliferation was 1 μM (**Figure 4**). Genistein, daidzein, and resveratrol showed higher induction-fold of cell proliferation than both urolithins at the concentration of 1 μM, whereas enterolactone did not induce proliferation at this concentration. Therefore, by comparing IC_{50} values (**Table 1**) and the hormone-dependent proliferation of MCF-7 cells (**Figure 4**), it is obvious that estrogenic activity is not simply related to the higher or lower affinity of phytoestrogens for ERs.

It is noted that both urolithins exerted high estrogenic response at higher concentrations (from 10 μM). This is important because the other phytoestrogens inhibited cell proliferation at higher concentrations in accordance with the dual effect of these phytoestrogens, that is, estrogenic/antiestrogenic activities at low concentrations but antiproliferative effect at higher concentrations (*3*). In the case of urolithins, no antiproliferative effect, cytotoxicity, apoptosis, or effect on cell cycle was observed at 40 μM, the highest concentration assayed (results not shown). With regard to the antiestrogenic activity, that is, prevention of the estradiol-induced cell proliferation, both urolithins showed slightly higher antiestrogenic activities than the rest of the

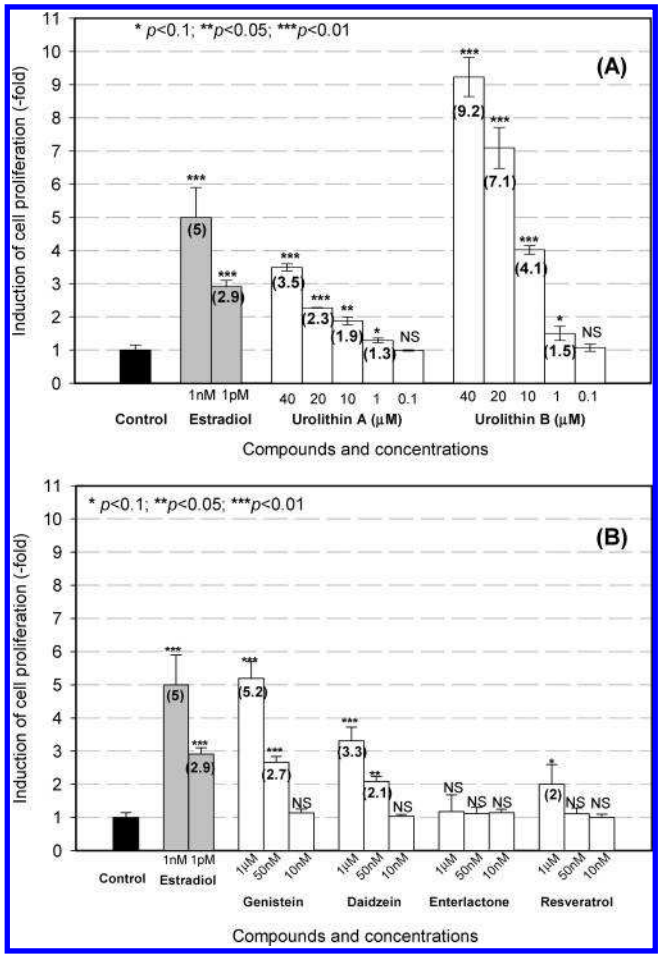

Figure 4. Estrogenic activity in the MCF-7 cells model: (**A**) estrogenic activity of urolithins; (**B**) estrogenic activity of the rest of the phytoestrogens. Estrogenic activity was measured as the induction-fold of cell proliferation of treated cells versus the nontreated (control) cells. A higher induction means a higher estrogenic activity. Assay conditions are detailed under Materials and Methods. Cell proliferation was measured after 7 days of incubation with the different compounds. Mean fold-induction value over the control is indicated in parentheses. Asterisks indicate significant difference from controlL ***, $p < 0.01$; **, $p < 0.05$; *, $p < 0.1$. NS, not significant.

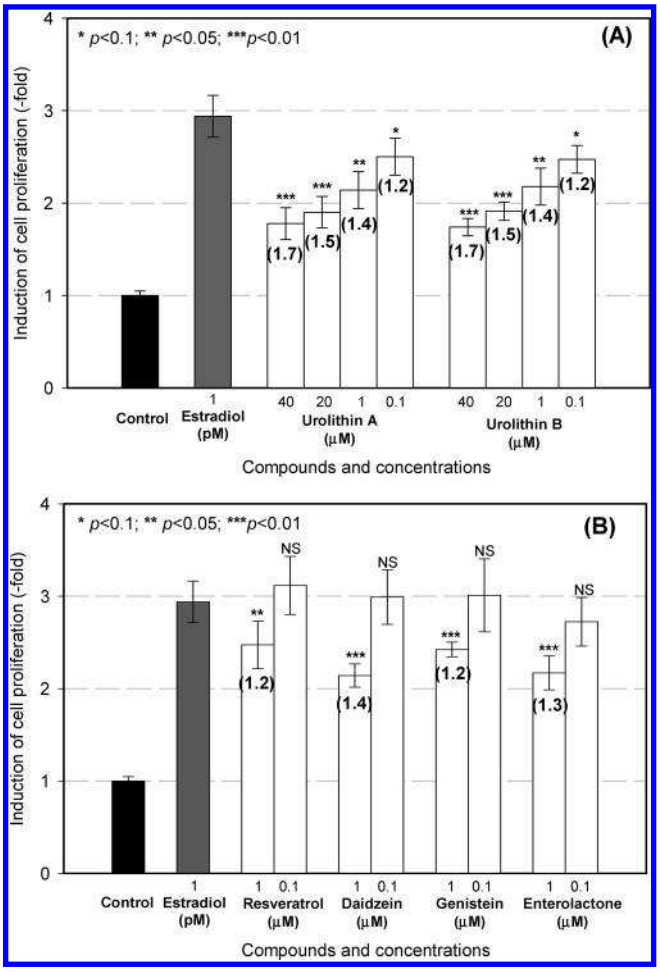

Figure 5. Antiestrogenic activity in the MCF-7 cells model: (**A**) antiestrogenic activity of urolithins; (**B**) antiestrogenic activity of the rest of phytoestrogens. Activity was measured as the induction-fold of cell proliferation of treated cells versus the nontreated (control) cells, in the presence of estradiol 1 pM. A lower induction means a higher antiestrogenic activity of the test compound. Assay conditions are detailed under Materials and Methods. Cell proliferation was measured after 7 days of incubation with the different compounds. Mean value of reduction in fold-induction is indicated in parentheses. Asterisks indicate significant difference from estradiol: ***, $p < 0.01$; **, $p < 0.05$; *, $p < 0.1$. NS, not significant.

phytotestrogens assayed because the lowest concentration assayed (0.1 μM) partially inhibited estradiol-induced cell proliferation (**Figure 5**). Both urolithins A and B, like the other phytoestrogens assayed, displayed in vitro estrogenic and antiestrogenic activities. These results suggest that they could potentially act as SERMs.

As mentioned before, most of the estrogen receptors are cytosolic or located in the nucleus. To this purpose our next step was to determine the uptake and metabolism of urolithins by MCF-7 breast cancer cells as well to evaluate the time-course accumulation of the metabolites produced.

Do Urolithins Enter MCF-7 Cells? We have mentioned above that urolithins are ellagitannins and ellagic acid-derived metabolites produced by human gut microflora (*14*, *20*, *21*). The study of the uptake and metabolism of dietary polyphenols by cells is not usually approached (*38*, *39*), and this study is even less frequent of the in-iivo-generated and blood-circulating metabolites derived from dietary polyphenols. In the present study we examined the LC-MS/MS profile of both cell media and MCF-7 cell extracts in the absence (control, **Figure 6A**) and in the presence of either 40 μM urolithin A or urolithin B (**Figure 6B**−**E**). Both urolithins were very stable in the cell media (without cells) without significant degradation during the assay time (results not shown).

In addition to urolithin B (peak 1, **Figure 6B**), six derived metabolites were identified according to their UV spectra and ion mass in the cell media of MCF-7 cells previously incubated with urolithin B, that is, urolithin A (peak 2, m/z^- 227), urolithin A structural isomer (peak 3, m/z^- 227), urolithin B-sulfate (peak 4, m/z^- 291), urolithin A structural isomer-sulfate (peak 5, m/z^- 307), urolithin B-glucuronide (peak 6, m/z^- 388), and urolithin A-sulfate (peak 7, m/z^- 307).

In addition to urolithin A (peak 2, **Figure 6C**), three derived metabolites were identified in the cell media of MCF-7 cells previously incubated with urolithin A, that is, urolithin A-sulfate (peak 7, m/z^- 307), urolithin A-glucuronide (peak 8, m/z^- 403), and urolithin A-disulfate (peak 9, m/z^- 307).

To assess the uptake of both urolithins A and B by MCF-7 cells, the corresponding cell lysates were analyzed (**Figure 6D**,**E**). In the lysates from MCF-7 cells previously incubated with urolithin B, this metabolite was the most abundant molecule

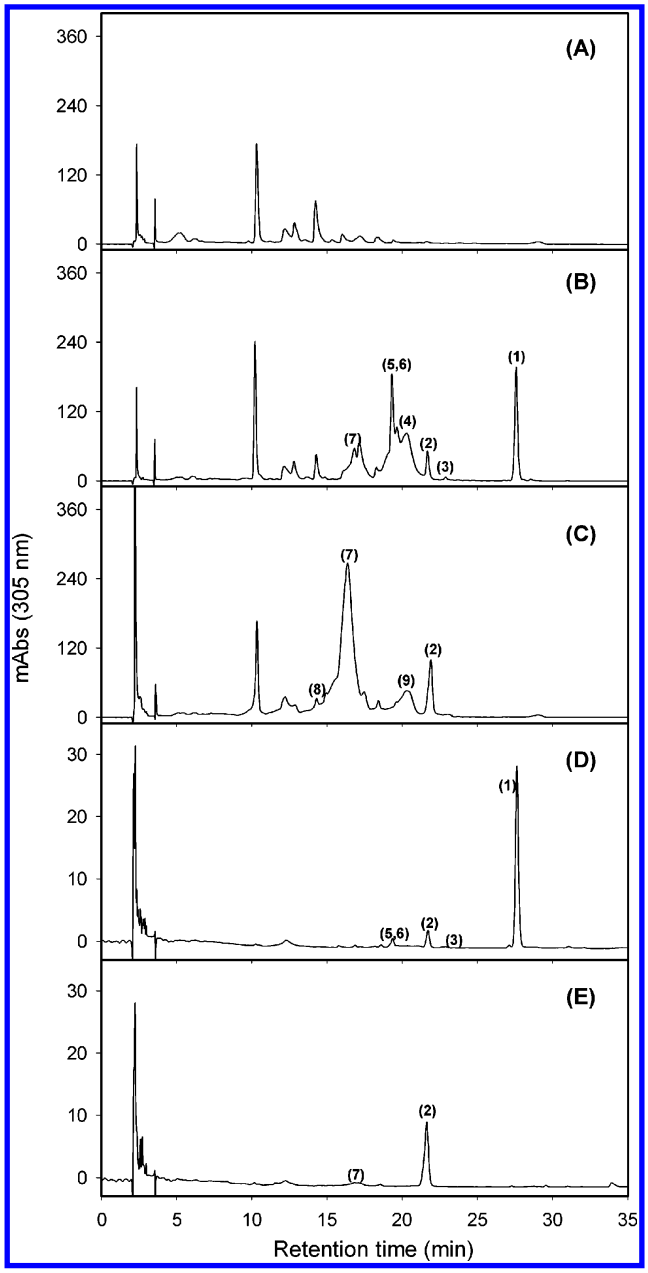

Figure 6. (**A**–**C**) HPLC profiles at 305 nm of culture media and (**D**, **E**) cell extract: (**A**) control culture medium after 48 h; (**B**, **C**) cell medium in the presence of 40 μM urolithin B and 40 μM urolithin A, respectively, for 48 h; (**D**, **E**) HPLC profiles of lysed MCF-7 cells after treatment with 40 μM urolithin B and 40 μM urolithin A, respectively, for 24 h. Peaks: 1, urolithin B; 2, urolithin A; 3, urolithin A structural isomer; 4, urolithin B-sulfate; 5, urolithin A structural isomer-sulfate; 6, urolithin B-glucuronide; 7, urolithin A-sulfate; 8, urolithin A-glucuronide; 9, urolithin A-disulfate.

(**Figure 6D**). Other derived metabolites (compounds **2**, **3**, **5**, and **6**) were also detected in lesser amounts (**Figure 6D**).

In the case of lysates from MCF-7 cells previously treated with urolithin A, this metabolite was clearly detected (peak 2, **Figure 6E**) as was the monosulfate derivative, although in much lesser amount (peak 7, **Figure 6E**).

These results indicated that urolithins entered MCF-7 cells, which mainly produced sulfate derivatives that were further excreted to the medium. The incubation of cell media and cell lysates with either sulfatase or glucuronidase selectively removed sulfate and glucuronide derivatives, respectively (results not shown), which further confirmed the identification of the metabolites as urolithin sulfate and glucuronide derivatives. In

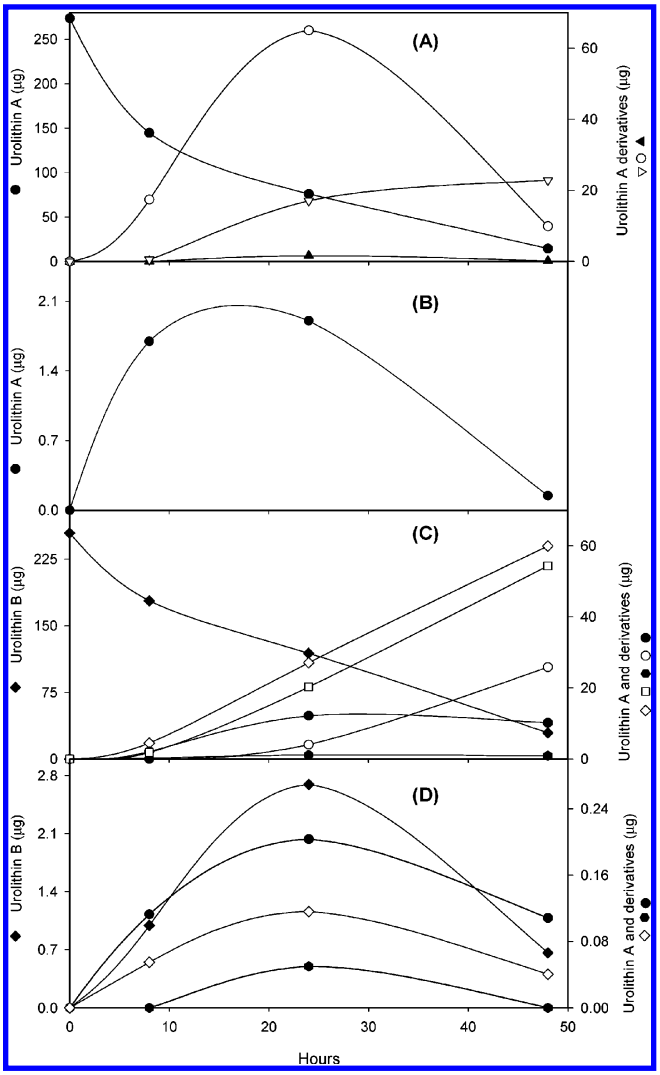

Figure 7. Time course evolution of the stability and metabolism of urolithins in the MCF-7 cell medium (**A**, **C**) and MCF-7 lysates (**B**, **D**): (**A**, **B**) incubation with urolithin A; (**C**, **D**) incubation with urolithin B; (●) urolithin A; (◆) urolithin B; (○) urolithin A-sulfate; (⬢) urolithin A structural isomer; (◇) urolithin B-sulfate; (▲) urolithin A-glucuronide; (▽) urolithin A-disulfate; (□) urolithin A structural isomer-sulfate. Urolithin B-glucuronide could not be quantified in all of the samples. The mean value ($n = 3$) is shown. CV was always <10%. MCF-7 cells were incubated with 40 μM urolithins A and B (273.6 and 254.4 μg, respectively).

addition, the capacity of MCF-7 cells to hydroxylate urolithin B to produce urolithin A is also noteworthy (**Figure 6B**,**D**). The sulfation and glucuronidation of urolitins by MCF-7 cells suggests the involvement of the phase II (detoxifying) enzymes glucuronyl and sulfate transferases. The hydroxylation of urolithin B to yield urolithin A enables more conjugations of the molecule to increase its excretion mainly through the conjugation of sulfates in the case of MCF-7 cells. However, the hydroxylation of urolithin B to yield urolithin A may be involved in the higher estrogenic activity of urolithin B observed in the E-Screen assay (**Figure 4A**) because a synergistic estrogenic action in the mixture of both urolithins A and B cannot be ruled out (and this mixture is not present in the incubation of MCF-7 cells with urolithin A because no dehydroxylation was observed).

The analysis of cell media to study the time-course evolution of the urolithin-derived metabolites described above showed that urolithin A (solid circle, **Figure 7A**) and urolithin B (solid diamond, **Figure 7C**) disappeared in the medium concomitant

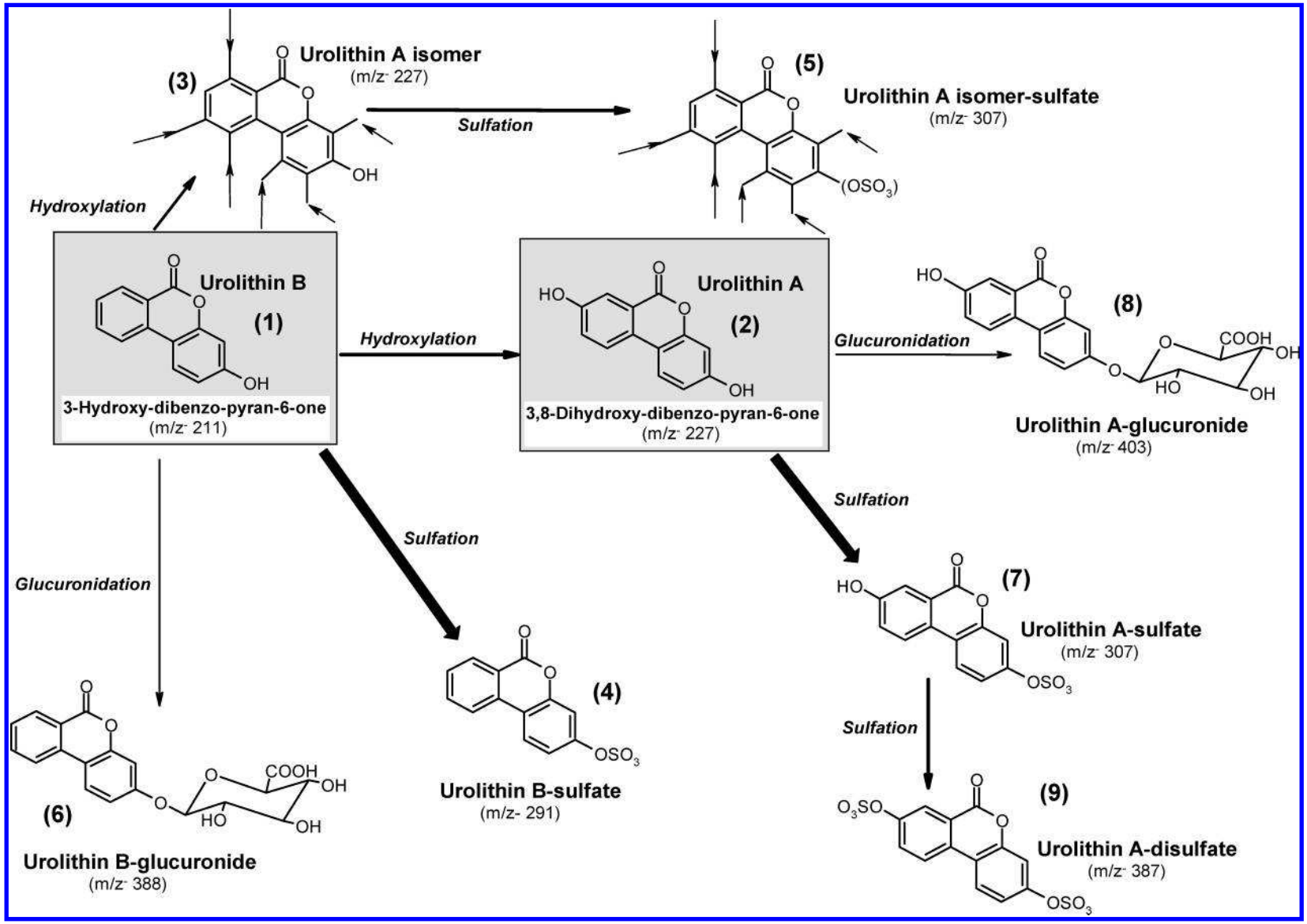

Figure 8. Tentative metabolism pathway of urolithins A and B by MCF-7 cells. Thicker arrows designate the preferential pathway. Long-dash arrows are minor pathways. Short-dash arrows designate possible hydroxylation places. Numbers that follow the name of compounds are referred to the peaks in **Figure 6**.

with the accumulation of their derived metabolites (**Figure 7A**,**C**). The main derived metabolite excreted in the medium of MCF-7 cells treated with urolithin A was urolithin A-sulfate (open circle, **Figure 7A**). Urolithin B-sulfate (open diamond, **Figure 7C**) and urolithin A-sulfate were the main metabolites detected when the cells were treated with urolithin B.

The main compound detected in the cell lysates was urolithin A in urolithin A-treated cells (**Figure 7B**), whereas both urolithins A and B were detected in lysates of urolithin B-treated cells (**Figure 7D**). The analysis of these results led us to propose a tentative pathway followed by MCF-7 cells to metabolize both urolithins (**Figure 8**). According to our results, urolithin B (compound **1**, **Figure 8**) is mainly sulfated to yield urolithin B-sulfate (compound **4**, **Figure 8**), although it can be also hydroxylated in the 8-position to yield urolithin A (compound **2**, **Figure 8**) or in other positions to yield a tentative urolithin A structural isomer (compound **3**, **Figure 8**) to facilitate the excretion of the molecule. Another minor pathway is the direct glucuronidation of urolithin B to yield the corresponding glucuronide derivative (compound **6**, **Figure 8**). Both hydroxylated derivatives from urolithin B, that is, urolithin A (compound **2**) and urolithin A structural isomer (compound **3**), are sulfated to yield the corresponding sulfate derivatives (compounds **7** and **5**, respectively, **Figure 8**).

When MCF-7 cells were incubated with urolithin A, the main derivative detected was urolithin A-sulfate (compound **7**, **Figure 8**). The disulfate derivative was detected in lesser amount (compound **9**, **Figure 8**). The direct glucuronidation of urolithins A and B was a minor pathway in MCF-7 cells (compound **8**, **Figure 8**).

In summary, our results suggest that urolithins A and B, the human colonic microflora metabolites produced from dietary ellagic acid and the related molecules ellagitannins, display both estrogenic and antiestrogenic activities under our assay conditions. These results suggest that both urolithins could be potentially considered as enterophytoestrogens like equol, that is, gut microflora derived metabolites with estrogenic/antiestrogenic activities derived from plant food constituents. It should be noted that the production of urolithins (as in the case of equol, for instance) greatly depends on the microflora of each human subject (*14*, *20*, *21*), and thus the potential biological effect could be also different between individuals. According to our results, consumption of ellagitannin-containing foodstuffs such as pomegranate, walnuts, berries, and oak-aged wines may exert some proestrogenic/antiestrogenic effects. In this context, further research is warranted to evaluate the possible role of ellagitannins and ellagic acid as dietary "pro-phytoestrogens".

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