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The Ellagic Acid Derivative 4,4′-Di-*O*-Methylellagic Acid Efficiently Inhibits Colon Cancer Cell Growth through a Mechanism Involving WNT16

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ABSTRACT

Ellagic acid (EA) and some derivatives have been reported to inhibit cancer cell proliferation, induce cell cycle arrest, and modulate some important cellular processes related to cancer. This study aimed to identify possible structure-activity relationships of EA and some in vivo derivatives in their antiproliferative effect on both human colon cancer and normal cells, and to compare this activity with that of other polyphenols. Our results showed that 4,4′-di-*O*-methylellagic acid (4,4′-DiOMEA) was the most effective compound in the inhibition of colon cancer cell proliferation. 4,4′-DiOMEA was 13-fold more effective than other compounds of the same family. In addition, 4,4′-DiOMEA was very active against colon cancer cells resistant to the chemotherapeutic agent 5-fluoracil, whereas no effect was observed in nonmalignant colon cells. Moreover, no correlation between antiproliferative and antioxidant activities was found, further supporting that structure differences might result in dissimilar molecular targets involved in their differential effects. Finally, microarray analysis revealed that 4,4′-DiOMEA modulated Wnt signaling, which might be involved in the potential antitumor action of this compound. Our results suggest that structural-activity differences between EA and 4,4′-DiOMEA might constitute the basis for a new strategy in anticancer drug discovery based on these chemical modifications.

Introduction

According to the American Cancer Society, cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells (http://www.cancer.org). Colorectal cancer (CRC) is one of the most common cancers worldwide. According to the European Colorectal Cancer Patient Organization, CRC represents the second most common malignant tumor in Europe, killing 230,000 people each year (http://www.europacolon.com). There are different factors associated with a high risk of developing CRC, such as obesity, physical inactivity, diet high in red or processed meat, alcohol consumption, and long-term smoking. Interestingly, a diet rich in vegetables and fruits was demonstrated to be significantly associated with a reduced risk of developing colon cancer (American Cancer Society, 2011, 2012).

Chemotherapy administration is one of the most important decisions to make in the management of cancer patients (American Cancer Society, 2012). The vast majority of chemotherapeutic treatments produce adverse side effects that habitually persist after a long-term period. The antimetabolite 5-fluorouracil (5-FU) is the most commonly used chemotherapeutic agent in CRC. In addition to its side effects, resistance to 5-FU is relatively frequent and new strategies to overcome resistance are urgently needed to gain effectiveness of the treatment. Consequently, identification of new

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ABBREVIATIONS: 3,3′-DiOMEA, 3,3′-di-*O*-methylellagic acid; 4,4′-DiOMEA, 4,4′-di-*O*-methylellagic acid; 5-FU, 5-fluorouracil; CRC, colorectal cancer; DMSO, dimethylsulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EA, ellagic acid; FRAP, ferric reducing antioxidant power; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; TROLOX, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Uro-A, urolithin A, 3,8-dihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one; Uro-B, urolithin B, 3-hydroxy-6*H*-dibenzo[*b,d*]pyran-6-one.

compounds and structures with anticancer properties with the aim of reducing adverse events and/or useful in overcoming drug resistance is necessary for patients and current health systems.

Nature constitutes an important source of cancer chemopreventive compounds. Indeed, a number of important chemotherapeutic drugs have been obtained from natural sources or derived from natural structures (González-Vallinas et al., 2013a). In fact, chemoprevention through dietary intervention is an emerging option to delay or reduce the mortality of cancer and minimize the adverse effects of chemotherapeutic treatments (Coates et al., 2007; González-Vallinas et al., 2013b). Berries, pomegranates, muscadine grapes, walnuts, almonds, and pecans contain bioactive compounds that are directly related to cancer prevention, including polyphenols with interesting properties modulating cell signaling cascades (Espin et al., 2013). Ellagitannins are a class of hydrolysable tannins that have been reported to exert antioxidant, anti-inflammatory, and antitumorigenic properties and to inhibit angiogenesis and prevent the genomic instability that leads to cancer development (Stoner et al., 2007; Umesalma and Sudhandiran, 2011).

Ellagitannins are found naturally in foods as hexahydroxydiphenoyl-glucose esters, whose hydrolysis releases ellagic acid (EA) that is poorly absorbed in the stomach and small intestine but is highly metabolized by the intestinal microbiota to produce urolithin A (Uro-A; 3,8-dihydroxy-6*H*-dibenzo[*b,d*]pyran-6-one) and urolithin B (Uro-B; 3-hydroxy-6*H*-dibenzo[*b,d*]pyran-6-one) (Cerdá et al., 2004; Sharma et al., 2010). These two in vivo metabolites are then conjugated with glucuronic acid and/or methyl ethers and are the main products absorbed and detected in plasma, urine, and prostate tissue (González-Sarrías et al., 2010a; Larrosa et al., 2010a). Whereas conjugated metabolites are more abundant in the systemic circulation, EA derivatives aglycones, including urolithins, can reach relevant concentrations in the colonic mucosa of patients with CRC (Nuñez-Sánchez et al., 2014).

EA exerts antiproliferative and antioxidant properties as described in a multitude of in vitro and in vivo studies and in different cancer cell lines (Narayanan et al., 1999; Seeram et al., 2005; González-Sarrías et al., 2009; Chung et al., 2013; Qiu et al., 2013; Santos et al., 2013; Vanella et al., 2013; Umesalma et al., 2014; Zhang et al., 2014). The antiproliferative action of EA could be mediated for its ability to directly inhibit the DNA binding of certain carcinogens, including nitrosamines (Mandal et al., 1988; Mandal and Stoner, 1990) and polycyclic aromatic hydrocarbons (Teel et al., 1986).

Likewise, urolithins (mainly Uro-A) inhibit colon cancer cell proliferation, induce cell cycle arrest, and modulate some important cellular processes involved in colon cancer development such as the inflammatory process, transformation, hyperproliferation, initiation of carcinogenesis, angiogenesis, and metastasis (Aggarwal and Shishodia, 2006; Larrosa et al., 2010b; Li et al., 2012).Therefore, EA and derivatives (including urolithins) available in natural compounds contribute to colon cancer chemoprevention and might constitute a complementary therapeutic approach for the treatment of colon cancer (González-Sarrías et al., 2009, 2010b).

We compared the antiproliferative properties of different polyphenols, including EA and in vivo derivatives with reported biologic properties, which can exert potential beneficial effects by inhibiting cancer cell growth (Table 1). Because EA has been described as an antioxidant and anti-inflammatory compound (Huang et al., 2012), we also analyzed the potential correlation between this biologic activity and the inhibition of colon cancer cell growth. This study aimed to reveal the structural modifications that result in a potentiation of the activity of this compound in colon cancer prevention, as well as to gain new insights regarding its mechanism of action.

Materials and Methods

Phenolic Compounds and Derived Metabolites

EA, resveratrol, homovanillic acid, dihydrocaffeic acid, gallic acid, 4-*O*-methylgallic acid, and 3-*O*-methylgallic acid were purchased from Sigma-Aldrich (St. Louis, MO). 3,3′-Di-*O*-methylellagic acid (3,3′-DiOMEA; 99% purity) and 4,4′-di-*O*-methylellagic acid (4,4′-DiOMEA; 99% purity) were purchased from Bertin Pharma (Montigny le Bretonneux, France). Uro-A and Uro-B, both with purity higher than 95%, were provided by CEBAS-CSIC (Murcia, Spain).

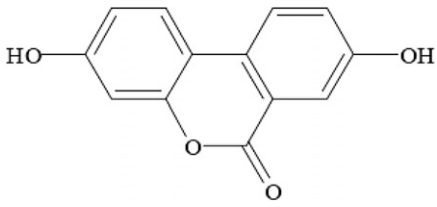
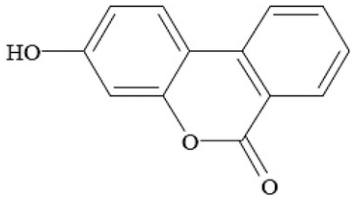
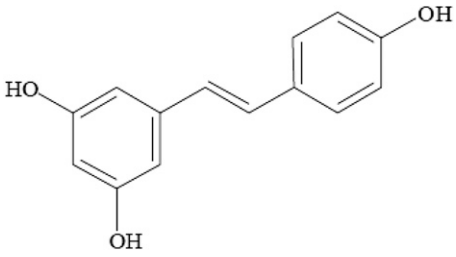
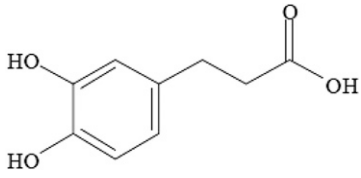
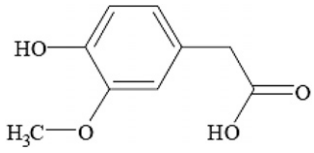
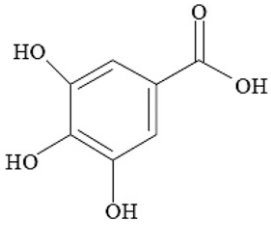
Cell Culture

SW-620 and HT-29 human colon cancer cells, as well as CCD18Co normal human colon cells, were obtained from American Type Culture Collection (Manassas, VA) and were maintained in their respective culture media at 37°C with 5% CO_2 and 95% humidity. SW-620-5FuR corresponds to a cell line derived from SW-620, in which acquired resistance to 5-FU was reached by continuously exposing cells to increasing concentrations of 5-FU up to 150 μM over 15 months, as previously described (González-Vallinas et al., 2013b). SW-620, SW-620-5FuR, and HT-29 were cultured in Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco/Invitrogen), 2 mM glutamine (BioWhittaker; Lonza Group, Basel, Switzerland) and 1% antibiotics/antifungal agents (containing 10,000 U/ml penicillin base, 10,000 μg/ml streptomycin base, and 25,000 ng/ml amphotericin B; Gibco/Invitrogen). CCD18Co cells were cultured in Eagle's minimal essential medium (American Type Culture Collection) supplemented with 10% fetal bovine serum (Gibco/Invitrogen) and 1% antibiotics/antifungal agents (containing 10,000 U/ml penicillin base, 10,000 μg/ml streptomycin base, and 25,000 ng/ml amphotericin B; Gibco/Invitrogen). Cells were kept subconfluent, and media were changed every day. Stock solutions for each phenolic and derived metabolite were prepared by dissolving the corresponding compound in dimethylsulfoxide (DMSO).The maximum final concentration of DMSO in all culture media was 0.2 μl/ml, including nontreated cells.

Cell Viability Assay

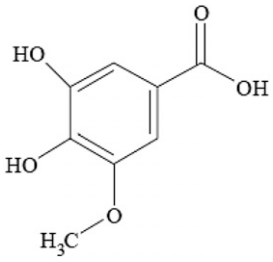
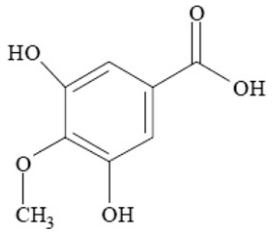
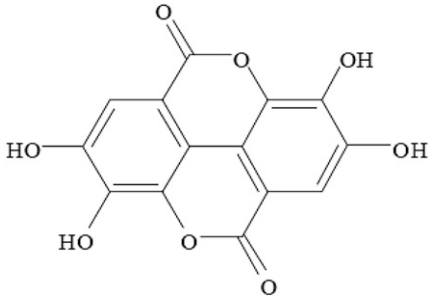
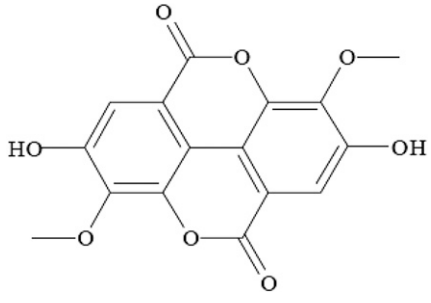
SW-620, SW-620-5FuR, HT-29, and CCD18Co cells were seeded in 24-well plates in the exponential growth phase using 500 μl cell suspension per well at a density between 15×10^3 and 60×10^3 cells. After 24 hours, media were replaced with 500 μl media containing serial concentrations of each polyphenol. After 72 hours of treatment, cells were subjected to an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with 50 μl/well of MTT (5 mg/ml in phosphate-buffered saline) (Sigma-Aldrich). After 3 hours of incubation at 37°C, MTT-containing media were removed and the MTT reduced to purple formazan by living cells was solubilized by the addition of 200 μl/well of DMSO. After 1 hour of incubation, quantities of the formazan product, which are directly related to the number of viable cells, were measured at 560 nm using a scanning spectrophotometer microplate reader (Biochrom Asys UVM 340 Microplate Reader; ISOGEN, De Meern, The Netherlands). At least two independent experiments each performed in triplicate were conducted in each case.

TABLE 1
Phenolic compounds and in vivo derivatives included in the study and the main reported properties related to antitumor potential.

Compound	Chemical Structure	CAS Number	Biologic Activities
Uro-A		1143-70-0	Decreases clonogenic efficiency and cell proliferation through cell cycle arrest in the G(0)/G(1) and G(2)/M stages, followed by induction of apoptosis in HT-29 cells (Kasimsetty et al., 2010) Inhibits Wnt signaling in the human 293T cell line (Sharma et al., 2010) Decreases inflammatory markers, including iNOS, COX-2, prostaglandin E synthase, and PGE_2, in colonic mucosa (Larrosa et al., 2010b) Inhibits aromatase activity in live cell assay (Adams et al., 2010) Inhibits cell proliferation and reduces oxidative stress status in bladder cancer (Qiu et al., 2013)
Uro-B		1139-83-9	Decreases clonogenic efficiency and cell proliferation through cell cycle arrest in the G(0)/G(1) and G(2)/M stages, followed by induction of apoptosis in HT-29 cells (Kasimsetty et al., 2010) Inhibits aromatase activity in live cell assay (Adams et al., 2010) Inhibits cell proliferation and reduces oxidative stress status in bladder cancer (Qiu et al., 2013)
Resveratrol		501-36-0	In combination with quercetin, decreases the generation of ROS and increases the antioxidant capacity in HT-29 colon cancer cells (Del Follo-Martinez et al., 2013) Exhibits anticancer activity through caspase-3 cleavage and PARP cleavage induction in HT-29 colon cancer cells (Del Follo-Martinez et al., 2013) Inhibits cell proliferation in HCT116 and Caco2 colon cancer cells (Fouad et al., 2013)
Dihydrocaffeic acid		1078-61-1	Reduces the cytotoxicity and proinflammatory cytokine production (IL-6 and IL-8) in human keratinocyte cell line HaCaT (Poquet et al., 2008)
Homovanillic acid and derivatives		306-08-1	Induces apoptosis in leukemic cells through oxidative stress (Ito et al., 2004)
Gallic acid		149-91-7	Decreases Caco-2 cell viability through cell cycle arrest at G(0)/G(1), caspase-3 activation, DNA fragmentation, and nuclear condensation (Forester et al., 2014) Inhibits transcription factors NF-κB, AP-1, STAT-1, and OCT-1, which are known to be activated in CRC (Forester et al., 2014) Protects against DNA oxidation by activation of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, and glutathione-*S*-transferase-π) and a decrease of intracellular ROS concentrations in lymphocytes (Ferk et al., 2011) Exhibits antioxidant and anticarcinogenic activity against 1,2-dimethyl hydrazine–induced rat colon carcinogenesis (Giftson et al., 2010)

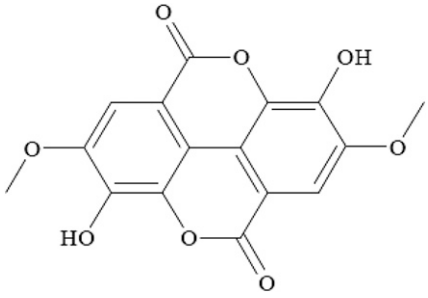
(*continued*)

TABLE 1—*Continued*

Compound	Chemical Structure	CAS Number	Biologic Activities
3-*O*-Methylgallic acid		3934-84-7	Decreases Caco-2 cell viability through cell cycle arrest at G(0)/G(1), caspase-3 activation, DNA fragmentation, and nuclear condensation (Forester et al., 2014) Inhibits transcription factors NF-κB, AP-1, STAT-1, and OCT-1, which are known to be activated in CRC (Forester et al., 2014)
4-*O*-Methylgallic acid		4319-02-2	Inhibits VEGF production under hypoxic conditions, as well as production of ROS in the endothelial cells stimulated with VEGF (Jeon et al., 2005) Inhibits endothelial cell invasion and tube formation stimulated with bFGF (Jeon et al., 2005) Inhibits the expression and production of inflammatory genes and mediators such as NO, PGE_2 (as well as the expression of iNOS, COX-2, and TNF-α), and IL-1β in mouse leukemic monocyte macrophage cell line RAW264.7 and in primary macrophages stimulated with LPS (Na et al., 2006)
EA		476-66-4	Prevents rat colon carcinogenesis induced by 1,2-dimethylhydrazine through inhibition of the AKT/phosphoinositide-3 kinase pathway (Umesalma and Sudhandiran, 2011) Exhibits anti-inflammatory property by iNOS, COX-2, TNF-α, and IL-6 downregulation due to inhibition of NF-κB and exerts its chemopreventive effect on colon carcinogenesis (Umesalma and Sudhandiran, 2010) Induces apoptosis via mitochondrial pathways in colon cancer Caco-2 cells but not in normal colon cells (Larrosa et al., 2006) Induces downregulation of the mitogenic IGF-II, activates p21 (waf1/Cip1), mediates a cumulative effect on the G1/S transition phase, and causes apoptotic cell death in SW480 colon cancer cells (Narayanan and Re, 2001) Inhibits Wnt signaling in a human 293T cell line (Sharma et al., 2010) Inhibits cell proliferation and reduces oxidative stress status in bladder cancer (Qiu et al., 2013) Reduces cancer cell viability by apoptosis induction associated with decreased ATP production in Caco-2, MCF-7, Hs 578T, and DU 145 cancer cells without any toxic effect on the viability of normal human lung fibroblast cells (Losso et al., 2004) Exhibits anti-PLA_2 activity, an enzyme that stimulates the growth of the human pancreatic cancer cell line, and correlates with HER2 overexpression and mediates estrogen-dependent breast cancer cell growth (Da Silva et al., 2008) Exhibits antimutagenic activity in *Salmonella typhimurium* (Smart et al., 1986)
3,3′-DiOMEA		2239-88-5	Exhibits anti-PLA_2 activity, an enzyme that stimulates the growth of the human pancreatic cancer cell line, and correlates with HER2 overexpression and mediates estrogen-dependent breast cancer cell growth (Da Silva et al., 2008) Exhibits antimutagenic activity in *S. typhimurium* (Smart et al., 1986)

(*continued*)

TABLE 1—*Continued*

Compound	Chemical Structure	CAS Number	Biologic Activities
4,4′-DiOMEA		3374-77-4	Exhibits antimutagenic activity in *S. typhimurium* (Smart et al., 1986)

bFGF, basic fibroblast growth factor; CAS, Chemical Abstracts Service; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NO, nitric oxide; PARP, ADP ribose polymerase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial cell growth factor.

Determination of Antioxidant Capacity

Ferric Reducing Antioxidant Power Assay. The ferric reducing antioxidant power (FRAP) assay was used for determining the antioxidant capacity. One hundred-fifty microliters of FRAP reagent prepared daily and preincubated at 37°C 10 minutes was mixed with 40 μl of test sample, standards, or methanol (for the reagent blank). The standard curve was constructed using serial dilutions of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) in DMSO between 0 and 500 μM. The FRAP reagent was prepared from 300 mM sodium acetate buffer (pH 3.6), 20 mM ferric chloride, and 10 mM ferric-2,4,6-trypyridyl-*s*-triazine (Sigma-Aldrich) made up in 40 mM hydrochloric acid. All three of the above solutions were mixed together in a 10:1:1 (v/v/v) ratio. The absorbance of the reaction mixture was measured spectrophotometrically at 550 nm after incubation at 37°C for 15 minutes. Experiments were repeated three times and all measurements were taken in triplicate. Values were derived from the TROLOX standard curve.

2,2-Diphenyl-1-Picrylhydrazyl Assay. Scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals is the basis of this antioxidant capacity assay. DPPH was purchased from Sigma-Aldrich, and a working solution at 200 μM as well as dilutions of the assayed polyphenols were prepared in methanol. The standard curve was constructed using serial dilutions of TROLOX in methanol between 0 and 100 μM. Fifty microliters of each concentration of TROLOX and polyphenols was put in each 96-well plate in triplicate and then 200 μl DPPH was added. The absorbance of the reaction mixture was measured at 520 nm after 15 minutes of incubation at room temperature in the dark. Experiments were repeated three times and all measurements were taken in triplicate. Values were derived from the TROLOX standard curve.

RNA Isolation

SW-620 colon cancer cells (1.7×10^5 cells per well) were seeded in six-well plates and maintained under standard culture conditions. After overnight incubation, cells were treated with different concentrations of 4,4′-DiOMEA [0 (nontreated), 5, 20, and 50 μM] with three replicates per test concentration. After 72 hours of treatment, the culture medium was discarded and total RNA was isolated from each plate using the RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. RNA quantity and quality were checked by UV spectroscopy (NanoDrop 2000 Spectrophotometer; Thermo Scientific, Waltham, MA).

The experiment was independently repeated four times in the same conditions and total RNA from each experiment was independently analyzed.

Gene Expression Assays

A comparative microarray gene expression analysis between nontreated (control) and 5 μM 4,4′-DiOMEA–treated SW-620 colon cancer cells for 72 hours was performed at the Genomic Service Facility at the Spanish National Center for Biotechnology (Madrid, Spain). RNA integrity was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and 200 ng total RNA from each sample was reverse transcribed and fluorescently labeled using the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's protocol. The complementary RNAs were prepared for hybridization in an Agilent SurePrint G3 Human 8 × 60 K (Whole Human Genome Microarray Kit) platform using the one-color gene expression system following the manufacturer's protocol (Agilent Technologies).

Quantitative Real-Time Polymerase Chain Reaction Analysis

Validation of microarray data was performed using quantitative real-time polymerase chain reaction (PCR) analysis for measuring the transcript levels in the selected group of differentially regulated genes. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions, and 1 μg total RNA was reverse transcribed by a High Capacity cDNA Archive Kit (Applied Biosystems) for 2 hours at 37°C. TaqMan assays for gene expression (Applied Biosystems, Foster City, CA), which contain the specific primer and TaqMan probe for each gene, were used. Quantitative PCR was performed in real time and in triplicate on the 7900HT Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase gene expression in each sample was used as an endogenous reference for the relative quantification of transcripts. RQ Manager software (Applied Biosystems) was used for data analysis. To calculate the relative expression of each gene, we applied the $2^{-\Delta\Delta}$ threshold cycle method as previously described (Ramírez de Molina et al., 2007, 2008).

Statistical Analysis

Dose-response curves of the cell viability assays were analyzed by analysis of variance with Bonferroni and Tukey post hoc tests. Data were presented as means ± S.E.M. of at least two independent experiments each performed in triplicate. Statistical significance was defined as $P < 0.05$. The statistical analyses were performed with R statistical software (version 2.15, http://www.r-project.org; R Project for Statistical Computing, Vienna, Austria).

Data from microarray analysis were extracted and analyzed with FIESTA viewer software (version 1.0, http://bioinfogp.cnb.csic.es/tools/FIESTA; Centro Nacional de Biotecnología, Madrid, Spain). Statistical significance to determine differences in gene expression between groups (nontreated and 5 μM 4,4′-DiOMEA–treated cells in four independent experiments) was determined by the Limma package (linear models for microarray data) (Smyth, 2004), using

a P value <0.05 as the level of significance. We set a minimum change of gene expression (either overexpression or repression) of 2-fold of the control (nontreated cells) to define that a gene is differentially regulated. Differentially expressed genes were classified and used for computational analysis to identify potential functional pathways and networks using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA) as well as the Gene Ontology, KEGG Pathway, and Gene Set Enrichment Analysis databases. The results are presented as P values of the hypergeometric test and P values of the hypergeometric test adjusted by false discovery rate correction, using <0.05 as the level of significance.

Results

4,4′-DiOMEA Strongly Inhibits Colon Cancer Cell Growth. We studied the growth inhibitory effect of 11 different phenolic compounds and derived metabolites (Table 1) in a preliminary screening on HT-29 cells based on the MTT assay. Results showed that 6 of the 11 compounds (homovanillic acid, dihydrocaffeic acid, gallic acid, 4-*O*-methylgallic acid, 3-*O*-methylgallic acid, and Uro-B) had no effect on cell growth in HT-29 colon cancer cells at the assayed concentrations (1–100 μM) (Table 2).

By contrast, 5 of 11 the analyzed compounds (EA, 3,3′-DiOMEA, 4,4′-DiOMEA, Uro-A, and resveratrol) displayed antiproliferative activity in the HT-29 colon cancer cell line under these assay conditions (Fig. 1; Table 2).

Interestingly, the effects of EA, 3,3′-DiOMEA, and 4,4′-DiOMEA on colon cancer cell viability were significantly different despite belonging to the same family of polyphenols (Table 2). The structural variation of 4,4′-DiOMEA was related to the highest antiproliferative activity, (IC_{50} of 7.6 $\pm$ 1.5 μM in HT-29 cells), which was 12-fold higher than that of its EA precursor. These results suggest that 4,4′-DiOMEA is the most effective agent against colon cancer cells within the members of the EA family tested, which was confirmed using an additional human colon cancer–derived cell line (SW-620) (Fig. 2; Table 2), in which the growth inhibitory effect of this compound was around 13-fold higher than that exerted by the precursor EA.

4,4′-DiOMEA Is an Effective Agent against Colon Cancer Cells Resistant to the Chemotherapeutic Drug 5-FU. With the aim of determining whether 4,4′-DiOMEA could be helpful in the management of chemoresistance of colon cancer cells, its effect on the proliferation of SW-620 cells resistant to 5-FU (SW-620-5FuR) was evaluated. Cell sensitivity of SW-620 colon cancer cells and SW-620-5FuR cells with acquired resistance to 5-FU treatment was assayed. SW-620-5FuR resistance to 5-FU was previously verified. We observed that the IC_{50} value of SW-620-5FuR for 5-FU was higher than 5000 μM, whereas the IC_{50} value of parental SW-620 was 7.1 $\pm$ 1.3 μM (Table 2). Our results showed that SW-620-5FuR cells were sensitive to 4,4′-DiOMEA (Table 2), which suggests that this compound might be helpful in treatment strategies aimed at overcoming 5-FU resistance.

Furthermore, to determine the potential specificity of this compound against cancer cells, its antiproliferative action on normal human colon epithelial CCD18Co cells was also determined. Table 2 shows that normal colon cells were not affected under conditions in which cancer cell growth was totally abrogated by 4,4′-DiOMEA. Thus, EA and 3,3′-DiOMEA displayed relatively low sensitivity against colon cancer cells ($IC_{50} > 70$ μM) and their growth inhibitory activity was almost 2-fold higher than that for normal cells (IC_{50} = approximately 40 μM). By contrast, 4,4′-DiOMEA displayed high activity against both HT-29 and SW-620 colon cancer cells ($IC_{50} \leq 10$ μM) under conditions in which normal cells were almost not affected by this compound ($IC_{50} \geq 55$ μM) (Table 2), which further pointed to this EA derivate as a promising agent in colon cancer therapy.

The Antiproliferative Activity of 4,4′-DiOMEA Is Not Related to Its Antioxidant Activity and Might Be Mediated by Wnt Signaling Inhibition. To evaluate whether the reported antioxidant activity of these compounds was related to their antiproliferative activity against colon cancer cell growth, the antioxidant capacity of EA, 3,3′-DiOMEA, and 4,4′-DiOMEA was determined by the FRAP assay at the same concentration in which antiproliferative activity was observed. Figure 3A shows that EA and 3,3′-DiOMEA exhibited a dose-dependent effect, but 4,4′-DiOMEA did not show antioxidant activity at any assayed concentration.

In fact, the ferric reducing power of EA was higher than that exerted by the other compounds, whereas its antiproliferative activity against colon cancer cells was similar to that of 3,3′-DiOMEA (with almost 100-fold lower antioxidant

TABLE 2

Sensitivity of different human cell lines to selected compounds

Data are presented as IC_{50} values (concentration of an inhibitor that is needed for 50% inhibition of cell proliferation at 72 hours), with means $\pm$ S.E.M. of at least two independent experiments each performed in triplicate.

Compound	Cell Line			
	HT-29	SW-620	SW-620-5FuR	CCD18Co
			μM	
5-FU	—	7.1 ± 1.3	>5000.0	<3.0
EA	95.0 ± 10.4	79.0 ± 4.0	45.0 ± 5.0	37.5 ± 2.5
3,3′-DiOMEA	106.0 ± 3.3	72.5 ± 2.5	145.0 ± 5.0	47.5 ± 2.5
4,4′-DiOMEA	7.6 ± 1.5	5.8 ± 1.6	28.8 ± 3.2	59.5 ± 4.55
Uro-A	38.5 ± 3.5	26.0 ± 1.0	—	—
Uro-B	ns	ns	—	—
Resveratrol	90.0 ± 10.0	35.0 ± 1.1	—	—
Homovanillic acid	ns	—	—	—
Gallic acid	ns	—	—	—
Dihydrocaffeic acid	ns	—	—	—
4-*O*-Methylgallic acid	ns	—	—	—
3-*O*-Methylgallic acid	ns	—	—	—

—, not determined; ns, no significant activity found at assayed concentrations.

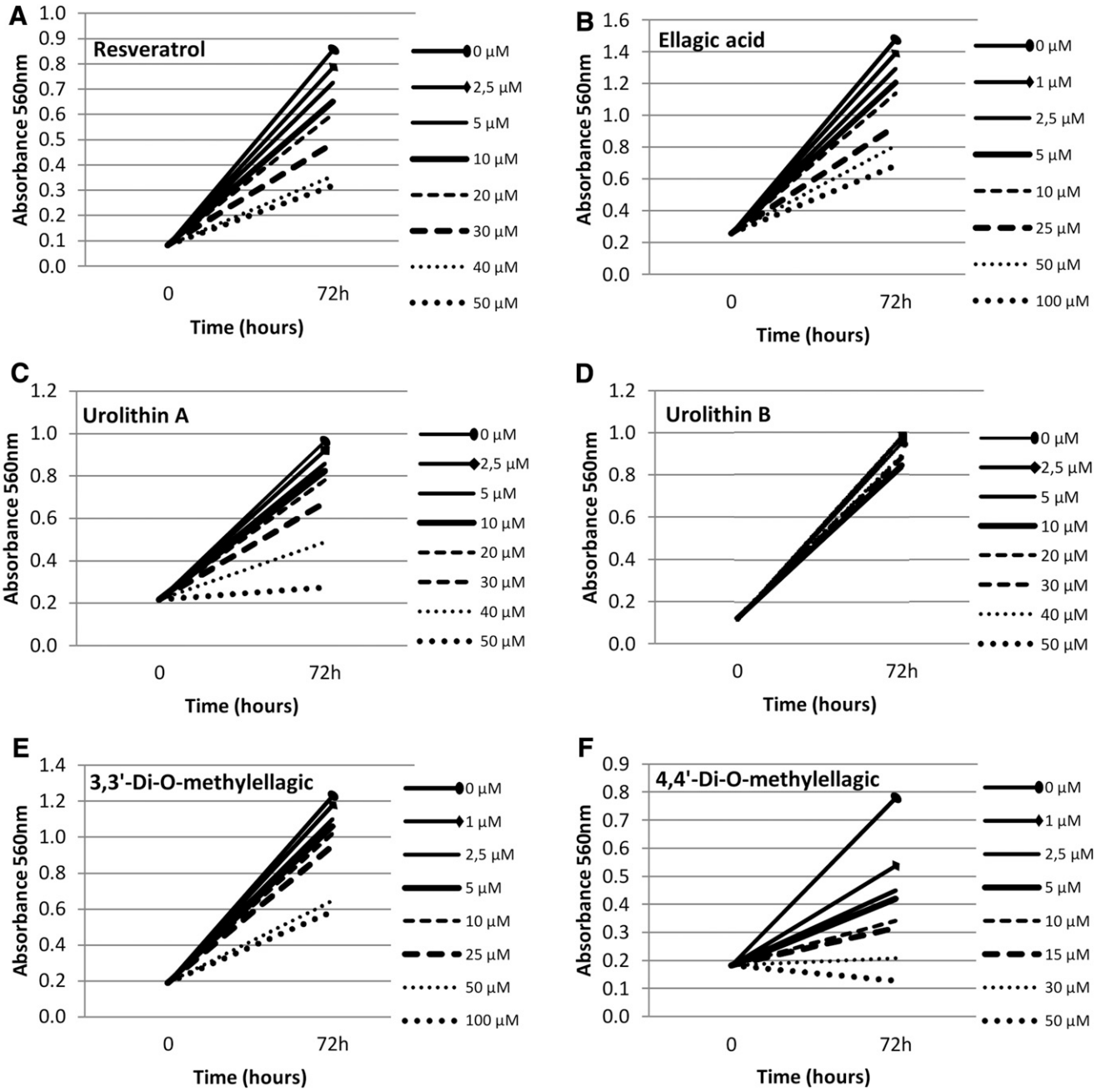


Fig. 1. Effects of different phenolic compounds on colon cancer cell viability. Cell proliferation assay of resveratrol (A), EA (B), Uro-A (C), Uro-B (D), 3,3′-DiOMEA (E), and 4,4′-DiOMEA (F) in a representative experiment of at least two independent assays performed in HT-29 and SW-620 cells.

power) and was more than 10-fold lower than that of 4,4′-DiOMEA (with no antioxidant activity). To validate these results, the antioxidant capacity of these polyphenols was further determined by the DPPH assay. The antioxidant capacity of these three polyphenols was confirmed by this alternative method, showing a dose-dependent effect for EA and lower antioxidant power for 3,3′-DiOMEA, whereas 4,4′-DiOMEA did not show activity at any concentration tested (Fig. 3B).

To gain insight in the molecular mechanisms involved in the antiproliferative activity of 4,4′-DiOMEA, a comparative microarray gene expression analysis between nontreated (control) and 5 μM 4,4′-DiOMEA–treated SW-620 colon cancer cells was performed. Our results showed that only 11 genes were differentially expressed between nontreated and 4,4′-DiOMEA–treated cells; of these genes, 5 were down-regulated and 6 were upregulated (Table 3).

Gene Ontology analysis of differentially expressed genes was performed to identify the most relevant networks and cellular functions involved in the antiproliferative activity of this molecule (Table 4). This analysis showed that Wnt signaling was the most relevant pathway in cancer modulated by this compound, which could be mediating its antiproliferative effect in colon cancer cells. WNT16 was highlighted as the main differentially expressed gene after 4,4′-DiOMEA treatment. This gene is involved in important processes such

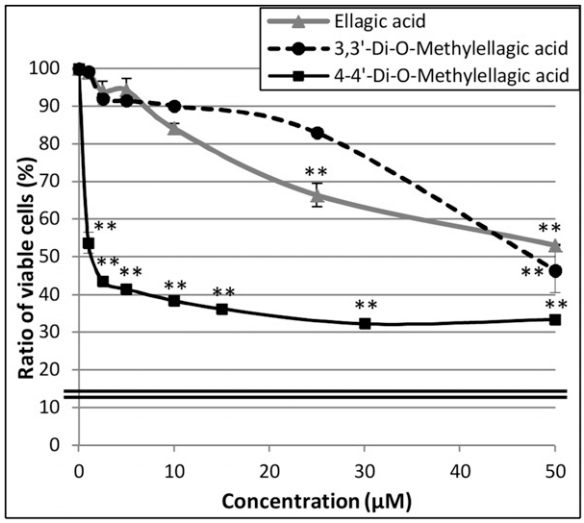


Fig. 2. 4,4′-DiOMEA induces human colon cancer cell growth inhibition. Dose-response curves of the cell viability assays after 72 hours treatment of SW-620 colon cancer cells with increasing concentrations of EA and its derivatives (3,3′-DiOMEA and 4,4′-DiOMEA). Data represent means ± S.E.M. of at least two independent experiments each performed in triplicate. Asterisks indicate statistically different values in treated cells with respect to the control (nontreated cells); $^{**}P < 0.01$. The double line indicates the ratio of viable cells at time zero.

as the response to oxidative stress and pathways related to cancer, including both Wnt and Hedgehog signaling pathways (Table 4). The modulation of WNT16 by 4,4′-DiOMEA was validated by quantitative real-time PCR (Fig. 4) in which a dose-dependent downregulation of WNT16 by 4,4′-DiOMEA was observed, showing decreased levels of WNT16 mRNA versus nontreated cells by 36, 50, and 81% after 5, 20, and 50 μM 4,4′-DiOMEA treatment, respectively.

Discussion

Ellagitannins constitute a diverse group of polyphenols with known biologic activity (Larrosa et al., 2010a). However, this activity is greatly affected by the low bioavailability of both ellagitannins and their hydrolysis product EA, which is further metabolized to urolithins by the colon microbiota to yield urolithins (Espin et al., 2013). In fact, the occurrence of urolithins, EA, and a number of derivatives, including dimethylellagic acid, was recently described in both normal and malignant colon tissues from patients with CRC after consumption of pomegranate extracts (Nuñez-Sánchez et al., 2014). This study suggested that these metabolites could be the real active molecules involved in the reported biologic effects for ellagitannins and EA, especially those effects related to gastrointestinal pathologies such as CRC (Nuñez-Sánchez et al., 2014). In addition, this study established the basis for the investigation of different EA-derived metabolites, including urolithins, as possible antitumor compounds. In this regard, our study considers EA and in vivo metabolites with the aim of elucidating possible structure-activity relationships that could be involved in the antiproliferative effect of these molecules on colon cancer cells, an approach that could be useful in the design and development of new antitumor agents. Our results showed strong differences within the EA family of compounds regarding effects on colon

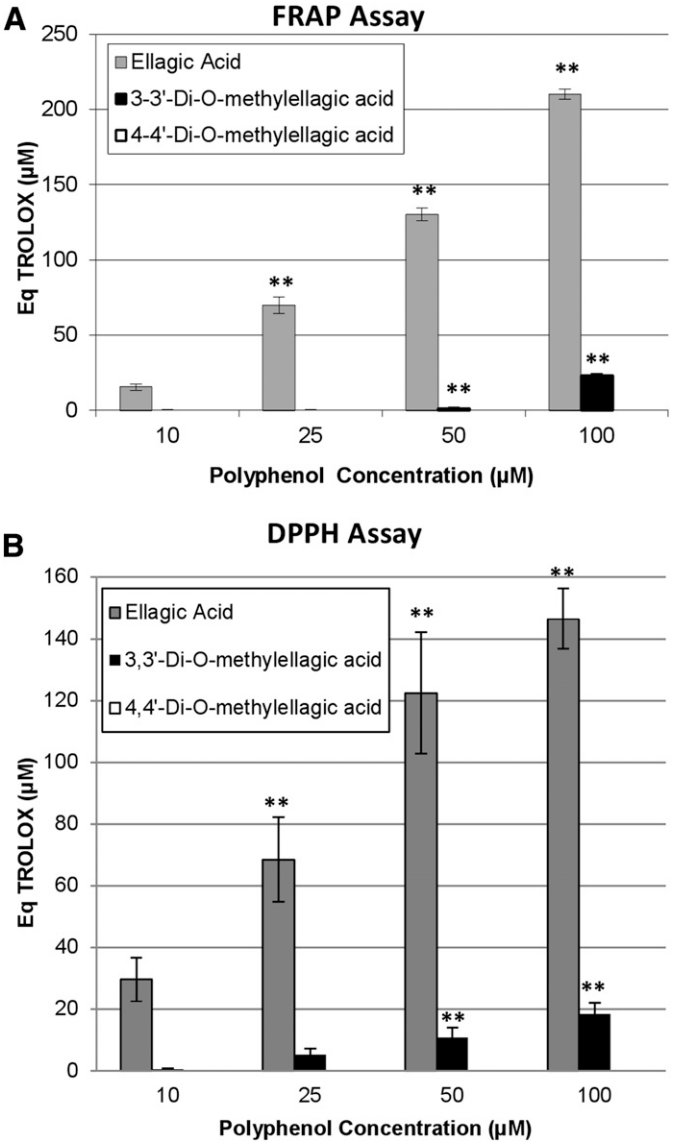


Fig. 3. (A) FRAP assay after addition of EA and its derivatives. (B) Validation of the antioxidant capacity of EA and its derivatives by DPPH assay. Data represent means ± S.E.M. of the equivalent of TROLOX (Eq TROLOX) from three independent experiments, with three replicates per test concentration. Asterisks indicate statistically different values of the equivalent of TROLOX in polyphenol concentrations of 25, 50, and 100 μM with respect to the lowest concentration (10 μM); $^{**}P < 0.01$.

cancer cell viability, with 4,4′-DiOMEA being the most effective compound and inducing cell growth inhibition in a dose-dependent manner (Fig. 2). To our knowledge, these results are the first to show the potent antiproliferative activity of 4,4-DiOMEA as a promising chemotherapeutic drug and also confirm previous studies regarding the effect of EA and Uro-A on the growth rate of premalignant cells in different types of cancer (Stoner et al., 2007; González-Sarrías et al., 2009; Li et al., 2012; Chung et al., 2013; González-Vallinas et al., 2013b; Qiu et al., 2013; Santos et al., 2013; Vanella et al., 2013; Umesalma et al., 2014; Zhang et al., 2014). In this regard, a recent study reported that Uro-A exerted the highest antiproliferative activity on a panel of colon cancer cell lines, followed by Uro-C, Uro-D, and Uro-B. This study also reported that HT-29 cells were

TABLE 3

Genes differentially expressed after 4,4′-DiOMEA treatment

Microarray data of differentially expressed genes after treatment of human colon cancer SW-620 cells with 5 μM 4,4′-DiOMEA for 72 hours (conditions in which antiproliferative activity is observed). The experiment was repeated four times, each performed in triplicate per test concentration. Genes showing a statistical significant differential expression ($P < 0.05$) and more than 2-fold absolute change variation are shown. Presented data include fold changes, intensity values (control, logControl; treated, logExperiment), S.E.M. values, and *P* values for the Limma package (linear models for microarray data).

Gene	Accession Number	Fold Change	logControl	S.E.M. (logControl)	log Experiment	S.E.M. (logExperiment)	*P* Value (Limma)	Description
RHBDL1	NM_001278720	−2.54	13.67	0.1	12.33	0.7	0.039	*Homo sapiens* rhomboid, veinlet-like 1 (*Drosophila*) (RHBDL1), transcript variant 1
A4GALT	NM_017436	−2.49	14.02	0.3	12.71	0.5	0.016	*H. sapiens* α1,4-galactosyltransferase (A4GALT)
WNT16	NM_057168	−2.3	7.14	0.7	5.94	0.1	0.05	*H. sapiens* wingless-type MMTV integration site family, member 16 (WNT16), transcript variant 1
PCSK1N	NM_013271	−2.19	14.66	0.3	13.53	0.5	0.029	*H. sapiens* proprotein convertase subtilisin/kexin type 1 inhibitor (PCSK1N)
PAPPA	NM_002581	−2.12	7.36	0.6	6.27	0.1	0.033	*H. sapiens* pregnancy-associated plasma protein A, pappalysin 1 (PAPPA)
WDR52	NM_001164496	4.5	6.4	0.1	8.57	1.2	0.039	*H. sapiens* WD repeat domain 52 (WDR52), transcript variant 1
MUSK	NM_005592	2.46	6.47	0.1	7.77	0.7	0.044	*H. sapiens* muscle, skeletal, receptor tyrosine kinase (MUSK), transcript variant 1
GRHL1	NM_198182	2.2	6.5	0.1	7.64	0.5	0.013	*H. sapiens* grainyhead-like 1 (*Drosophila*) (GRHL1)
GTF2I	ENST00000473333	2.1	6.17	0	7.24	0.3	0.002	General transcription factor Iii
SLC22A8	ENST00000451262	2.06	7.13	0.1	8.18	0.3	0.002	Solute carrier family 22 (organic anion transporter), member 8
S100A5	NM_002962	2.05	7.95	0.2	8.98	0.4	0.013	*H. sapiens* S100 calcium binding protein A5 (S100A5)

able to partially overcome the effects after 48 hours, which was related to the complete glucuronidation of urolithins that exerted lower anticancer activity (González-Sarrías et al., 2014). Therefore, this supports our results regarding the lack of an antiproliferative effect of Uro-B on HT-29 cells.

TABLE 4

Gene Ontology analysis of differentially expressed genes after 4,4′-DiOMEA treatment

Analysis of biologic processes and pathways significantly altered by 4,4′-DiOMEA in SW-620 colon cancer cells. *P* value (Hyp) represents the *P* value of the hypergeometric test used in this analysis. Data were obtained from the Gene Ontology, KEGG pathways, and Gene Set Enrichment Analysis databases.

	Reference Support	*P* Value (Hyp)	*P* Value (Hyp)*	Genes
Biologic process				
Tripeptide transport	2	0.0012	0.0377	SLC22A8
Regulation of synaptic growth at neuromuscular junction	2	0.0012	0.0377	MUSK
Stress-induced premature senescence	5	0.0031	0.0419	WNT16
Regulation of gene expression	3160	0.0021	0.0424	EIF3K,MUSK,GRHL1,BARX1, CHD4,GTF2I,WNT16
Response to methotrexate	4	0.0025	0.0431	SLC22A8
Vasculature development	468	0.0028	0.0433	FZD9,GTF2I,EPHB4
Oxidative stress-induced premature senescence	3	0.0018	0.0453	WNT16
Cardiovascular system development	693	0.0008	0.0467	FZD9,GTF2I,WNT16,EPHB4
KEGG pathways				
Basal cell carcinoma	55	0.0005	0.0047	WNT16,FZD9
Melanogenesis	98	0.0016	0.0074	WNT16,FZD9
Wnt signaling pathway	149	0.0037	0.0112	WNT16,FZD9
Glycosphingolipid biosynthesis - globo series	14	0.0086	0.0193	A4GALT
Pathways in cancer	324	0.0167	0.0300	WNT16,FZD9
Basal transcription factors	39	0.0237	0.0355	GTF2I
Hedgehog signaling pathway	56	0.0338	0.0435	WNT16
Bile secretion	71	0.0427	0.0480	SLC22A8
Transcription factors				
CP2	178	0.0002	0.0132	CHD4,RHBDL1,PAPPA
PAX4	164	0.0001	0.0208	RBMS3,CHD4,PAPPA
E12	1805	0.0006	0.0225	A4GALT,MUSK,GTF2I,WNT16, RHBDL1,SLC22A8
AP4	1119	0.0005	0.0250	RBMS3,MUSK,FZD9,CHD4, CRHBP
TEF-1	384	0.0016	0.0495	RBMS3,MUSK,CHD4

(Hyp)*, *P* value of the hypergeometric test adjusted by false discovery rate correction.

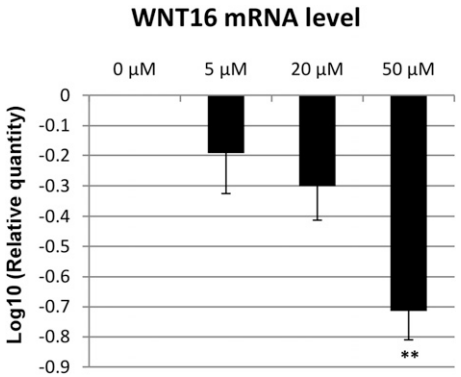


Fig. 4. WNT16 mRNA expression in human SW-620 colon cancer–derived cells treated with different concentrations of 4,4′-DiOMEA. Relative quantification for WNT16 in SW-620 cells treated with 5, 20, and 50 μM 4,4′-DiOMEA in relation to nontreated cells normalized to the endogenous control (glyceraldehyde 3-phosphate dehydrogenase). Four independent experiments were performed (each concentration in triplicate), and all samples were amplified in triplicate. Means ± S.E.M. are shown. Asterisks indicate statistically different values in treated cells (5, 20, and 50 μM) with respect to the control (nontreated cells; 0 μM); $^{**}P < 0.01$.

It is important to highlight that the differential hydroxyl (–OH) substitution of Uro-A and Uro-B resulted in drastically different antiproliferative activity of these compounds (Fig. 5), suggesting that additional –OH at the 8 position in Uro-A is essential for this biologic activity.

In this regard, a previous study also supported the potential role of –OH groups in urolithins in the interaction with breast cancer resistant protein transporter BCRP. This study suggested that the presence of an –OH group at the 8 position, but not at the 3 position, might favor the interaction with BCRP (González-Sarrías et al., 2013). It is important to take into account that urolithins are dibenzopyran-6-one derivatives produced by the opening and decarboxylation of one of the lactone rings of EA and the sequential removal of different hydroxyls. Since Uro-A was more active than both EA and its 3,3′-DiOMEA derivative, this result suggests that the presence of a lactone ring of EA is not relevant for its antiproliferative activity in cancer cells.

Regarding DiOMEA derivatives, these compounds have a methoxy group replacing an alcohol group with respect to EA structure, and this change produces a decrease in the molecular polarity (Cichocki et al., 2008; Paul et al., 2009). The significant increase in the activity of 4,4′-DiOMEA with respect to the 3,3′ derivative further confirmed the important role of –OH groups in these positions, which could establish the basis for structural-based EA drug design with enhanced anticancer activity (Fig. 5). In addition, results showed that CCD18Co normal human colon epithelial cells were more sensitive to EA and 3,3′-DiOMEA treatments than cancer cells. On the contrary, 4,4′-DiOMEA was more selective against colon cancer cells, which further supported its use as a potential cancer chemopreventive agent. It is also important to note that 4,4′-DiOMEA exerted significant antiproliferative activity in SW-620-5FuR cells, a cell line that does not respond to 5-FU treatment, which is one of the most commonly used treatments for CRC. Clinical studies have demonstrated that only 10–15% of patients with advanced CRC respond to administration of 5-FU alone; these response rates modestly increase to near 50% when this drug is combined with other antitumoral agents (Zhang et al., 2008). Consequently, drug resistance represents one of the main problems of current chemotherapy failure. In this regard, anticancer therapies based on the combination of agents targeting different molecules, either within the same signaling pathway or involved in different pathways, may more likely avoid resistance to therapy. Our results indicate that 4,4′-DiOMEA might constitute a promising coadjuvant agent in CRC therapy, although future additional preclinical and clinical experiments will be required.

EA has been reported to exert antioxidant effects (Huang et al., 2012), which could be mediating its anticancer activity. Thus, we studied the potential correlation between the antiproliferative and antioxidant activities for EA and its derivatives 3,3′-DiOMEA and 4,4′-DiOMEA. The results showed that EA exerted the highest antioxidant activity using the FRAP method. The activity of 3,3′-DiOMEA was 100-fold lower than that of EA at the maximum concentration assayed, whereas 4,4′-DiOMEA did not show activity at any concentration tested. These results of antioxidant capacity were further confirmed by using the DPPH assay, an alternative method for determination of antioxidant power. This suggests that the antioxidant activity of these EA derivatives does not seem to be directly related to their antiproliferative effects on colon cancer cell lines; however, additional research is needed, including other related methods such as lipid peroxidation, to further understand the molecular events leading to the potential antitumor action of this compound and the putative involvement of its additional biologic activities. In this sense, microarray analysis revealed that modulation of Wnt signaling might be involved in the antiproliferative action of this compound. This result is in agreement with other studies in which Wnt and Hedgehog pathway regulation by Uro-A and EA has been observed in cancer cells (Anitha et al., 2013; Espin et al., 2013). In this sense, Wnt proteins are reported to be extensively involved in oncogenesis and their expression is regulated by the nuclear factor κ-light-chain-enhancer of activated B cells after DNA damage. Specifically, the overexpression of WNT16 in nearby

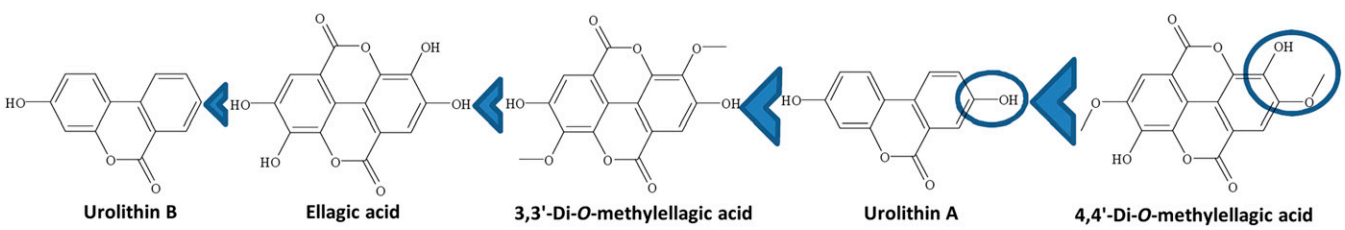


Fig. 5. Rank of the order of antiproliferative activity against colon cancer cells of EA and derivatives from lowest to the highest regarding their chemical structures.

normal cells was suggested to be responsible for the development of chemotherapy resistance in cancer cells (Sun et al., 2012). The expression of WNT16 in the tumor microenvironment attenuates the cytotoxic effects of chemotherapy in vivo, promoting tumor cell survival and disease progression. This suggests a mechanism by which consecutive cycles of genotoxic chemotherapy might increase drug resistance in subsequent treatment in the tumor microenvironment (Sun et al., 2012), further supporting the potential interest in its downregulation by 4,4′-DiOMEA in colon cancer therapy.

In conclusion, we report here that the most effective compound in colon cancer cell growth inhibition of this family of polyphenols was 4,4′-DiOMEA, which was also effective in colon cancer cells resistant to the chemotherapeutic agent 5-FU and had almost imperceptible activity on normal cells. Our study reveals that the small structural variations of EA conducting to the 4,4′-DiOMEA derivative results in a promising strategy to develop new structural-based EA anticancer drugs for CRC. The antiproliferative activity observed does not seem to be related to the antioxidant power of this compound; rather, it seems to be related to the modulation of Wnt signaling pathways.

Authorship Contributions

Participated in research design: Ramírez de Molina, Vargas, Reglero.

Conducted experiments: Vargas, Molina, Sánchez, Martínez-Romero, González-Vallinas, Martín-Hernández, Gómez de Cedrón.

Contributed new reagents or analytic tools: Dávalos, Calani, Del Rio, González-Sarrías, Espín, Tomás-Barberán.

Performed data analysis: Ramírez de Molina, Vargas, Molina, Martínez-Romero, Martín-Hernández, Del Rio, González-Sarrías, Espín, Tomás-Barberán, Reglero.

Wrote or contributed to the writing of the manuscript: Ramírez de Molina, Vargas, Dávalos, González-Sarrías, Espín, Tomás-Barberán, Reglero.

References

Adams LS, Zhang Y, Seeram NP, Heber D, and Chen S (2010) Pomegranate ellagitannin-derived compounds exhibit antiproliferative and antiaromatase activity in breast cancer cells in vitro. *Cancer Prev Res (Phila)* **3**:108–113.

Aggarwal BB and Shishodia S (2006) Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol* **71**:1397–1421.

American Cancer Society (2011) *Colorectal Cancer Facts and Figures 2011–2013*, American Cancer Society, Atlanta, GA.

American Cancer Society (2012) *Cancer Facts and Figures 2012*, American Cancer Society, Atlanta, GA.

Anitha P, Priyadarsini RV, Kavitha K, Thiyagarajan P, and Nagini S (2013) Ellagic acid coordinately attenuates Wnt/β-catenin and NF-κB signaling pathways to induce intrinsic apoptosis in an animal model of oral oncogenesis. *Eur J Nutr* **52**:75–84.

Cerdá B, Espín JC, Parra S, Martínez P, and Tomás-Barberán FA (2004) The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur J Nutr* **43**:205–220.

Chung YC, Lu LC, Tsai MH, Chen YJ, Chen YY, Yao SP, and Hsu CP (2013) The inhibitory effect of ellagic acid on cell growth of ovarian carcinoma cells. *Evid Based Complement Alternat Med* **2013**:306705.

Cichocki M, Paluszczak J, Szaefer H, Piechowiak A, Rimando AM, and Baer-Dubowska W (2008) Pterostilbene is equally potent as resveratrol in inhibiting 12-O-tetradecanoylphorbol-13-acetate activated NFkappaB, AP-1, COX-2, and iNOS in mouse epidermis. *Mol Nutr Food Res* **52** (Suppl 1):S62–S70.

Coates EM, Popa G, Gill CI, McCann MJ, McDougall GJ, Stewart D, and Rowland I (2007) Colon-available raspberry polyphenols exhibit anti-cancer effects on in vitro models of colon cancer. *J Carcinog* **6**:4.

Da Silva SL, Calgarotto AK, Chaar JS, and Marangoni S (2008) Isolation and characterization of ellagic acid derivatives isolated from Casearia sylvestris SW aqueous extract with anti-PLA(2) activity. *Toxicon* **52**:655–666.

Del Follo-Martinez A, Banerjee N, Li X, Safe S, and Mertens-Talcott S (2013) Resveratrol and quercetin in combination have anticancer activity in colon cancer cells and repress oncogenic microRNA-27a. *Nutr Cancer* **65**:494–504.

Espin JC, Larrosa M, Garcia-Conesa MT, and Tomas-Barberan F (2013) Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. *Evid Based Complement Alternat Med* **2013**:270418.

Ferk F, Chakraborty A, Jäger W, Kundi M, Bichler J, Mišík M, Wagner KH, Grasl-Kraupp B, Sagmeister S, Haidinger G, et al. (2011) Potent protection of gallic acid against DNA oxidation: results of human and animal experiments. *Mutat Res* **715**:61–71.

Forester SC, Choy YY, Waterhouse AL, and Oteiza PI (2014) The anthocyanin metabolites gallic acid, 3-O-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde decrease human colon cancer cell viability by regulating pro-oncogenic signals. *Mol Carcinog* **53**:432–439.

Fouad MA, Agha AM, Merzabani MM, and Shouman SA (2013) Resveratrol inhibits proliferation, angiogenesis and induces apoptosis in colon cancer cells: calorie restriction is the force to the cytotoxicity. *Hum Exp Toxicol* **32**:1067–1080.

Giftson JS, Jayanthi S, and Nalini N (2010) Chemopreventive efficacy of gallic acid, an antioxidant and anticarcinogenic polyphenol, against 1,2-dimethyl hydrazine induced rat colon carcinogenesis. *Invest New Drugs* **28**:251–259.

González-Sarrías A, Espín JC, Tomás-Barberán FA, and García-Conesa MT (2009) Gene expression, cell cycle arrest and MAPK signalling regulation in Caco-2 cells exposed to ellagic acid and its metabolites, urolithins. *Mol Nutr Food Res* **53**: 686–698.

González-Sarrías A, Giménez-Bastida JA, García-Conesa MT, Gómez-Sánchez MB, García-Talavera NV, Gil-Izquierdo A, Sánchez-Alvarez C, Fontana-Compiano LO, Morga-Egea JP, Pastor-Quirante FA, et al. (2010a) Occurrence of urolithins, gut microbiota ellagic acid metabolites and proliferation markers expression response in the human prostate gland upon consumption of walnuts and pomegranate juice. *Mol Nutr Food Res* **54**:311–322.

González-Sarrías A, Giménez-Bastida JA, Núñez-Sánchez MA, Larrosa M, García-Conesa MT, Tomás-Barberán FA, and Espín JC (2014) Phase-II metabolism limits the antiproliferative activity of urolithins in human colon cancer cells. *Eur J Nutr* **53**:853–864.

González-Sarrías A, Larrosa M, Tomás-Barberán FA, Dolara P, and Espín JC (2010b) NF-kappaB-dependent anti-inflammatory activity of urolithins, gut microbiota ellagic acid-derived metabolites, in human colonic fibroblasts. *Br J Nutr* **104**: 503–512.

González-Sarrías A, Miguel V, Merino G, Lucas R, Morales JC, Tomás-Barberán F, Alvarez AI, and Espín JC (2013) The gut microbiota ellagic acid-derived metabolite urolithin A and its sulfate conjugate are substrates for the drug efflux transporter breast cancer resistance protein (ABCG2/BCRP). *J Agric Food Chem* **61**: 4352–4359.

González-Vallinas M, González-Castejón M, Rodríguez-Casado A, and Ramírez de Molina A (2013a) Dietary phytochemicals in cancer prevention and therapy: a complementary approach with promising perspectives. *Nutr Rev* **71**:585–599.

González-Vallinas M, Molina S, Vicente G, de la Cueva A, Vargas T, Santoyo S, Garcia-Risco MR, Fornari T, Reglero G, and Ramirez de Molina A (2013b) Antitumor effect of 5-fluorouracil is enhanced by rosemary extract in both drug sensitive and resistant colon cancer cells. *Pharmacol Res* **72**:61–68.

Huang WY, Zhang HC, Liu WX, and Li CY (2012) Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing. *J Zhejiang Univ Sci B* **13**:94–102.

Ito K, Nakazato T, Yamato K, Miyakawa Y, Yamada T, Hozumi N, Segawa K, Ikeda Y, and Kizaki M (2004) Induction of apoptosis in leukemic cells by homovanillic acid derivative, capsaicin, through oxidative stress: implication of phosphorylation of p53 at Ser-15 residue by reactive oxygen species. *Cancer Res* **64**:1071–1078.

Jeon KS, Na HJ, Kim YM, and Kwon HJ (2005) Antiangiogenic activity of 4-O-methylgallic acid from Canavalia gladiata, a dietary legume. *Biochem Biophys Res Commun* **330**:1268–1274.

Kasimsetty SG, Bialonska D, Reddy MK, Ma G, Khan SI, and Ferreira D (2010) Colon cancer chemopreventive activities of pomegranate ellagitannins and urolithins. *J Agric Food Chem* **58**:2180–2187.

Larrosa M, García-Conesa MT, Espín JC, and Tomás-Barberán FA (2010a) Ellagitannins, ellagic acid and vascular health. *Mol Aspects Med* **31**:513–539.

Larrosa M, González-Sarrías A, Yáñez-Gascón MJ, Selma MV, Azorín-Ortuño M, Toti S, Tomás-Barberán F, Dolara P, and Espín JC (2010b) Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. *J Nutr Biochem* **21**:717–725.

Larrosa M, Tomás-Barberán FA, and Espín JC (2006) The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J Nutr Biochem* **17**: 611–625.

Li WW, Li VW, Hutnik M, and Chiou AS (2012) Tumor angiogenesis as a target for dietary cancer prevention. *J Oncol* **2012**:879623.

Losso JN, Bansode RR, Trappey A, 2nd, Bawadi HA, and Truax R (2004) In vitro anti-proliferative activities of ellagic acid. *J Nutr Biochem* **15**:672–678.

Mandal S, Shivapurkar NM, Galati AJ, and Stoner GD (1988) Inhibition of N-nitrosobenzylmethylamine metabolism and DNA binding in cultured rat esophagus by ellagic acid. *Carcinogenesis* **9**:1313–1316.

Mandal S and Stoner GD (1990) Inhibition of N-nitrosobenzylmethylamine-induced esophageal tumorigenesis in rats by ellagic acid. *Carcinogenesis* **11**:55–61.

Na HJ, Lee G, Oh HY, Jeon KS, Kwon HJ, Ha KS, Lee H, Kwon YG, and Kim YM (2006) 4-O-Methylgallic acid suppresses inflammation-associated gene expression by inhibition of redox-based NF-kappaB activation. *Int Immunopharmacol* **6**: 1597–1608.

Narayanan BA, Geoffroy O, Willingham MC, Re GG, and Nixon DW (1999) p53/p21 (WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. *Cancer Lett* **136**:215–221.

Narayanan BA and Re GG (2001) IGF-II down regulation associated cell cycle arrest in colon cancer cells exposed to phenolic antioxidant ellagic acid. *Anticancer Res* **21** (1A):359–364.

Nuñez-Sánchez MA, García-Villalba R, Monedero-Saiz T, García-Talavera NV, Gómez-Sánchez MB, Sánchez-Álvarez C, García-Albert AM, Rodríguez-Gil FJ, Ruiz-Marín M, Pastor-Quirante FA, et al. (2014) Targeted metabolic profiling of pomegranate polyphenols and urolithins in plasma, urine and colon tissues from colorectal cancer patients. *Mol Nutr Food Res* **58**:1199–1211.

Paul S, Rimando AM, Lee HJ, Ji Y, Reddy BS, and Suh N (2009) Anti-inflammatory action of pterostilbene is mediated through the p38 mitogen-activated protein kinase pathway in colon cancer cells. *Cancer Prev Res (Phila)* **2**:650–657.

Poquet L, Clifford MN, and Williamson G (2008) Effect of dihydrocaffeic acid on UV irradiation of human keratinocyte HaCaT cells. *Arch Biochem Biophys* **476**: 196–204.

Qiu Z, Zhou B, Jin L, Yu H, Liu L, Liu Y, Qin C, Xie S, and Zhu F (2013) In vitro antioxidant and antiproliferative effects of ellagic acid and its colonic metabolite, urolithins, on human bladder cancer T24 cells. *Food Chem Toxicol* **59**:428–437.

Ramírez de Molina A, Gallego-Ortega D, Sarmentero-Estrada J, Lagares D, Gómez Del Pulgar T, Bandrés E, García-Foncillas J, and Lacal JC (2008) Choline kinase as a link connecting phospholipid metabolism and cell cycle regulation: implications in cancer therapy. *Int J Biochem Cell Biol* **40**:1753–1763.

Ramírez de Molina A, Sarmentero-Estrada J, Belda-Iniesta C, Tarón M, Ramírez de Molina V, Cejas P, Skrzypski M, Gallego-Ortega D, de Castro J, Casado E, et al. (2007) Expression of choline kinase alpha to predict outcome in patients with early-stage non-small-cell lung cancer: a retrospective study. *Lancet Oncol* **8**: 889–897.

Santos IS, Ponte BM, Boonme P, Silva AM, and Souto EB (2013) Nanoencapsulation of polyphenols for protective effect against colon-rectal cancer. *Biotechnol Adv* **31**: 514–523.

Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, and Heber D (2005) In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem* **16**:360–367.

Sharma M, Li L, Celver J, Killian C, Kovoor A, and Seeram NP (2010) Effects of fruit ellagitannin extracts, ellagic acid, and their colonic metabolite, urolithin A, on Wnt signaling. *J Agric Food Chem* **58**:3965–3969.

Smart RC, Huang MT, Chang RL, Sayer JM, Jerina DM, and Conney AH (1986) Disposition of the naturally occurring antimutagenic plant phenol, ellagic acid, and its synthetic derivatives, 3-O-decylellagic acid and 3,3′-di-O-methylellagic acid in mice. *Carcinogenesis* **7**:1663–1667.

Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**:Article3.

Stoner GD, Wang LS, Zikri N, Chen T, Hecht SS, Huang C, Sardo C, and Lechner JF (2007) Cancer prevention with freeze-dried berries and berry components. *Semin Cancer Biol* **17**:403–410.

Sun Y, Campisi J, Higano C, Beer TM, Porter P, Coleman I, True L, and Nelson PS (2012) Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. *Nat Med* **18**:1359–1368.

Teel RW, Babcock MS, Dixit R, and Stoner GD (1986) Ellagic acid toxicity and interaction with benzo[a]pyrene and benzo[a]pyrene 7,8-dihydrodiol in human bronchial epithelial cells. *Cell Biol Toxicol* **2**:53–62.

Umesalma S, Nagendraprabhu P, and Sudhandiran G (2014) Antiproliferative and apoptotic-inducing potential of ellagic acid against 1,2-dimethyl hydrazine-induced colon tumorigenesis in Wistar rats. *Mol Cell Biochem* **388**:157–172.

Umesalma S and Sudhandiran G (2010) Differential inhibitory effects of the polyphenol ellagic acid on inflammatory mediators NF-kappaB, iNOS, COX-2, TNF-alpha, and IL-6 in 1,2-dimethylhydrazine-induced rat colon carcinogenesis. *Basic Clin Pharmacol Toxicol* **107**:650–655.

Umesalma S and Sudhandiran G (2011) Ellagic acid prevents rat colon carcinogenesis induced by 1, 2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. *Eur J Pharmacol* **660**:249–258.

Vanella L, Di Giacomo C, Acquaviva R, Barbagallo I, Li Volti G, Cardile V, Abraham NG, and Sorrenti V (2013) Effects of ellagic acid on angiogenic factors in prostate cancer cells. *Cancers (Basel)* **5**:726–738.

Zhang N, Yin Y, Xu SJ, and Chen WS (2008) 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules* **13**:1551–1569.

Zhang T, Chen HS, Wang LF, Bai MH, Wang YC, Jiang XF, and Liu M (2014) Ellagic acid exerts anti-proliferation effects via modulation of Tgf-β/Smad3 signaling in MCF-7 breast cancer cells. *Asian Pac J Cancer Prev* **15**:273–276.

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