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Oak Ellagitannins Suppress the Phosphorylation of the Epidermal Growth Factor Receptor in Human Colon Carcinoma Cells

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The ellagitannins castalagin and vescalagin, and the *C*-glycosides grandinin and roburin E as well as ellagic acid were found to potently inhibit the growth of human colon carcinoma cells (HT29) in vitro. In a cell-free system these compounds were identified as potent inhibitors of the protein tyrosine kinase activity of the epidermal growth factor receptor (EGFR) with IC_{50} values in the low nanomolar range. To address the question of whether the interference with the activity of the isolated EGFR also plays a role within intact cells, effects on the phosphorylation status of the EGFR, as a measure for its activity, were determined in HT29 cells. As exemplified for castalagin and grandinin, both the nonglycosylated and the glycosylated ellagitannins effectively suppressed EGFR phosphorylation, but only at concentrations $\geq$10 μM, thus, in a concentration range where growth inhibition was observed. These results indicate that the suppression of EGFR-mediated signaling might contribute to the growth inhibitory effects of these compounds present in oak-matured wines and spirits such as whiskey. In contrast, despite substantial growth inhibitory properties, ellagic acid did not significantly affect EGFR phosphorylation in HT29 cells up to 100 μM.

KEYWORDS: Ellagic acid; grandinin; castalagin; roburin E; vescalagin

INTRODUCTION

Ellagitannins are hydrolyzable tannins that belong to the class of plant polyphenols. They have common properties with procyanidins, including their water-soluble character, their high molecular weight, and their ability to precipitate proteins and alkaloids (*1, 2*). Ellagitannins are esters of hexahydroxydiphenic acid, supposed to originate from oxidative C−C coupling of phenolic galloyl groups in vivo and a polyol, usually glucose or quinic acid (*3, 4*). When ellagitannins are exposed to acids or bases, ester bonds are hydrolyzed and the hexahydroxydiphenic acid spontaneously rearranges into the water-insoluble bislactone ellagic acid, after which these natural food constituents are named (*3, 4*). The dietary intake of ellagitannins from common foodstuff is limited to a few fruit and nut species, such as raspberries, blackberries, walnuts, and hazelnuts (*3, 5*). Ellagic acid is naturally present together with ellagitannins in plant tissues or formed during food processing (*6, 7*). Castalagin, vescalagin, grandinin, and roburins A−E were identified as constituents of oak wood (*8, 9*). Dissolved in hydroalcoholic mediums such as wine or whiskey, these tannins possess oxidizing potential and taste properties that give them a potential role in the aging process in oak barrels (*10*). Their concentration in oak wood depends on the respective species and the geographical origin, as well as the seasoning and toasting processes used for barrel production (*8*). Little is known so far on the bioavailability of ellagitannins. Ellagitannins have been reported to disappear in the stomach of rats 1 h after ingestion without accumulation of ellagic acid (*11*).

Ellagitannins and ellagic acid have been shown to mediate antiproliferative, apoptotic, and antioxidative effects (*5, 7, 12, 13*), but little is known so far about the underlying mechanisms of action. A spectrum of polyphenols has already been reported to target receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) (*14–19*). The EGFR stirs one of the key signaling cascades in the regulation of cell growth (*14–16, 20*). Thus, the suppression of EGFR activity is not only of interest in terms of chemotherapy but might also be regarded as a promising mechanism of action with respect to chemoprevention.

In the present study we investigated whether ellagitannins and ellagic acid affect the isolated EGFR activity and whether the interference with the receptor is of relevance within intact cells. The tested ellagitannins represent the two stereoisomeric

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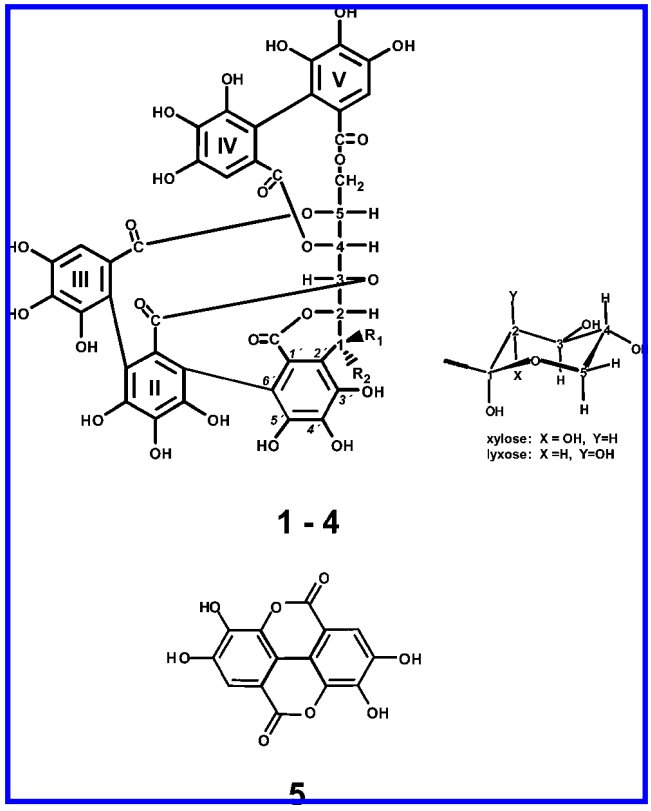

Figure 1. Structures of the ellagitannins (**1**−**4**) castalagin (**1**; R_1 = H, R_2 = OH), vescalagin (**2**; R_1 = OH, R_2 = H), grandinin (**3**; R_1 = lyxose, R_2 = H), and roburin E (**4**; R_1 = xylose, R_2 = H) and the ellagitannin degradation product ellagic acid (**5**).

aglycones castalagin and vescalagin (**Figure 1**) and the two corresponding *C*-glycosides roburin E and grandinin (**Figure 1**). For comparison, ellagic acid (**Figure 1**), an ellagitannin degradation product formed upon acid hydrolysis, was included in the testing.

MATERIALS AND METHODS

Chemicals and Materials. Ellagic acid was obtained from Extrasynthese (Genay, France). The EGFR-specific inhibitor tyrphostin AG1478 was purchased from Sigma-Aldrich (Taufkirchen, Germany). For all assays, the compound solutions were freshly prepared in DMSO (final concentration maximum 1%) before starting the respective experiments.

Chips from a seasoned and 2 years air-dried oak wood (*Quercus robur* L. and *Quercus alba* L.) were obtained from the cooperage industry (United States). The oak wood ellagitannins castalagin and vescalagin were isolated from oak chips closely following the procedure reported recently (*9*).

Preparative Isolation of Grandinin and Roburin E. Oak wood chips (500 g) were extracted with ethanol/water (62.5:37.5, v/v; 3 × 1.5 L) for 12 h at 20 °C with stirring. After removal of the ethanol at reduced pressure, the extract obtained was freeze-dried to give the ethanolic oak wood extract (15 g). Aliquots (5 g) of the extract were dissolved in methanol/water (40:60, v/v; 50 mL) and placed onto the top of a water-cooled 100 × 5 cm glass column XK50 (Amersham Pharmacia Biotech, Uppsala, Sweden) filled with a slurry of Sephadex LH 20 material (Amersham Pharmacia Biotech), which was conditioned with a mixture (60:40, v/v) of water (adjusted to pH 4.5 with 0.1% formic acid) and methanol. Chromatography was performed with methanol/water (40:60, v/v; pH 4.5; 2 L), followed by methanol/water (60:40, v/v; pH 4.5; 2 L), methanol/water (80:20, v/v; pH 4.5; 2 L), and, finally, methanol (2 L). The flow rate was kept constant at 3 mL/min by means of a P1-type peristaltic pump (Amersham Pharmacia Biotech). The effluent was monitored by means of a UV 2070/2075-type UV−vis detector (Jasco, Gross-Umstadt, Germany) operating at 272 nm, and the effluents of eight peaks (I−VIII) were collected, freed from solvent under vacuum, and freeze-dried twice. Aliquots (200 mg) of fraction V were dissolved in formic acid (0.3% in water; 20 mL) and, after membrane filtration, aliquots (2 mL) were applied on a 250 × 21.2 mm i.d., 5 μM, RP-18 column, ODS-Hypersil, (ThermoHypersil, Kleinostheim, Germany). With the effluent monitored at 272 nm, chromatography was performed with aqueous formic acid (0.3% in water) for 5 min, increasing the acetonitrile content to 5% over 15 min and then to 15% over another 15 min, thereafter increasing the acetonitrile content to 60% within 10 min at a flow rate of 18.0 mL/min and then held constant for 3 min. The *C*-lyxoside grandinin and the *C*-xyloside roburin E were collected, freed from solvents under vacuum, and freeze-dried three times, followed by NMR and MS spectroscopic structure determination to give the target compounds as pale gray, amorphous powders in a purity of >99%.

Grandinin: $C_{46}H_{34}O_{30}$; UV−vis (water) λ_{max} = 229 nm; LC-MS (ESI$^-$): 532 (100, $[M - 2H]^{2-}$, 1065 (85, $[M - H]^-$); ^{1}H NMR (400 MHz; D_2O): δ 3.52 [d, 1H, J = 13.0 Hz, H−C(5″)], 3.54 [s, 1H, H−C(1)], 3.89 [d, 1H, J = 13 Hz, H−C(5″)], 3.96 [m, 2H, H−C(3″), H−C(4″)], 4.11 [d, 1H, J = 13.0 Hz, H−C(6)], 4.25 [s, 1H, H−C(2″)], 4.70 [d, 1H, J = 7 Hz, H−C(3)], 4.86 [d, 1H, J = 12 Hz, H−C(6)], 5.08 [t, 1H, J = 6.0 Hz, H−C(4)], 5.44 [s, 1H, H−C(2)], 5.50 [d, 1H, J = 6.0 Hz, H−C(5)], 6.68 [s, 1H, H−C(7′)(V)], 6.88 [s, 1H, H−C(7′)(IV)], 6.98 [s, 1H, H−C(7′)(III)]; ^{13}C NMR (100 MHz; D_2O): δ 45.2 [C(1)], 62.1 [C(5″)], 65.1 [C(6)], 65.9 [C(4″)], 69.7 [C(4)], 70.7 [C(3)], 70.9 [C(5)], 71.2 [C(3″)], 71.3 [C(2″)], 72.3 [C(2)], 100.7 [C(1″)], 107.2 [C(2′)(V)], 109.5 [C(2′)(IV)], 109.5 [C(2′)(III)], 112.0 [C(6′)(I)], 113.4 [C(6′)(IV)], 113.6 [C(6′)(V)], 114.0 [C(2′)(II)], 114.2 [C(2′)(I)], 115.3 [C(6′)(II)], 115.3 [C(6′)(III)], 123.3 [C(1′)(III)], 124.0 [C(1′)(IV)], 125.4[C(1′)(V)], 125.6 [C(1′)(I)], 126.3 [C(1′)(II)], 134.4 [C(4′)(I)], 135.2 [C(4′)(V)], 136.2 [C(4′)(IV)], 136.8 [C(4′)(III)], 137.5 [C(4′)(II)], 143.0–146.5 [10C, C(3′)(I−V), C(5′)(I−V)], 165.9 [C(7′)(II)], 166.8 [C(7′)(I)], 167.4 [C(7′)(IV)], 167.4 [C-(7′)(III)], 170.0 [C(7′)(V)].

Roburin E: $C_{46}H_{34}O_{30}$; UV−vis (water) λ_{max} = 229 nm; LC-MS (ESI$^-$): 532 (100, $[M - 2H]^{2-}$, 1065 (52, $[M - H]^-$); ^{1}H NMR (400 MHz; D_2O): δ 3.48 [s, 1H, H−C(1)], 3.52 [m, 1H, J = 5, 9 Hz, H−C(4″)], 3.59 [d, 1H, J = 11.0 Hz, H−C(5″)], 3.69 [m, 1H, J = 9 Hz, H−C(3″)], 3.71 [d, 1H, J = 9 Hz, H−C(2″)], 3.76 [dd, 1H, J = 5, 9 Hz, H−C(5″)], 4.06 [d, 1H, J = 12.9 Hz, H−C(6)], 4.70 [d, 1H, J = 7 Hz, H−C(3)], 4.84 [d, 1H, J = 12.8 Hz, H−C(6)], 5.05 [t, 1H, J = 7.0 Hz, H−C(4)], 5.47 [d, 1H, J = 7 Hz, H−C(5)], 5.76 [s, 1H, H−C(2)], 6.65 [s, 1H, H−C(7′)(V)], 6.74 [s, 1H, H−C(7′)(IV)], 6.86 [s, 1H, H−C(7′)(III)]; ^{13}C NMR (100 MHz; D_2O): δ 45.9 [C(1)], 62.1 [C(5″)], 65.4 [C(6)], 68.9 [C(4)], 69.1 [C(4″)], 70.4 [C(3)], 70.6 [C(5)], 71.7 [C(2)], 73.2 [C(3″)], 74.0 [C(2″)], 99.4 [C(1″)], 107.0 [C(2′)(V)], 108.7 [C(2′)(IV)], 109.3 [C(2′)(III)], 112.1 [C(6′)(I)], 113.4 [C(6′)(III)], 113.6 [C(6′)(II)], 114.1 [C(6′)(V)], 115.2 [C(2′)(II)], 115.2 [C(2′)(I)], 115.2 [C(6′)(IV)], 123.4 [C(1′)(IV)], 123.9 [C(1′)(III)], 125.2 [C(1′)(I)], 125.5 [C(1′)(V)], 126.3 [C(1′)(II)], 134.4 [C(4′)(II)], 135.1 [C(4′)(V)], 136.2 [C(4′)(III)], 136.6 [C(4′)(IV)], 137.3 [C(4′)(I)], 143.6–146.7 [10C, C(3′)(I−V), C(5′)(I−V)], 165.9 [C(7′)(II)], 167.0 [C(7′)(IV)], 167.4 [C(7′)(III)], 167.5 [C(7′)(I)], 170.0 [C(7′)(V)].

Cell Culture. The human colon carcinoma cell line HT29 was cultivated in Dulbecco's modified Eagle's medium (DMEM with 4.5 g/L glucose, without sodium pyruvate) (Invitrogen Life Technologies, Karlsruhe, Germany) in humidified incubators (37 °C, 5% CO_2). The cell culture medium was supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.

Sulforhodamine B (SRB) Assay. The sulforhodamine B assay was performed according to a modified method of Skehan et al. (*21*) as reported previously (*14–16*).

Tyrosine Kinase Assay. The EGFR was isolated from A431 cells and purified by affinity chromatography using wheat germ lectin agarose (Pharmacia Biotech, Uppsala, Sweden) according to the method of Kern et al. (*15*). The ELISA was carried out as described previously (*14–16*).

Western Blot Analysis. HT29 cells (1.2 × 10^6) were seeded per Petri dish and allowed to grow for 48 h. Thereafter, cells were serum-reduced (1% FCS) for 24 h and incubated with the respective ellagitannins and tyrphostin AG1478 for 45 min in serum-free medium,

respectively. Stimulation with EGF (100 ng/mL) was performed within the last 15 min of incubation. Cells were abraded at 4 °C in 0.2 mL of RIPA buffer [50 mM Tris-HCl, pH 7.4, 250 mM sodium chloride, 1 mM EDTA, 1 mM sodium fluoride, 1% (v/v) Igepal, 1 mM PMSF, 1 mM sodium orthovanadate, and 40 μL protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) freshly added to 2 mL of RIPA buffer]. Thereafter, the cell lysate was homogenized thoroughly and subsequently centrifuged for 10 min (20000*g*, 4 °C). The supernatant was separated by SDS-PAGE (7% polyacrylamide gel), and the proteins were transferred onto a nitrocellulose membrane. Detection was performed using rabbit polyclonal antibodies against human EGFR/phospho-EGFR (Tyr1173, 175 kDa) (New England Biolabs GmbH, Frankfurt, Germany). An anti-rabbit IgG peroxidase conjugate (New England Biolabs GmbH) was used as secondary antibody. α-Tubulin (56 kDa) (Santa Cruz Biotechnology, Heidelberg, Germany) was used as loading control. The respective chemoluminescent signals (Lumi-GLO, New England Biolabs GmbH) were analyzed using the LAS 3000 with AIDA Image Analyzer 3.52 software for quantification (Raytest, Straubenhardt, Germany). Arbitrary light units were plotted as test over control (percent). At least three independent experiments were performed, presented as the mean ± SD.

High-Performance Liquid Chromatography (HPLC). Liquid chromatography was performed on a HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisting of two pumps (PU 2086/2087), a gradient mixer (1 mL), a Rheodyne injector with a 200 μL loop, and an MD 2010 plus diode array detector (Jasco) monitoring the effluent in a wavelength range between 220 and 500 nm.

LC−Time-of-Flight Mass Spectrometry (LC-TOF-MS). High-resolution mass spectra of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer and referenced on sodium formate.

Liquid Chromatography−Mass Spectrometry (LC-MS). Electrospray ionization (ESI) spectra were acquired on an API 4000 Q-Trap LC-MS/MS system (AB Sciex Instruments, Darmstadt, Germany) with an Agilent 1100 HPLC system operating at a flow rate of 200 μL/min with direct loop injection of the sample (2–20 μL). The spray voltage was set at −4500 V in ESI^- mode and at 5500 V in ESI^+ mode. Nitrogen served as curtain gas (20 psi); the declustering potential was set at −10 to −40 V in ESI^- mode and at 30 V in ESI^+ mode. The mass spectrometer was operated in the full-scan mode, monitoring positive and negative ions. Fragmentation of $[M - H]^-$ and $[M + H]^+$ pseudomolecular ions into specific product ions was induced by collision with nitrogen (4×10^{-5} torr) and a collision energy of −40 V.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^1H, ^{13}C, COSY, HMQC, and HMBC spectroscopic experiments were performed on a DPX 400 NMR spectrometer from Bruker (Rheinstetten, Germany). Samples were dissolved in D_2O and placed into NMR tubes (Schott Professional 178 × 5 mm, Mainz, Germany) prior to measurement. Spectrum analysis was done with NMR Software Mestre-C.

RESULTS

With the aim of investigating the influence of the oak ellagitannins on the growth of human colon carcinoma cells, castalagin and vescalagin as well as the ellagitannin *C*-pentosides grandinin and roburin E were isolated from oak wood chips by solvent extraction and purified by means of iterative chromatography.

Effects on Cell Growth. Growth inhibitory properties of ellagitannins and ellagic acid were determined using the SRB assay. Incubation of HT29 cells with the test compounds for 72 h led to substantial inhibition of cell growth. The stereoisomeric aglycones castalagin and vescalagin inhibited cell growth with IC_{50} values of 11.1 ± 1.8 and 22.4 ± 2.6 μM, respectively (**Table 1**; **Figure 2**), whereas the glycosylated stereoisomers roburin E and grandinin showed growth inhibitory properties with IC_{50} values of 16.7 ± 1.4 and 28.9 ± 2.6 μM (**Figure 2**). Also, ellagic acid was found to inhibit cell growth (IC_{50} = 66.7 ± 9.6 μM; **Table 1**; **Figure 2**), albeit less effectively than the ellagitannins. On the basis of the IC_{50} values the potency of ellagitannins for cell growth inhibition in HT29 cells can be summarized as castalagin > roburin E > vescalagin > grandinin ≫ ellagic acid (**Table 1**).

Table 1. Growth Inhibitory Properties of Ellagitannins and Ellagic Acid in the Sulforhodamine B Assay and Inhibition of the EGFR Activity in the ELISA in Comparison to Effects on the Phosphorylation Status of the Receptor in HT29 Cells

compound	growth inhibition IC_{50} (μM)	EGFR ELISA IC_{50} (nM)	EGFR autophosphorylation IC_{50}[a] (μM)
castalagin	11.1 ± 1.8	49.9 ± 2.5	10
vescalagin	22.4 ± 2.6	61.8 ± 9.5	nt
roburin E	16.7 ± 1.4	59.2 ± 5.4	nt
grandinin	28.9 ± 2.6	71.2 ± 3.2	35
ellagic acid	66.7 ± 9.6	65.5 ± 5.6	−
tyrphostin AG1478	0.9 ± 0.2	2400 ± 100 (*14,16*)	17.5 ± 5.6

[a] nt, not tested; −, no significant inhibition up to 100 μM (Figure 6).

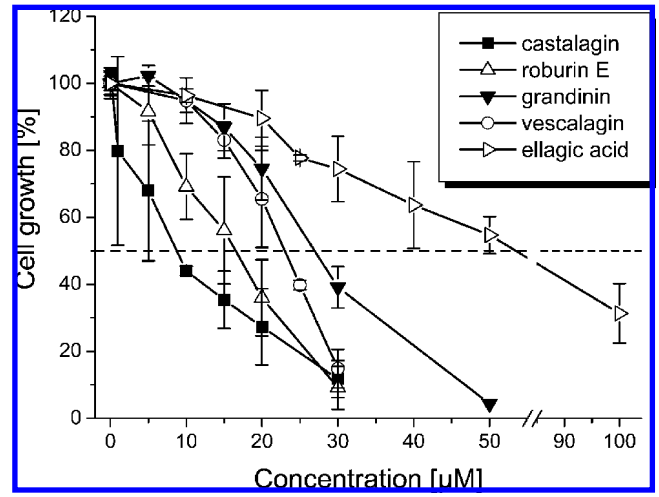

Figure 2. Inhibition of cell growth in HT29 cells by ellagitannins and ellagic acid determined in the sulforhodamine B assay. Growth inhibition was calculated as percent survival of treated cells over control cells (treated with the solvent 1% DMSO) × 100 [T/C%]. The values given are the mean ± SD of at least three independent experiments, each performed in quadruplicate.

Inhibition of the Protein Tyrosine Kinase Activity of the Isolated EGFR. Effects on the protein tyrosine kinase (PTK) activity of isolated EGFR were determined using an enzyme-linked immunosorbent assay (ELISA). All tested ellagitannins were found to effectively inhibit the PTK activity of the isolated EGFR already in nanomolar concentrations without significant differences in inhibitory potency between the stereoisomers or glycosylated versus nonglycosylated analogues (**Table 1**; **Figure 3**). In a similar concentration range also ellagic acid was identified as a potent inhibitor of the isolated EGFR (**Table 1**; **Figure 3**). With respect to the inhibition of the PTK activity of the EGFR, the inhibitory properties of the test compounds can be summarized as castalagin > roburin E ≥ vescalagin ≥ ellagic acid ≥ grandinin (**Table 1**).

Modulation of EGFR Autophosphorylation. We further addressed the question of whether the potent inhibitory effects of ellagitannins and ellagic acid are limited to the isolated EGFR preparation or are also of relevance within intact colon tumor cells. The impact of the test compounds on the phosphorylation status of the EGFR in HT29 cells was determined by Western blot analysis. With respect to the limited availability of

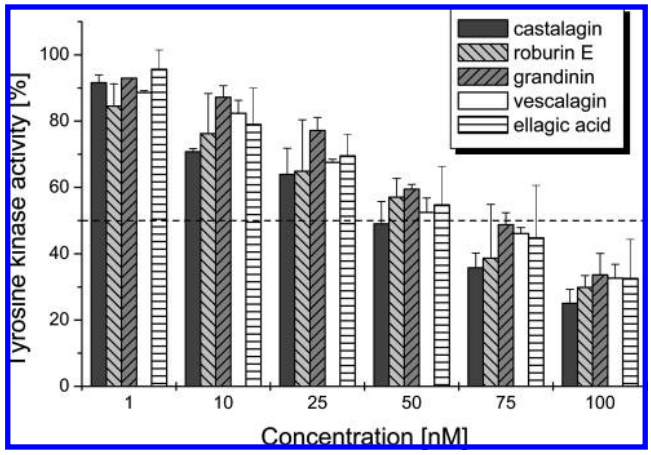

Figure 3. Inhibition of the tyrosine kinase activity of the EGF receptor by ellagitannins and ellagic acid. The phosphorylation of tyrosine residues of a peptide poly (Glu/Tyr) was determined by ELISA using an anti-phosphotyrosine antibody linked to peroxidase. The data presented are the mean ± SD of at least three independent experiments, each performed in triplicate.

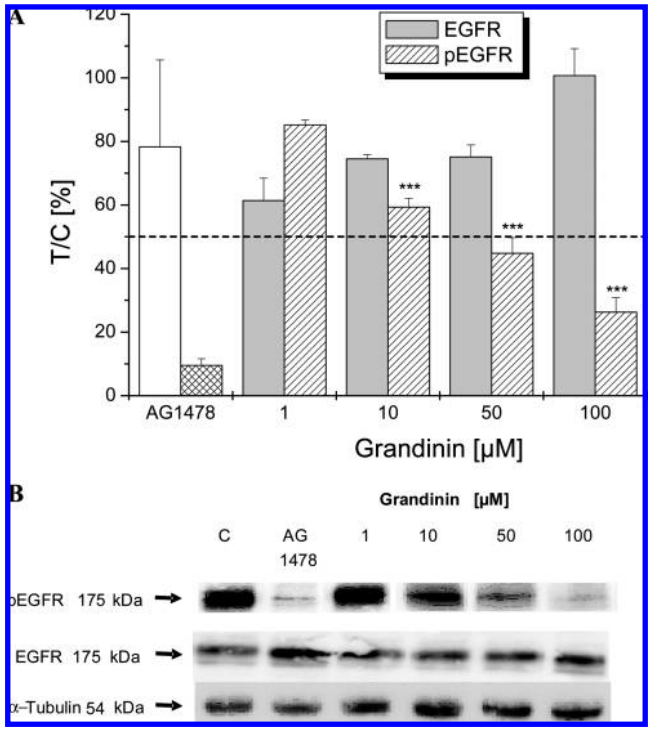

Figure 4. (**A**) Western blot analysis of the EGFR receptor protein in HT29 cells after 45 min of treatment with grandinin. The data are presented as percent of the solvent control stimulated by 100 ng/mL EGF. The data presented are the means ± SD of three independent experiments. The indicated significances were referred to the lowest control (*** = $p < 0.001$). (**B**) Representative Western blot experiment; C, solvent control (1% DMSO as final concentration); tyrphostin AG1478, EGFR-specific inhibitor (1 μM).

ellagitannins, castalagin and grandinin, as representatives for nonglycosylated and glycosylated analogues, were selected for further testing in comparison to ellagic acid.

Grandinin was found to potently suppress EGFR phosphorylation (**Figure 4**) in a concentration-dependent manner with an IC_{50} value of 35 ± 0.3 μM. Also, castalagin effectively diminished the phosphorylation status of the EGFR, reaching

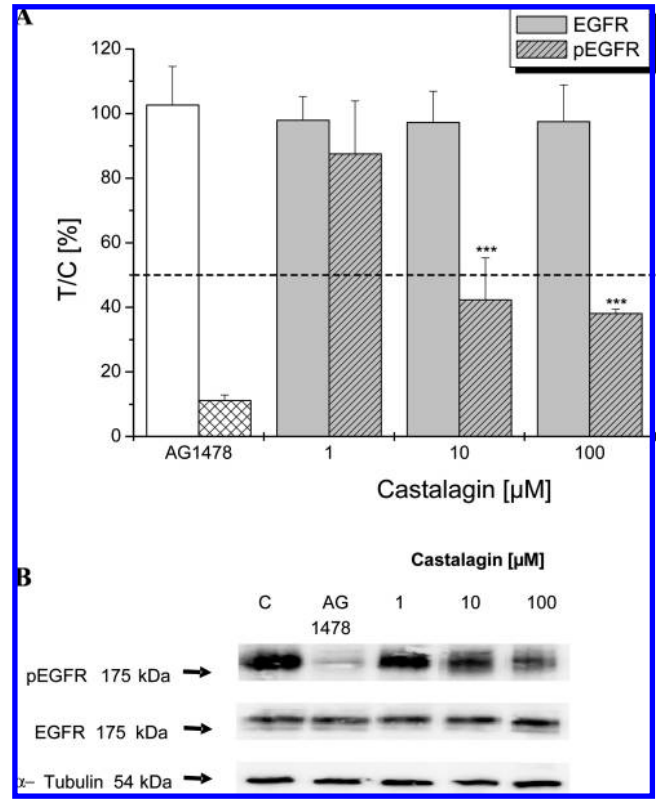

Figure 5. (**A**) Western blot analysis of the EGFR receptor protein in HT29 cells after 45 min of treatment with castalagin. The data are presented as percent of the solvent control stimulated by 100 ng/mL EGF. The data presented are the means ± SD of three independent experiments. The indicated significances were referred to the lowest control (*** = $p < 0.001$). (**B**) Representative Western blot experiment; C, solvent control (1% DMSO as final concentration); tyrphostin AG1478, EGFR specific inhibitor (1 μM).

50% inhibition of receptor phosphorylation at about 10 μM (**Figure 5**). In contrast, ellagic acid did not significantly affect EGFR autophosphorylation in HT29 cells up to 100 μM (**Figure 6**).

DISCUSSION

Ellagitannins have recently been reported as a promising class of bioactive compounds, potently suppressing the growth of tumor cells in vitro (*5, 7, 12, 13*). However, little is known so far about the underlying mechanisms of action. In the evaluation of the usefulness of ellagitannins in the prevention of carcinogenesis, the question has to be addressed whether enhanced intake of these ellagitannins might also be associated with undesired health effects. Some ellagitannins have been reported to inhibit the catalytic activity of topoisomerases (*22–26*). Depending on the mode of interaction with the target enzyme, effective inhibition of topoisomerases might impair the maintenance of DNA integrity (*27, 28*).

Castalagin, vescalagin, roburin E, and grandinin were found to potently inhibit the growth of HT29 cells (**Figure 2**). The hydrolyzation product ellagic acid was slightly less effective, albeit substantial growth inhibitory properties were observed. Also, Losso et al. (*7*) reported cytotoxicity and antiproliferative activity of ellagic acid against human colon carcinoma cells (Caco-2) in a comparable concentration range. This is in line with results obtained in other cell models (*5, 12*).

The growth inhibitory properties of the test compounds in HT29 cells can be summarized as castalagin > roburin E >

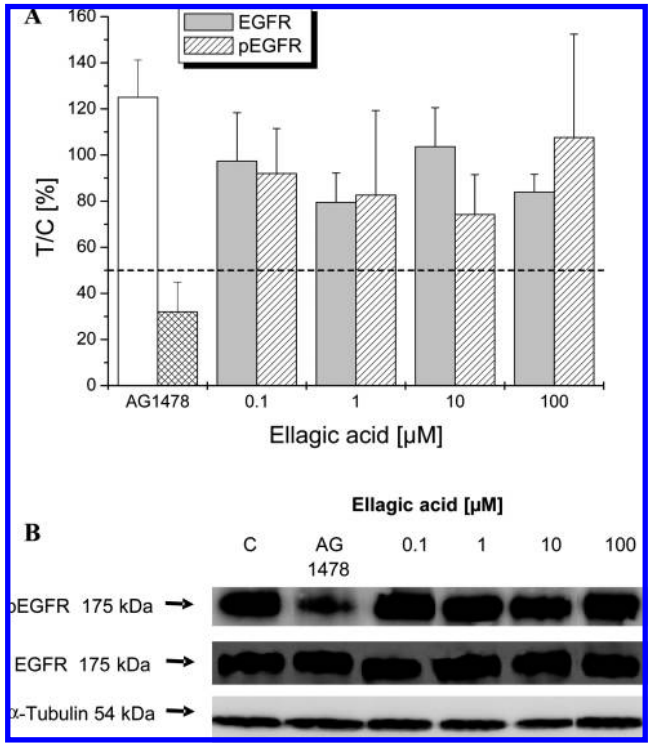

Figure 6. (**A**) Western blot analysis of the EGFR receptor protein in HT29 cells after 45 min of treatment with ellagic acid. The data are presented as percent of the solvent control stimulated by 100 ng/mL EGF. The data presented are the means ± SD of five independent experiments. (**B**) Representative Western blot experiment; C, solvent control (1% DMSO as final concentration); tyrphostin AG1478, EGFR-specific inhibitor (1 μM).

vescalagin > grandinin ≫ ellagic acid. These results indicate some structural dependency of the growth inhibitory effects. Among the free aglycones, castalagin showed higher potency compared to vescalagin, which might be due to stereoselective interaction with respective cellular target structures. Interestingly, also the ellagitannin glycosides roburin E and grandinin exhibited substantial growth inhibitory properties. The glycoside roburin E even significantly exceeded the growth inhibitory effects of the free aglycone vescalagin. In contrast to other polyphenol classes, glycosylation of ellagitannins is apparently not associated with a substantial loss in effectiveness.

With respect to the potential mechanism of action, the selected ellagitannins, ellagitannin glycosides, and the free ellagic acid were found to suppress the PTK activity of the EGFR in a cell-free system already in the nanomolar range. The inhibitory potency can be summarized as castalagin > roburin E ≥ vescalagin ≥ ellagic acid ≥ grandinin (**Figure 3**). However, a discrepancy in effectiveness of several orders of magnitude was observed between the concentration of test compounds inhibiting the activity of the isolated EGFR and substance concentrations mediating growth inhibition. Therefore, we addressed the question of whether the inhibition of EGFR activity by ellagitannins and ellagic acid is also of relevance within intact cells. The activity of the EGFR is mirrored by its phosphorylation status, which is crucial for the interaction of the intracellular domain with downstream elements of the MAPK cascade (*29–31*). Suppression of the autophosphorylation of the EGFR has already been shown for several flavonoids (*14, 16–19, 32, 33*). Indeed, treatment of HT29 cells with grandinin or castalagin was found to effectively suppress EGFR autophosphorylation (**Figures 4** and **5**). However, these inhibitory effects on the EGFR activity in cell culture experiments were achieved in only the micromolar range. Thus, up to a factor of 500 was observed between the effects of ellagitannins on the isolated receptor and the respective response in the cell system. It might speculated that due to the relatively high molecular weight of these compounds compared to many other foodborne polyphenols limited cellular uptake might play a role. These results indicate that at least for this class of polyphenols the EGFR−ELISA system is not predictive for the cellular response.

However, with respect to the mechanism of action, the results clearly show that in growth inhibitory concentrations castalagin and grandinin effectively suppress the phosphorylation of the EGFR in HT29 cells. The interference of ellagitannins with the EGFR within intact cells might indeed be of relevance for the growth inhibitory properties of these compounds. Interestingly, as already observed for the growth inhibitory effects, the suppression of EGFR phosphorylation is not limited to the free aglycone castalagin, but is also observed with the glycoside grandinin, albeit with slightly minor potency.

The potent inhibitory potential of castalagin is mirrored in all of the applied test systems (**Figures 2**, **3**, and **5**), indicating that probably the ellagitannin aglycone form with an α-oriented C(1)-OH group is of relevance for the biological activity. However, for detailed structure–activity studies, further experiments including a higher number of analogues have to be performed.

In contrast to the ellagitannins, ellagic acid did not affect the phosphorylation status of the EGFR in HT29 cells in growth inhibitory concentrations. These results show that the interference with other so far unknown cellular targets is responsible for the substantial growth inhibitory properties of this natural food constituent.

In summary, ellagitannins appear to possess several bioactive properties that affect cellular signaling cascades regulating cell proliferation. In particular, castalagin and grandinin effectively blocked the activity of the EGFR in human colon carcinoma cells, an effect that might be interesting in terms of chemoprevention. However, to estimate the relevance of these findings for the in vivo situation, further studies on the bioavailability and metabolism of these compounds are indispensable. Furthermore, the question has to be addressed as to whether also potentially adverse effects such as the interference with topoisomerases have to be considered, which potentially limit the usefulness of these compounds.

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