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Perturbation of the EphA2−EphrinA1 System in Human Prostate Cancer Cells by Colonic (Poly)phenol Catabolites

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Supporting Information

ABSTRACT: The Eph tyrosine kinase receptors and their ephrin ligands play a central role in human cancer as their deregulated expression induces tumorigenesis with aggressive phenotypes. To evaluate their potential contribution to EphA2−ephrinA1 modulation, several colonic catabolites of dietary (poly)phenolics, known to be generated in vivo, were screened using an ELISA-based binding assay. Some of the catabolites inhibited the binding in a dose-dependent manner (IC_{50} values from 0.26 to 43 μM). Functional studies on prostate adenocarcinoma cells revealed that pyrogallol and protocatechuic acid specifically antagonized ephrinA1-Fc-induced EphA2 phosphorylation at concentrations that were not cytotoxic. The active concentrations of pyrogallol appear to be close to what can be reached in vivo under physiological conditions. Finally, because of the roles played by the Eph-ephrin system not only in cancer development but also in neurodegeneration and diabetes, pyrogallol and protocatechuic acid are candidates for more detailed functional studies to elucidate their role in these pathophysiological processes.

KEYWORDS: *Eph kinases, ephrin, dietary (poly)phenols, colonic catabolites, human microbiome, cancer*

INTRODUCTION

The Eph receptors represent the largest family of protein−tyrosine kinases, and their ligands are membrane-anchored proteins called ephrins. Both Eph receptors and ephrins are divided into A and B classes. The ephrinA ligands are glycosylphosphatidylinositol (GPI)-anchored molecules, whereas the ephrinB ligands are transmembrane proteins having cytoplasmic domains that can engage in various signaling activities.[1,2] The Eph/ephrin system modulates many biological processes, including cell−substrate adhesion, cell−cell interactions and cell migration/invasion. It plays a critical role in orchestrating cell movement during morphogenetic processes such as gastrulation, segmentation, angiogenesis, axonal pathfinding, and neural crest cell migration.[1,2] Eph/ephrin expression and activity in normal adult tissues are less prominent than in embryonic tissues, but recent data suggest that up-regulated expression and deregulated function of the Eph/ephrin system in a diversity of human cancers play important roles in tumor progression, for example, by promoting tumor angiogenesis and, in some cases, cancer cell malignancy.[3−5]

Many Eph receptors and ephrins have been implicated in tumorigenesis, but the EphA2 receptor and the ephrinA1 ligand are the most widely expressed and extensively investigated. EphA2 is highly expressed in most types of cancers, and its levels correlate with tumor stage and progression.[6−8] In some cancers, EphA2−ephrinA1 interaction promotes malignancy and vasculogenic mimicry.[9] In addition, although EphA2 is not expressed in embryonic or quiescent adult blood vessels, this receptor is extensively up-regulated in tumor blood vessels, where it plays an important role in tumor angiogenesis.[4,5,10−12]

Eph−ephrin engagement leads not only to a "forward signal", mediated by Eph kinase activation, but also to the transduction of a "reverse signal" into the ephrin-expressing cell. Moreover, Eph receptors and ephrins can also signal independently of each other, through cross-talk with other signaling systems, which produces more distinctive outcomes. Both tumor suppression and tumor promotion effects are reported in the literature for this system, depending upon cells and microenvironments. As the Eph−ephrin system plays an important role in epithelial homeostasis by promoting contact-dependent growth inhibition and decreasing motility and invasiveness, it is not surprising that interactions between ephrins and Eph receptors can inhibit oncogenic signaling via the HRAS-Erk, PI3K-Akt, and Abl-Crk pathways. Similarly, ephrin reverse signaling can contribute to tumor suppression, promoting tight

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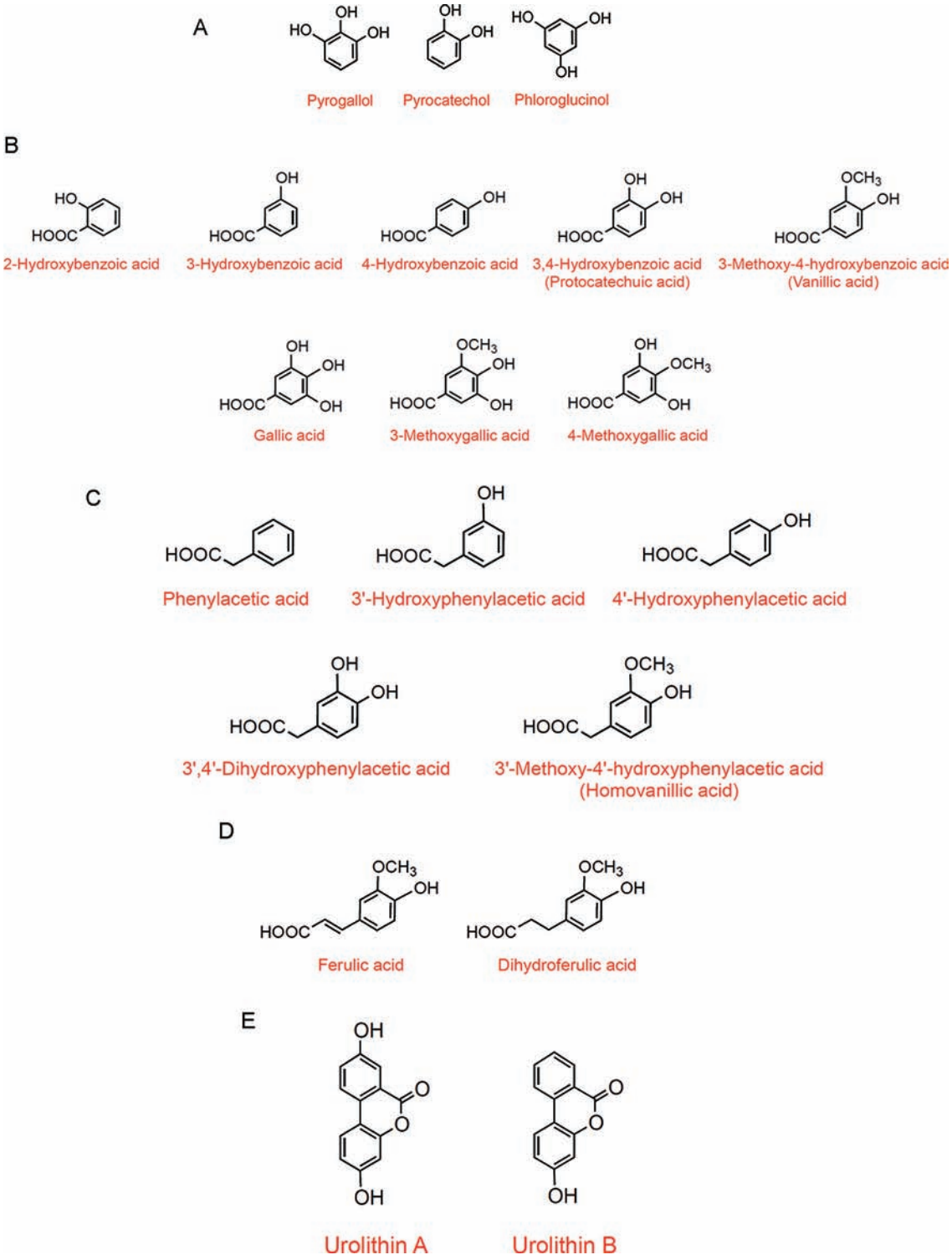

Figure 1. Chemical structures of (A) hydroxybenzenes, (B) benzoic acid derivatives, (C) phenylacetic acid derivatives, (D) ferulic acid and dihydroferulic acid, and (E) urolithins A and B.

junction formation, inhibiting the migratory and invasive effects of the CXCR4 G protein-coupled chemokine receptor, and/or down-regulating EGFR levels in glioblastoma cells. Conversely, this system can promote tumor angiogenesis and enhance malignant transformation in some cases, promoting cell proliferation by ABL1 activation, supporting ameboid-type migration of cancer cells by RHOA downstream, and activating Src family kinase FYN, integrin-mediated adhesion, and Erk MAP kinases in fibroblasts.[5] Finally, imbalance of Eph/ephrin function may contribute to neurodegeneration,[4] and up-regulation of multiple Eph receptors and ephrins has been detected at sites of nervous system injury.[13] Moreover, a recent study showed that β cells communicate via EphA and ephrinsA regulating insulin release.[14] These findings suggest that pharmacological modulation of forward and/or reverse signaling could open promising perspectives, not only in cancer

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chemoprevention and/or chemotherapy but also in pathologies where the Eph−ephrin system is unbalanced.

Mohamed et al.[15] reported the interference of (poly)phenol-rich botanicals with the EphA2−ephrinA1 activity. The research was conducted with the aim of further clarifying the possible involvement of this system in the association between the adherence to specific diets and the incidence of various human chronic diseases including prostate, liver, colon, lung, stomach, bladder, and breast cancers.[16] However, (poly)phenols are now recognized to undergo extensive metabolism after ingestion by humans. The small intestine has long been regarded as a key site in (poly)phenol absorption and metabolism, and whereas dietary (poly)phenols are indeed modified and absorbed during passage through the small intestine before entering the systemic circulation, the majority of the intake passes from the small to the large intestine.[17,18] In the colon, the human microbiome induces substantial transformations that are very different from those induced by human intestinal and hepatic enzymes.[19] The human colon hosts a highly complex microbial ecosystem that interacts with the host through a symbiotic relationship and operates as a bioreactor with a virtually unlimited metabolic potential.[20] The gut microbiota carries out chemical reactions to modify flavonoid and phenolic structures, resulting in the production and absorption of a range of low molecular weight catabolites. Microbial enzymes can hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones, induce ring fission reactions, and operate reduction, decarboxylation, demethylation, and dehydroxylation reactions.[19]

In the present study we tested the ability of several (poly)phenol catabolites of colonic origin (Figure 1) to interact with the EphA2−ephrinA1 system, as in vivo these are the compounds that are absorbed, reaching the internal compartments of the human organism after ingestion of (poly)phenol-rich foods and beverages.

MATERIALS AND METHODS

Reagents. All culture media and supplements were purchased from Lonza (Basel, Switzerland). Recombinant proteins and antibodies were from R&D Systems. Cells were purchased from ECACC (Porton Down, U.K.). Leupeptin, aprotinin, NP40, MTT, Tween20, BSA, and salts for solutions were from Applichem (Darmstadt, Germany); analytical grade extraction solvents, EDTA, and sodium orthovanadate were from Sigma (St. Louis, MO, USA).

Gallic acid (3,4,5-trihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid), 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, phenylacetic acid, 3′-hydroxyphenylacetic acid, 4′-hydroxyphenylacetic acid, 3′,4′-dihydroxyphenylacetic acid, ferulic acid, and dihydroferulic acid were purchased from Sigma as well as pyrogallol (1,2,3-trihydroxybenzene), pyrocatechol (1,2-dihydroxybenzene), and phloroglucinol (1,3,5-trihydroxybenzene). Homovanillic acid (3′-methoxy-4′-hydroxyphenylacetic acid), 3-*O*-methylgallate, and 4-*O*-methylgallate were purchased from Extrasynthese (Genay Cedex, France).

Urolithins were synthesized by Cu(II)-mediated coupling of 1,3-dihydroxybenzene and 2-bromo-5-hydroxybenzoic acid (urolithin A) or 2-bromobenzoic acid (urolithin B) in concentrated aqueous NaOH according to already reported procedures.[21] 2-Bromo-5-hydroxybenzoic acid was obtained from commercially available 2-bromo-5-methoxybenzoic acid through demethylation by BBr_3 (3 equiv) in CH_2Cl_2/hexane (3:2, v/v) at low temperature (−20 to 0 °C). Urolithins were isolated by precipitation, and their purity was checked by HPLC analysis. Their NMR and MS characteristics were in agreement with the literature.[21]

ELISA Screening and IC_{50} Determination. This method was as previously described.[22] Briefly, 96-well ELISA high binding plates (Costar 2592) were incubated overnight at 4 °C with 100 μL/well of 1 μg/mL EphA2-Fc (R&D 639-A2) diluted in sterile PBS (0.2 g/L KCl, 8.0 g/L NaCl, 0.2 g/L KH_2PO_4, 1.15 g/L Na_2HPO_4, pH 7.4), washed three times with washing buffer (PBS + 0.05% Tween 20, pH 7.5), and blocked with 300 μL of blocking solution (PBS + 0.5% BSA) for 1 h at 37 °C. Compounds (dissolved as 1 mM stock solutions in PBS) were added to the wells at appropriate concentrations and incubated at 37 °C for 1 h. Biotinylated ephrinA1-Fc (R&D BT602) was added for 4 h at 100 ng/mL in displacement studies. Wells were washed three times and incubated with 100 μL/well streptavidin−HRP (Sigma S5512) solution (0.05 μg/mL in PBS supplemented with 0.5% BSA, pH 7.4) for 20 min at room temperature, then washed again three times and incubated at room temperature with 0.1 mg/mL tetramethylbenzidine (Sigma T2885) reconstituted in stable peroxide buffer (11.3 g/L citric acid, 9.7 g/L sodium phosphate, pH 5.0) and 0.1% H_2O_2 (30% m/m in water) added immediately before use. The reaction was stopped with 3 N HCl 100 μL/well, and absorbance at 450 nm was measured using an ELISA plate reader (Sunrise, TECAN, Switzerland). The IC_{50} value was determined using one-site competition nonlinear regression with Prism software (GraphPad Software Inc.).

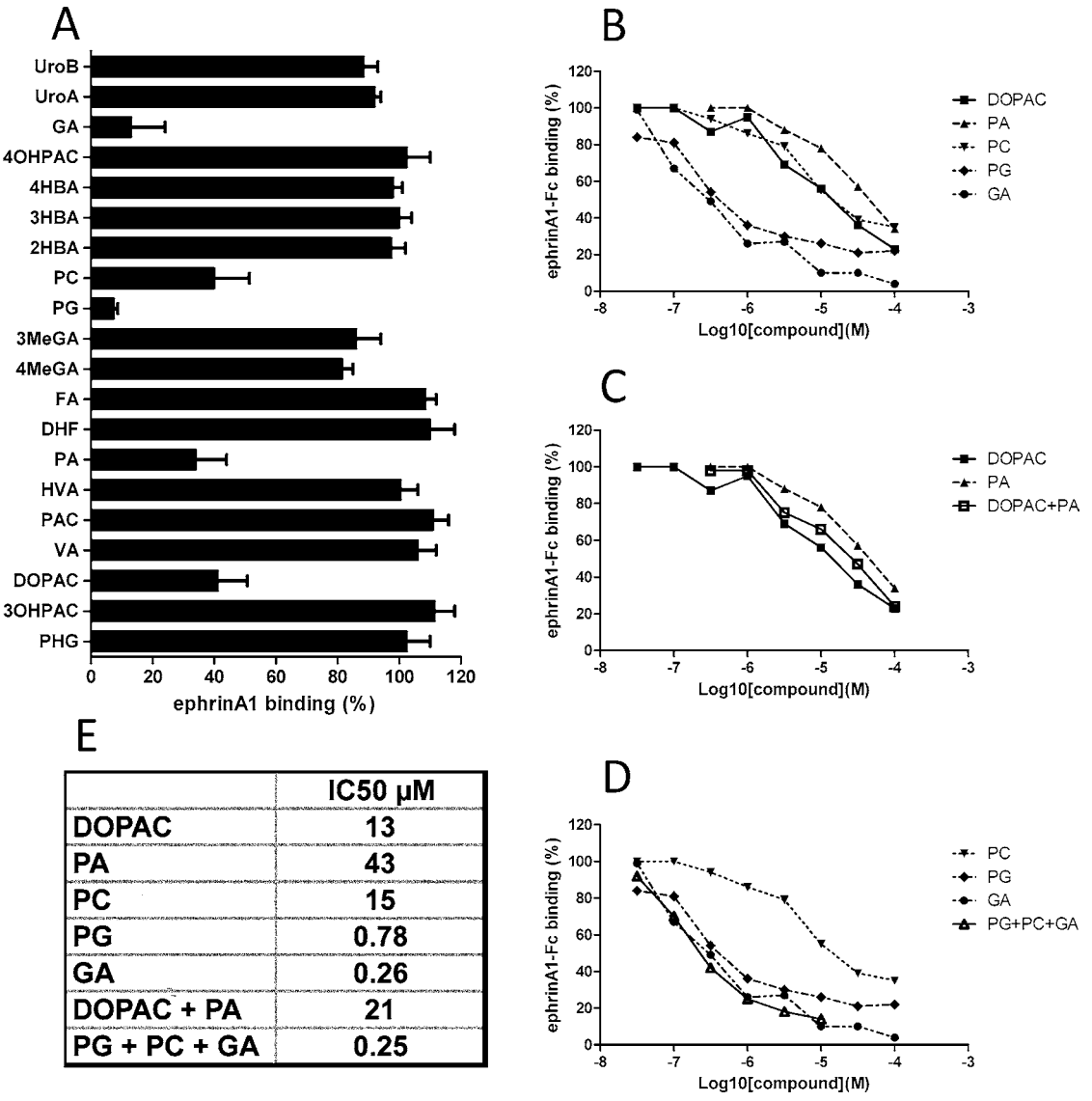
Cell Cultures. PC3 human prostate adenocarcinoma cells were grown in Ham F12 supplemented with 7% fetal bovine serum (FBS) and 1% penicillin−streptomycin and grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Cell Lysates. Cells were seeded in 12-well plates at a concentration of 10^5 cells/mL in complete medium until they reached about 40% confluence and then serum starved overnight. The day after cells were treated with test compounds, vehicle, or standard drug, stimulated with the proper agonist, rinsed with sterile PBS, and solubilized in lysis buffer. The lysates were suspended and rocked at 4 °C for 30 min and then centrifuged at 14000*g* for 5 min. The protein content of supernatant was measured with a BCA protein assay kit (Thermo Scientific), standardized to 200 μg/mL, and transferred into a clean test tube ready to be used.

Phosphorylation Assays. EphA2 and EGF receptor (EGFR) phosphorylation were measured in cell lysates using DuoSet IC Sandwich ELISA (RnD Systems, DYC4056 and DYC1095, respectively) following the manufacturer's protocol. Briefly, 96-well ELISA high binding plates (Costar 2592) were incubated overnight at room temperature with 100 μL/well of the specific capture antibody diluted in sterile PBS to the proper working concentrations, washed, and blocked for 1 h at room temperature. Wells were washed, and 100 μL/well of lysates was added at room temperature for 2 h, after which wells were again washed before being incubated with detection antibody at room temperature for 2 h. The receptor phosphorylation was revealed utilizing a standard HRP format with a colorimetric reaction read at 450 nm.

LDH Assay. Cytotoxicity of all compounds was evaluated with a CytoTox 96 Non-Radioactive Cytotoxicity Assay, following the manufacturer's protocol (Promega 1780). Briefly, cells were seeded in 96-well plates at a density of 10^5cells/mL and the day after treated with compounds or lysis buffer for 2 h. After incubation, released LDH in culture supernatants was measured with a 30 min coupled enzymatic assay, which resulted in conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed was proportional to the number of lysed cells and quantified by ELISA plate reader (Sunrise, TECAN, Switzerland) at 492 nm. The results were expressed as the ratio between the absorbance of the cells treated with the compounds and cells treated with lysis buffer.

Statistical Analysis. The IC_{50} value was determined using one-site competition nonlinear regression with Prism software (GraphPad Software Inc.). Theoretical curves of metabolites associations were calculated with the Webb equation.[23] Data are the means of at least three independent experiments ± standard error. Comparison for phospho-EphA2 and phospho-EGFR levels was performed using one-way ANOVA followed by Dunnet's post hoc test.



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	IC50 μM
DOPAC	13
PA	43
PC	15
PG	0.78
GA	0.26
DOPAC + PA	21
PG + PC + GA	0.25

Figure 2. Compounds dose-dependently displace ephrin-A1-Fc from the immobilized EphA2-Fc ectodomain: (A) binding of ephrin-A1-Fc to immobilized EphA2-Fc in the presence of 100 μM of test compounds; (B) increasing concentrations of protocatechuic acid (PA), 3′,4′-dihydroxyphenylacetic acid (DOPAC), pyrocatechol (PC), pyrogallol (PG), and gallic acid (GA) tested toward EphA2−ephrinA1 binding; (C, D) association of equimolar (1:1) amount of the indicated compounds (the indicated final concentration (e.g., 1 μM) comes from the sum of the concentration of the single molecules (e.g., 1 μM = 0.5 μM DOPAC + 0.5 μM PA or 0.33 μM PC + 0.33 μM PG + 0.33 μM GA); data are the mean of at least four independent experiments); (E) IC_{50} values (μM) of compounds and associations calculated from graphs B−D.

RESULTS AND DISCUSSION

The ability of several (poly)phenol catabolites of colonic origin to perturb the EphA2−ephrinA1 interaction was evaluated. The structures of the tested compounds are illustrated in Figure 1. Protocatechuic acid can be generated by ring fission of anthocyanins and flavan-3-ols, as well as hydroxycinnamates,[19] and is a common catabolite formed after the consumption of wine, berries, green tea, and chocolate products. Gallic acid can be derived from the action of microbial esterases on galloylated flavan-3-ols, theaflavins, and galloylquinic acid and is, therefore, present in the colon and in blood after the intake of green and black teas. However, this trihydroxybenzoic acid can also be generated from anthocyanins and hydrolyzable gallotannins[19] and is produced after the intake of wine, berries, and pomegranate. 3-*O*-Methylgallate has been identified as a microbial catabolite of red wine anthocyanins,[24] whereas 4-*O*-methylgallate has been reported to be a biomarker of the consumption of tea, red wine, and grape seed extract. It has also been identified in the circulatory system after the ingestion of (−)-epigallocatechin-3-*O*-gallate.[25−28] Pyrogallol and pyrocatechol can be formed by decarboxylation of gallic and protocatechuic acids.[29,30] Phloroglucinol is a breakdown product of the A ring of quercetin[29] and can therefore be considered a common flavonol metabolite, circulating after the consumption of a wide range of fruits, vegetables, and beverages, in particular, after intakes of onions, apples, or black tea. Phenylacetic acid and its mono- and dihydroxyl counterparts are ring fission products of flavan-3-ols and flavonols[30,31] and as such are particularly common after the intake of an extensive range of polyphenol-rich products. Monohydroxybenzoic acids have been reported to be formed after the consumption of berry anthocyanins, green tea, and cocoa flavan-3-ols, coffee chlorogenic acids, and rosmarinic acid.[32] Dihydroferulic acid is a microbial metabolite formed by

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dehydrogenation of ferulic acid, which is present as a quinic acid ester in coffee, cereal-based products, and flaxseeds.[19] Urolithins have been described as very specific colonic metabolites of ellagitannins and ellagic acid, typically present in a number of products including pomegranate, raspberry, and chestnut skins.[29]

To perform binding assays, all of the compounds were dissolved in PBS as 1 mM stock solution and immediately tested to assess their interference with EphA2−ephrinA1 binding as previously described.[22] Briefly, the EphA2-Fc ectodomain was immobilized on ELISA plates (Corning 9018), and binding of biotinylated-ephrin-A1-Fc was detected using a standard colorimetric reaction developed by streptavidin−HRP and tetramethylbenzidine. Selectivity and specificity of the assay were previously assessed using nonbiotinylated ephrinA1-Fc as a ligand of the EphA2-Fc receptor. Furthermore, Fc alone (AG714, Millipore) did not interfere with the binding process in the range of concentrations tested.[22] All of the compounds were tested three times, incubating at 100 μM for 1 h before the addition of biotinylated ephrinA1-Fc at a concentration corresponding to its K_D (100 ng/mL) (Figure 2A). Compounds displacing >40% binding, namely, gallic acid, 3′,4′-dihydroxyphenylacetic acid, protocatechuic acid, pyrogallol, and pyrocatechol, were further studied to calculate the inhibitory concentration that reduced binding by 50% (IC_{50}). Displacement curves (Figure 2B) using decreasing concentrations of the five phenolic acids toward biotinylated ephrinA1-Fc were constructed. All of the compounds inhibited EphA2−ephrinA1 binding in a dose-dependent manner with IC_{50} values ranging from 0.26 μM (gallic acid) to 43 μM (protocatechuic acid) (Figure 2E).

We studied some of the compounds that gave positive results in two small groups. The first group was an equimolar mixture of 3′,4′-dihydroxyphenylacetic acid and protocatechuic acid (final concentration = 1 μM), the two most commonly described colonic catabolites, as arguably this mimics the situation that is almost always present in vivo after the consumption of fruits and vegetables. The equimolar triplet, pyrocatechol + pyrogallol + gallic acid (final concentration = 1 μM), was chosen for the chemical similarity of the three catabolites, which will be found in vivo after the consumption of a wide range of dietary polyphenols. Moreover, in contrast to other catabolites that could undergo further modifications when in contact with the colonic microbiome, these molecules are catabolic end points and are unlikely to undergo further degradation by microbial enzymes in the colon.

The data presented in Figure 2C,D, as well as the Supporting Information (Figure S1), using the Webb equation,[23] showed a simple additive effect. The IC_{50} from experimental and theoretical data are 21 and 14 μM, respectively, for 3′,4′-dihydroxyphenylacetic acid and protocatechuic acid and 0.25 and 0.37 μM, respectively, for pyrocatechol + pyrogallol + gallic acid.

Functional studies were performed on PC3 human prostate adenocarcinoma cells, known for their ability to naturally express EphA2 and a well-established model to investigate the Eph−ephrin system.[33,34] In this model we evaluated agonist or antagonist properties of the compounds toward the EphA2 receptor. None of the compounds was able to induce EphA2 phosphorylation by PC3 cells when incubated for 20 min at 100 μM (Figure 3A), indicating a nonagonist behavior. On the other hand, 100 μM protocatechuic acid and pyrogallol significantly inhibited EphA2 phosphorylation induced by

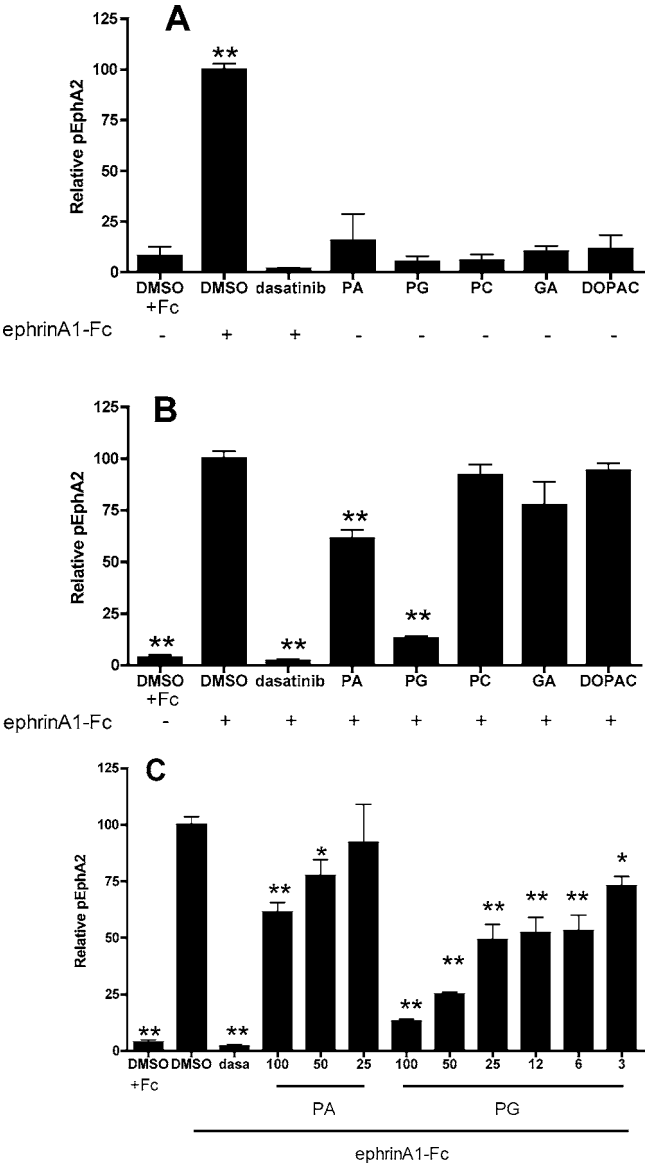

Figure 3. PG and PA dose-dependently inhibited Eph-kinases phosphorylation. Interference as agonists (A) or antagonists (B) of protocatechuic acid (PA), pyrocatechol (PC), pyrogallol (PG), gallic acid (GA), and 3′,4′-dihydroxyphenylacetic acid (DOPAC) toward EphA2 activity was tested inducing EphA2 receptor activation by 0.25 μg/mL ephrinA1-Fc in PC3 cells. Cells were pretreated for 20 min with 1% DMSO, or the indicated concentrations μM compounds, and then stimulated for 20 min with ephrinA1-Fc. Dasatinib was used as reference compound. (C) Dose-dependency of PA and PG was studied in the presence of ephrinA1-Fc. Test compound concentrations (μM) are indicated in the graph. Data are the mean of at least three independent experiments ± standard error. Phospho-EphA2 levels are relative to ephrinA1-Fc + DMSO. One-way ANOVA followed by Dunnet's post test was performed by comparing DMSO + ephrinA1-Fc to all other columns for panels B and C, and DMSO + Fc to all other columns for panel A. *, $p < 0.05$; **, $p < 0.01$.

treating PC3 cells with 0.25 μg/mL ephrinA1-Fc (Figure 3B) at noncytotoxic concentrations (Figure 4). Moreover, protocatechuic acid and pyrogallol reduced EphA2 phosphorylation in a dose-dependent manner, exhibiting IC_{50} values of 144 μM (extrapolated) and 9.2 μM (Figure 3C), respectively, in accordance with their different potencies in the binding assay. Dasatinib (1 μM) was used as a reference compound, being a

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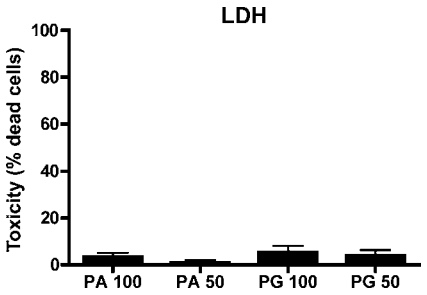

Figure 4. Active metabolites were not cytotoxic. PC3 cells were serum starved and incubated for 2 h with the indicated concentrations (μM) of metabolites. PA, protocatechuic acid; PG, pyrogallol. The supernatant was recovered, and LDH content was measured with a 30 min coupled enzymatic assay, which resulted in conversion of a tetrazolium salt (INT) into a red formazan product. The results were expressed as the ratio between absorbance of the cells treated with the compounds and cells treated with lysis buffer. Data are the mean of three independent experiments ± standard errors.

multikinase inhibitor endowed with a high potency toward Eph kinases.[35] Surprisingly, gallic acid, 3′,4′-dihydroxyphenylacetic acid, and pyrocatechol had no effect in this functional assay. Among the possible explanations is that these compounds may show higher affinity for proteins present in our experimental cell model, so becoming unavailable for the perturbation of the ephrin system. Alternatively, the presence of catechol methyltransferase activity in PC3 cells could be responsible of the methylation of specific hydroxyl groups, with a consequent inactivation of the tested catabolites.[36]

Because many catechols are known for their ability to promote many promiscuous interactions,[37] we tested whether they interfered with other kinase systems, choosing EGFR–EGF. This interference was studied inducing EGFR phosphorylation with 30 ng/mL EGF in PC3 cells, and gefitinib was used as a reference compound, being a standard EGFR kinase inhibitor. Despite their ability to promote promiscuous interactions, none of the active catabolites reduced EGFR activation (Figure 5), suggesting some degree of specificity of the interaction between these compounds and Eph–ephrin. From a physiological viewpoint, the concentration of

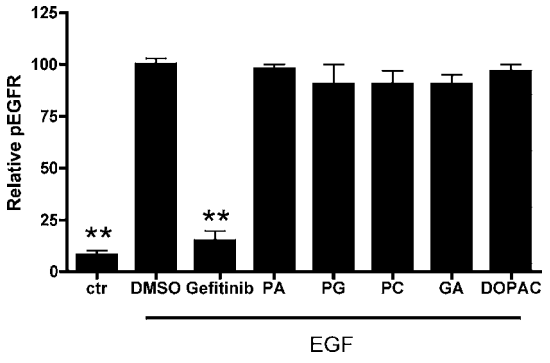

Figure 5. Metabolites do not affect EGFR activity. PC3 cells were pretreated for 20 min with 100 μM protocatechuic acid (PA), pyrogallol (PG), pyrocatechol (PC), gallic acid (GA), 3′,4′-dihydroxyphenylacetic acid (DOPAC), 10 μM gefitinib, or 1% DMSO as a control, and stimulated for 20 min with 30 ng/mL EGF. Phospho-EGFR levels are relative to EGF + DMSO. Data are the mean of at least three independent experiments ± standard error. **, $p < 0.01$ for comparison to EGF + DMSO by one-way ANOVA followed by Dunnet's multiple-comparison test.

protocatechuic acid in human plasma has been reported to be 0.18 μM[38] after the intake of 300 g of fresh strawberries and 0.49 μM after the consumption of 1 L of blood orange juice.[39] Therefore, the active concentrations observed in this study are somewhat higher than those that circulate in human plasma after a meal rich in (poly)phenols. The literature lacks information about its circulating concentrations of pyrogallol. However, its excretion in urine 0–24 h after the intake of green tea was reported to be significantly higher than the average excretion of intact flavan-3-ols glucuronide and sulfate metabolites.[30,40] Because the plasma concentration of flavan-3-ols has been reported to be >200 nM,[41] low micromolar plasma levels of pyrogallol would appear to be reasonably achievable. The active concentrations of this metabolite observed in this study, therefore, appear to be closely related to physiological conditions.

Although the current study made use of a prostate cell model, colonic epithelial cells, which are known to express the Eph–ephrin system in vivo, will almost certainly be exposed to higher concentrations of these catabolites after (poly)phenol intake. There is, for instance, a report of the presence of 51 μM pyrogallol and 13 μM protocatechuic acid in human fecal water after the intake of 200 g of raspberry puree.[42]

In conclusion, previous research has investigated the effects of phytochemicals on the EphA2–ephrinA1 protein–protein interaction.[15] However, as stated in the Introduction, (poly)-phenols are metabolized/catabolized after ingestion by humans, with most being degraded to simple phenolic acids by the action of the colonic microbiota. To our knowledge, this is the first study describing the effects of (poly)phenol colonic catabolites in this system. The screening of some of the most common catabolites allowed us to establish that pyrogallol and protocatechuic acid are able to functionally interfere with the EphA2–ephrinA1 system. The applied concentrations were high, but, at least for pyrogallol, seemingly reasonably attainable in vivo.

As described earlier, Eph kinase has a prominent role in tumorigenesis and in the formation of metastasis and is also involved in prostate carcinoma. The EphA2 receptor tyrosine kinase is overexpressed in human prostate cancers compared with benign prostate tissues[43] and is a candidate predictor of treatment efficacy for patients affected by prostate cancer and treated with dasatinib,[44] suggesting a positive role of Eph kinase inhibition in this kind of cancer. Moreover, considering the very high levels that could be reached by pyrogallol and, to a lesser extent, protocatechuic acid in the colon after the consumption of a wide array of different (poly)phenol-containing foods, in vivo interactions with colonic Eph–ephrin protein are feasible, and other studies have used these concentrations of (poly)-phenol metabolites in the evaluation of the colonic environment.[45,46]

In conclusion, because of the roles played by the Eph–ephrin system not only in cancer development but also in neurodegeneration and diabetes, pyrogallol and protocatechuic acid are candidates for more detailed functional studies to elucidate their role in these pathophysiological processes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1. Theoretical curve of combination drug using Webb equation (gray) and experimental data (black). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

PC3, prostate adenocarcinoma cells; Eph, ephrin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; BCA, bicinchoninic acid; LDH, lactate dehydrogenase; PG, pyrogallol; PC, pyrocatecol; PHG, phloroglucinol; 3MeGA, 3-methylgallate; 4MeGA, 4-methylgallate; GA, gallic acid; PA, protocatechuic acid; VA, vanillic acid; 2HBA, 2-hydroxybenzoic acid; 3HBA, 3-hydroxybenzoic acid; 4HBA, 4-hydroxybenzoic acid; PAC, phenylacetic acid ; HVA, homovanillic acid; 3OHPAC, 3-hydroxyphenylacetic acid; 4OHPAC, 4-hydroxyphenylacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; FA, ferulic acid; DHF, dihydroferulic acid; Uro A, urolithin A; Uro B, urolithin B.

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