Mol. Nutr. Food Res. 2005, *49*, 317 – 328 DOI 10.1002/mnfr.200400086

Inhibitors of the epidermal growth factor receptor in apple juice extract

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The polyphenol-rich extract of a consumer-relevant apple juice blend was found to potently inhibit the growth of the human colon cancer cell line HT29 *in vitro*. The epidermal growth factor receptor (EGFR) and its subsequent signaling cascade play an important role in the regulation of cell proliferation in HT29 cells. The protein tyrosine kinase activity of an EGFR preparation was effectively inhibited by the polyphenol-rich apple juice extract. Treatment of intact cells with this extract resulted in the suppression of the subsequent mitogen-activated protein kinase cascade. Amongst the so far identified apple juice constituents, the proanthocyanidins B1 and B2 as well as quercetin-3-glc (isoquercitrin) and quercetin-3-gal (hyperoside) were found to possess substantial EGFR-inhibitory properties. However, as to be expected from the final concentration of these potential EGFR inhibitors in the original polyphenol-rich extract, a synthetic mixture of the apple juice constituents identified and available so far, including both proanthocyanidins and the quercetin glycosides, showed only marginal inhibitory effects on the EGFR. These results permit the assumption that yet unknown constituents contribute substantially to the potent EGFR-inhibitory properties of polyphenol-rich apple juice extract. In summary, the polyphenol composition of apple juice possesses promising growth-inhibitory properties, affecting proliferation-associated signaling cascades in colon tumor cells.

Keywords: Apple polyphenols / Colon carcinoma / Epidermal growth factor receptor / Growth inhibition / Mitogen-activated protein kinase

Received: November 15, 2004; revised: January 25, 2005; accepted: January 26, 2005

1 Introduction

Dietary habits and lifestyle are more and more recognized to play an important role in the development of many different tumor types. Colorectal carcinomas represent the second most prevalent tumor in both genders in western industrialized countries. The aim of the present study was to identify nutritional factors which might be useful in the prevention of colon carcinogenesis. Malignant transformation is associated with changes in cellular signaling cascades regulating cell growth and differentiation. The extracellular signal-regulated/mitogen-activated protein kinase (ERK/MAPK) pathway represents one of the major intracellular signaling cascades in the control of cell proliferation [1, 2]. Activation of a respective cell surface receptor, such as the epidermal growth factor receptor (EGFR), initiates an exchange of GDP *versus* GTP at the G-protein Ras. GTP-loaded Ras recruits the serine/threonine kinase Raf-1 from the cytosol to the cell membrane, resulting in the activation of the kinase activity. Raf-1, an interface between cell surface receptors and nuclear transcription, is the entry point to the ERK/MAPK pathway (Fig. 1). Effective inhibition of the upstream located EGFR results in a suppression of the subsequent MAPK cascade, leading to the inhibition of cell growth [3].

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Abbreviations: **ABTS**, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonate); **AE02**, apple juice extract; **Cat**, (+)-catechin; **EC**, (−)-epicatechin; **ECG**, (−)-epicatechingallate; **EGC**, epigallocatechin; **EGCG**, epigallocatechingallate; **EGF**, epidermal growth factor; **EGFR**, epidermal growth factor receptor; **ELK1**, Ets like kinase 1; **ERK**, extracellular regulated kinase; **FCS**, fetal calf serum; **MAPK**, mitogen-activated protein kinase; **PDA**, photodiode array; **PE**, phloridzin equivalents; **PS**, penicillin/streptomycin; **PTK**, protein tyrosine kinase; **q-3-gal**, quercetin-3-galactoside; **q-3-glc**, quercetin-3-glucoside; **q-3-rha**, quercetin-3-rhamnoside; **q-3-rut**, quercetin-3-rutinoside; **SRB**, sulforhodamine B

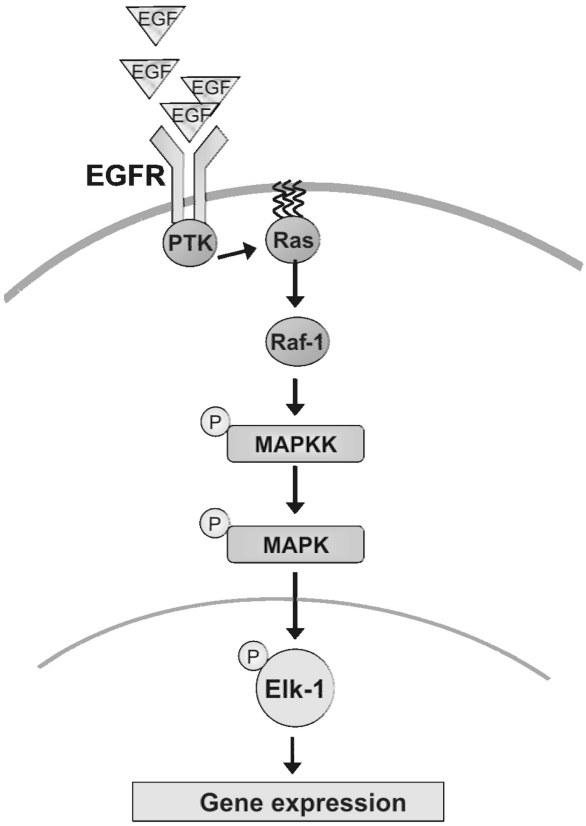


Figure 1. Simplified scheme of the EGFR/MAP kinase cascade. EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PTK, protein tyrosine kinase; Ras, GTP-binding protein; Raf-1, serine/threonine kinase; MAPKK, mitogen-activated protein kinase kinase; Elk-1, Ets-like kinase (transcription factor).

Food itself in its complex composition represents a rich source of so-called "bioactive" compounds. Especially within the huge class of plant polyphenols, several compounds have been reported to be of interest in terms of chemoprevention [4]. In the present study, we investigated the effect of apple polyphenols on the growth of human colon tumor cells. We further studied the impact of these compounds on signaling cascades, regulating cell proliferation with special emphasis on the EGFR/MAPK pathway.

2 Materials and methods

2.1 Chemicals

Chlorogenic acid, (−)-epicatechin (EC), (−)-epicatechin-gallate (ECG), proanthocyanidin B1 and B2 (PB1 and PB2), quercetin-3-rutinoside (q-3-rut), quercetin-3-rhamnoside (q-3-rha), quercetin-3-galactoside (q-3-gal), and quercetin-3-glucoside (q-3-glc) were purchased from Extrasynthèse (Genay, France). (+)-Catechin (Cat), (−)-epigallocatechin (EGC), caffeic acid, phloretin, and phloridzin, were obtained from Roth (Karlsruhe, Germany) and quercetin was received from Sigma (Taufkirchen, Germany). 4-Coumaroylquinic acid was a kind gift from Prof. Becker (Saarland University, Saarbruecken, Germany). Epigallocatechingallate (EGCG) was kindly provided by Nestlé (Vevey, Switzerland). For all assays the compound solutions were freshly prepared directly prior to the experiment, without the use of stored stock solutions. All compounds and mixtures were dissolved in DMSO with a final concentration in the different test systems of maximum 1%.

2.2 Production of the polyphenol-rich apple juice extract

An apple juice was produced from a mixture of table apples (20%) and the varieties Topaz (25%), Bohnapfel (17.5%), Winterrambour (22.5%), and Bittenfelder (15%). Polyphenols of 100 L of that juice were retained on 5 L adsorber resin (XAD 16 HP, Röhm & Haas, Frankfurt, Germany) packed onto a Pharmacia glass column (BPG 100, 100 × 10 cm). Water-soluble juice constituents like sugars, organic acids, and minerals were washed out with six bed volumes of distilled water. Polyphenols were eluted with three bed volumes of ethanol (96%). The ethanolic fraction was gently concentrated by evaporation, transferred into the water phase, and finally freeze-dried. The resulting polyphenol extract was kept in a cool, dry, and dark environment.

2.3 HPLC/photodiode array (PDA) detection of polyphenols

A solution of the polyphenolic apple extract was prepared in the concentration of 1 g/L in 20% methanol. The solution was filtered and 20 µL were injected on an HP 1090 HPLC system (Hewlett-Packard, Waldbronn, Germany) equipped with a PDA detector. A Phenomenex Aqua column (250/4 mm, protected by a corresponding 50/4 mm guard column: Phenomenex, Aschaffenburg, Germany) was used at ambient temperature. Gradient elution was applied with an acetonitrile/phosphoric acid gradient [5]. Detection wavelengths were 280 nm for flavonoids, 320 nm for phenolcarbonic acids, and 360 nm for the quercetin derivatives. Quantification was carried out using peak areas from external calibrations with commercially available reference substances. The content of 3- and 5-coumaroylquinic acid was calculated with the calibration curve based on preparatively isolated 4-coumaroylquinic acid. The amount of the two yet unidentified phloretin glycosides was calculated by the help of the phloridzin calibration curve.

2.4 Cell culture

The human vulva carcinoma cell line A431 [6] was cultured in humidified incubators (37°C, 5% CO_2) in minimum essential medium (MEM) containing L-glutamine (4.5 g/L) and the human colon carcinoma cell line HT29 was cultivated in Dulbecco's modified Eagle medium (DMEM with 4500 mg/L glucose, without sodium-pyruvate). Both cell culture media were supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS). MEM was obtained from Sigma (Taufkirchen, Germany), DMEM and the supplements were obtained from Invitrogen™ Life Technologies (Karlsruhe, Germany).

2.5 Sulforhodamine B assay

Effects on cell growth were determined according to the method of Skehan *et al.* [7]. Briefly, cells were seeded into 24-well plates and allowed to grow for 48 h before treatment. Cells were incubated with the respective drug for three days in serum-containing medium. Incubation was stopped by addition of trichloroacetic acid (50% solution). After 1 h at 4°C, plates were washed four times with water. The dried plates were stained with a 0.4% solution of sulforhodamine B (SRB). The dye was eluted with Tris-buffer (10 mM, pH 10.5) and quantified photometrically at 570 nm. Cytotoxicity was determined as percent survival, determined by the number of treated over control cells $\times 100$ (% T/C).

2.6 Tyrosine kinase assay

The EGFR was isolated from A431 cells and purified by affinity chromatography using wheat germ lectin agarose (Pharmacia Biotech, Uppsala, Sweden). 96-well plates were coated by incubation overnight at 37°C with 100 µL per well 0.1 mg/mL of the tyrosine kinase substrate poly (Glu : Tyr) 4 : 1 sodium salt in PBS. Excess poly (Glu : Tyr) 4 : 1 was removed by aspiration, and the plates were washed with wash buffer (0.1% Tween 20 in PBS). The kinase reaction was initiated by adding 50 µL ATP-solution (50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 200 µM ATP) to 40 µL of purified membrane fraction. 10 µL of the test compound (in 10% DMSO) was added to give a final DMSO concentration of 1%. Phosphorylation was proceeded at 37°C for 30 min. The kinase reaction was terminated by aspiration of the reaction mixture and the plate was washed with wash buffer (see above). Phosphorylated poly (Glu : Tyr) 4 : 1 was determined after 60 min incubation with an antiphosphotyrosine-peroxidase-conjugated antibody (Santa Cruz Biotechnology, Heidelberg, Germany), 0.6 U/mL in 1% BSA/PBS, 75 µL per well, at 37°C. The antibody was removed by aspiration, the plate washed again with wash buffer, and the peroxidase reaction started by addition of 100 µL ABTS® per well. After incubation at 37°C the absorbance was measured at 405 nm.

2.7 Reporter gene assay

2.7.1 Transfection of A431 cell line

Cells were plated in 24-well plates at a density of 3×10^5 cells in 1 mL medium containing 10% FCS and incubated at 37°C overnight. Thereafter, cells were held in serum-free medium for 24 h and then transfected by adding 150 ng pFR-Luc reporter plasmid and 2.5 ng pFA2-Elk-1 fusion transactivator plasmid (Stratagene, La Jolla, CA, USA) per well, together with 0.46 µL FuGENE 6® (Roche, Mannheim, Germany) in 10 µL serum-free medium (pre-equilibrated for 5 min). After gentle mixing and 15 min incubation at room temperature, cells were cultivated at 37°C for further 24 h.

2.7.2 Luciferase assay

Cells were incubated at 37°C for 0.5 h with substances or 1% DMSO prior to stimulation with 100 ng/mL EGF. After 4.5 h the incubation was stopped by washing each well with 1 mL PBS followed by cell treatment with lysis buffer (Promega, Mannheim, Germany). Lysates were transferred into 96-well plates and luciferase activity was measured using a luminometer.

2.8 Western blot analysis

10^6 HT29 cells were seeded per petri dish in DMEM supplemented with 10% FCS and 1% PS. After 48 h, the FCS concentration was reduced from 10 to 1% for 24 h. The test compounds were dissolved in DMSO and adopted in serum-free medium to a final DMSO concentration of 1%. Half an hour later, the cells were stimulated by adding 60 µL EGF (final concentration 100 ng/mL; Calbiochem, Schwalbach, Germany). After further 4.5 h, the medium was removed and dishes washed with cold PBS. Cells were abraded at 4°C with 0.2 mL lysis buffer (25 mM Tris-HCl, pH 7.4, 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 0.27 M sucrose, 10 mM Na-β-glycerophosphate, 5 mM Na-pyrophosphate, 0.5% v/v Triton-X 100, 5 mM Na_3VO_4). 40 µL protease inhibitory cocktail (Roche Diagnostics, Mannheim, Germany) and 2 µL β-mercaptoenthanol (0.1% v/v) were freshly added to 2 mL lysis buffer. Thereafter, the lysate was homogenized thoroughly and subsequently centrifuged for 10 min ($20\,000 \times g$, 4°C). The supernatant was subjected to SDS-PAGE (12% polyacrylamide gel). After electrophoresis the proteins were transferred onto a nitrocellulose membrane. Detection was performed using a rabbit polyclonal antibody against human p42/p44 (42,

44 kDa; Cell Signaling Technology, Beverly, MA, USA) and an anti-rabbit immunoglobulin G (IgG) peroxidase conjugate (New England Biolabs, Frankfurt, Germany) as a secondary antibody. The respective chemoluminescent signals (LumiGLO; Cell Signaling Technology) were analyzed using a Lumi-Imager™ with the LumiAnalyst 3.0 software for quantification (Roche Diagnostics). Arbitrary light units were plotted as test over control (%).

3 Results

From a mixture of table apples and traditional apple varieties (Topaz, Bohnapfel, Winterrambur, Bittenfelder) an apple juice blend was generated. An XAD absorber resin was used to separate the polyphenol fraction from other juice constituents, such as carbohydrates, the majority of organic acids and minerals, allowing the preparation of a polyphenol-rich extract (AE02) for biological testing. Per liter of apple juice about 0.5 g of the extract was generated. Characterization of the extract constituents was performed by HPLC with PDA detection. From 1 g of extract 533.9 mg of constituents were characterized (Table 1).

Table 1. Constituents of the polyphenol-rich apple juice extract and composition of synthetic mixtures

Known constituents	Apple juice extract (mg/g)	Mixture A (mg)	Mixture B (mg)
Proanthocyanidin B1	7	–	7
Proanthocyanidin B2	15.1	–	15.1
(−)-Epicatechin	19.2	19.2	19.2
Phloretinglycoside 1	24.7	–	–
Phloretinglycoside 2	9	–	–
Phloretin-xyloglucoside	66.2	–	–
Phloridzin	27.9	27.9	27.9
Chlorogenic acid	181.5	181.5	181.5
3-Coumaroylquinic acid	9.5	–	–
4-Coumaroylquinic acid	77.3	–	–
5-Coumaroylquinic acid	10.4	–	–
Caffeic acid	4.8	4.8	4.8
Quercetin-3-rhamnose	4.1	4.1	4.1
Quercetin-3-galactose	0.8	0.8	0.8
Quercetin-3-glucose	1.4	1.4	1.4
Quercetin-3-rutinose	2.6	2.6	2.6
Total	533.9	242.3	264.4

Of the apple juice polyphenols identified so far, chlorogenic acid represented by far the highest proportion followed by 4-coumaroylquinic acid. Glycosides of the dihydrochalcone phloretin accounted for about 10% of the total polyphenol mixture (about 20% of the identified constituents), with phloretin-xyloglycoside representing the highest proportion, followed by phloretin-2-glucoside (phloridzin). In addition, two compounds were detected, each consisting of a polyphenol moiety, exhibiting the characteristic PDA spectrum of phloretin, but possessing different sugar residues. The identification of the glycoside moiety is currently under investigation. In addition to substantial amounts of EC, the two dimers PB1 and PB2 were detected. Furthermore, minor contents of four different quercetinglycosides were found (Table 1). The polyphenol-rich apple juice extract was applied to further biological testing, addressing the question whether the polyphenol composition affects the growth of human colon tumor cells *in vitro*.

3.1 Inhibition of tumor cell growth

The effect of the polyphenol-rich apple juice extract and its constituents on the growth of the colon carcinoma cell line HT29 was determined in the SRB assay [7]. Incubation of HT29 cells with the polyphenol-rich apple juice extract (AE02) for 72 h led to a substantial inhibition of cell growth (IC_{50}-value of 134 ± 18 µg/mL) (Fig. 2A). To address the question which extract constituent(s) is (are) responsible for the observed growth inhibitory effect, the compounds identified so far and available in respective amounts (Table 1, Scheme 1) were applied to the SRB assay. Of the apple constituents tested so far, caffeic acid showed the strongest growth inhibitory properties, comparable in the extent to that of EC (Fig. 2B, Table 2). PB1 and PB2, as well as chlorogenic acid, were found to possess only marginal growth inhibitory properties. The dihydrochalcon glycoside

Table 2. Polyphenols identified in apple juice extract and structurally related compounds: growth inhibitory properties in the SRB assay (HT29 cells, 72 h incubation) and inhibition of the PTK activity of the EGFR (ELISA)

Compound	Growth inhibition IC_{50} (µM)	EGFR ELISA IC_{50} (µM)
Proanthocyanidin B1	235 ± 68	49.9 ± 10.2
Proanthocyanidin B2	191 ± 39	37.9 ± 2.8
(+)-Catechin[b)]	n.d.	> 300
(−)-Epicatechin	136 ± 4	> 300
Epigallocatechin[b)]	n.d.	297 ± 24
Epicatechin-3-gallate[b)]	74 ± 18	1.2 ± 0.4
Epigallocatechin-3-gallate[b)]	40 ± 6	0.2 ± 0.02
Caffeic acid	132 ± 19	> 300
Chlorogenic acid	205 ± 53	> 300
4-Coumaroylquinic acid	n.d.	> 300
Phloretin[b)]	89 ± 18	43.4 ± 5.1
Phloridzin	–[a)]	267 ± 50
Quercetin[b)]	52 ± 11	0.9 ± 0.4
Quercetin-3-glucose	> 300	6.7 ± 1.3
Quercetin-3-galactose	–[a)]	7.5 ± 1
Quercetin-3-rhamnose	–[a)]	225 ± 31
Quercetin-3-rutinose	–[a)]	> 300

n.d., not determined
a) No inhibition up to 300 µM
b) Analogues not present in the original extract, included for structure-activity comparison

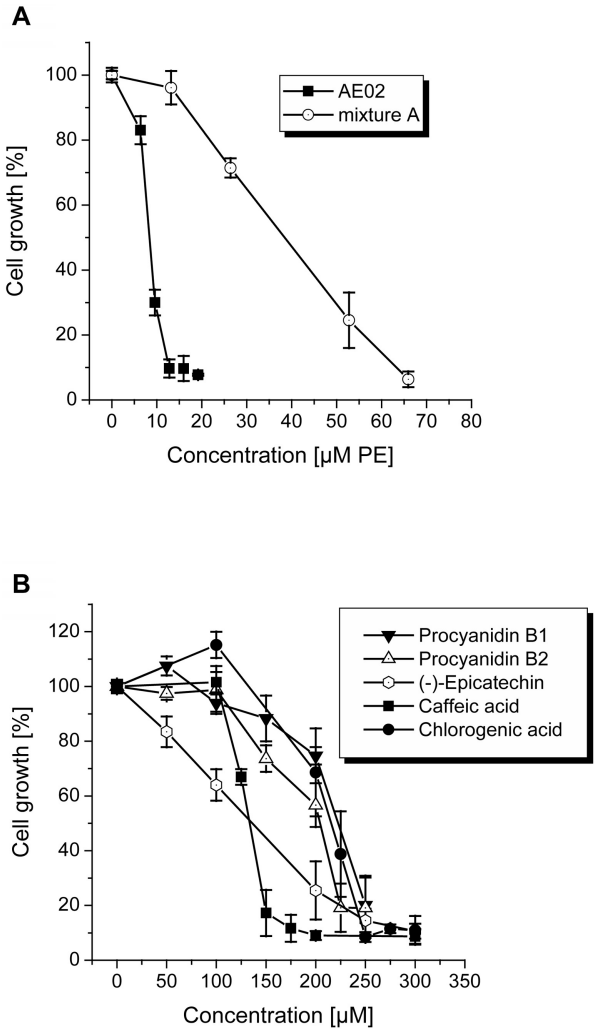
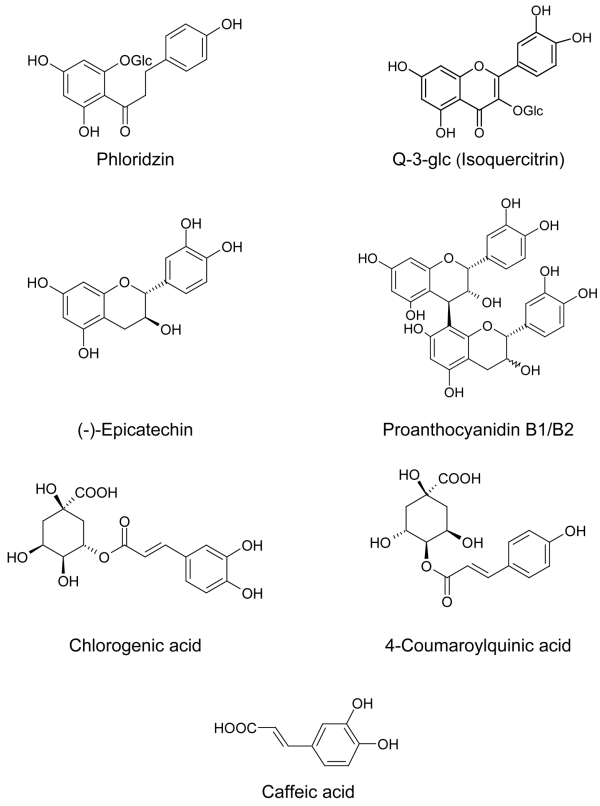


Figure 2. Inhibition of tumor cell growth *in vitro* by (A) apple juice extract AE02 in comparison to a synthetic blend of apple constituents (see Table 1). (B) Apple constituents. Growth inhibition was determined using the SRB assay [7]. HT29 cells were incubated for 72 h with the respective compound(s). Growth inhibition was calculated as survival of treated cells over control cells (treated with the vehicle 1% DMSO) × 100 (T/C %). The values given are the mean ± SD of at least three independent experiments, each performed in quadruplicate.

phloridzin did not affect cell growth up to 300 µM. In contrast, the free aglycon phloretin effectively inhibited the growth of HT29 cells (Table 2). A comparable pattern of activity was observed for the flavonols: the free aglycon quercetin potently inhibited tumor cell growth *in vitro*, whereas the respective glycosides, present in the apple juice extract, did not affect cell growth up to 300 µM, with the exception of q-3-glc, mediating at least slight growth inhibitory effects (33.3 ± 4.7% inhibition at 300 µM).

In order to investigate if the available extract constituents act differently when applied to the cells in complex poly-



Scheme 1. Polyphenols of different structural classes present in apple juice extract.

phenol compositions, a mixture of the apple constituents identified so far and available in respective amounts for testing in the SRB assay (mixture A, Table 1) was generated analogously to the respective relative contents of these compounds in the original extract AE02. To provide a similar molarity of the constituents in the testing, in spite of the partial availability of the constituents, the extract AE02 and the mixture were standardized with respect to the molarity of phloridzin (phloridzin equivalents, PE), a compound characteristic for apples.

Incubation of HT29 cells (72 h) with mixture A at a concentration of 9 µM PE (IC_{50} of AE02 = 9 ± 1 µM PE) resulted in an inhibition of cell growth of about 3% (Fig. 2A). The IC_{50}-value in the SRB-assay was achieved with mixture A at 34 ± 8 µM PE.

3.2 EGFR

Effects of test compounds on the protein tyrosine kinase activity of the EGFR was determined using an enzyme-linked immunosorbent assay (ELISA) adapted to a 96-well plate format. The specific EGFR inhibitor tyrphostin AG1478 was used as a positive control, showing an IC_{50}-value in this test system of 2.4 ± 0.1 µM [8, 9].

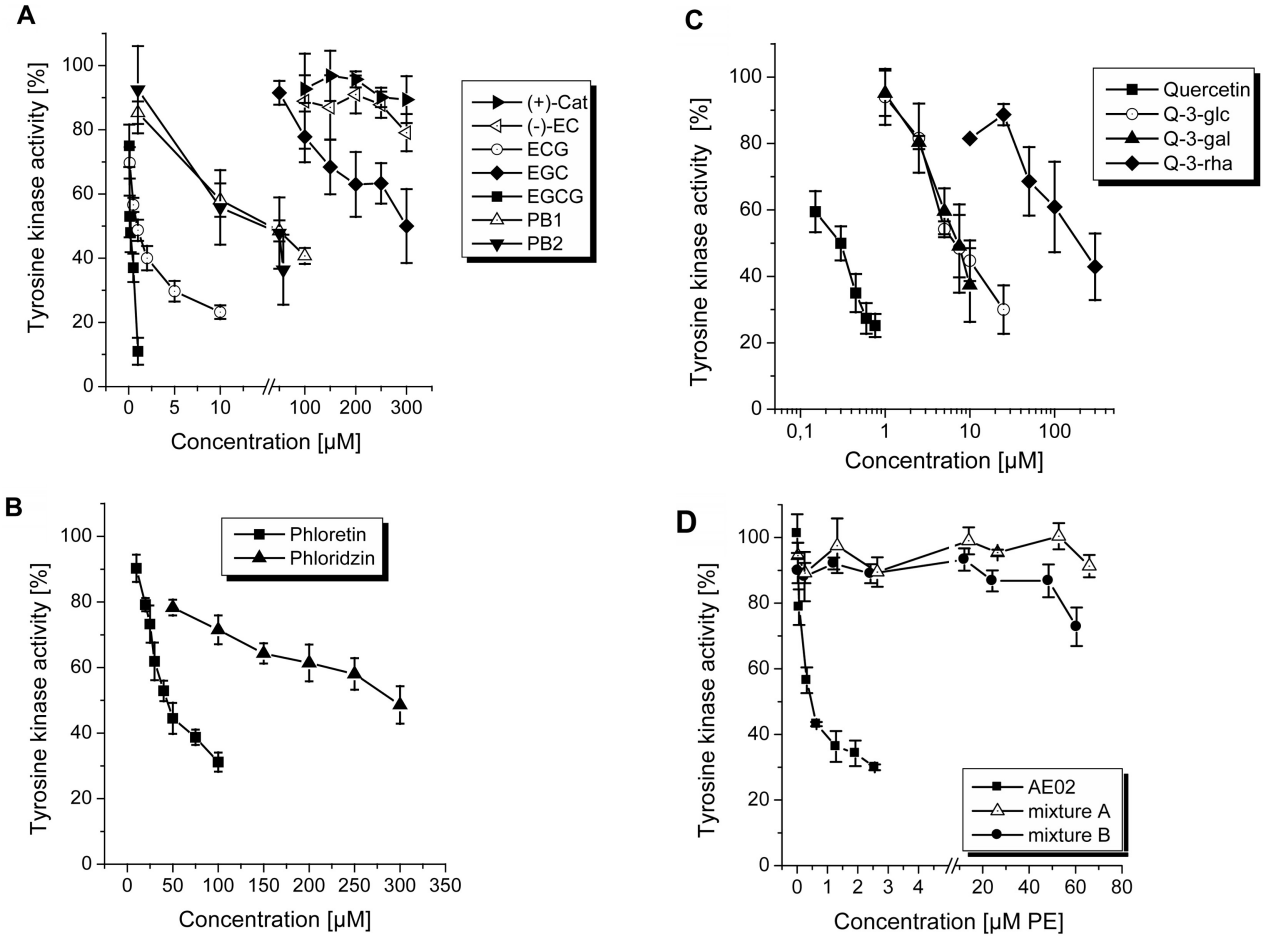


Figure 3. Inhibition of the tyrosine kinase activity of the EGFR. (A) Catechins, (B) dihydrochalcons, (C) flavonols, (D) apple juice extract AE02 in comparison to synthetic blends of apple constituents (see Table 1). The phosphorylation of tyrosine residues of a peptide poly (Glu/Tyr) was determined by ELISA using an antiphosphotyrosine antibody linked to peroxidase. The data presented are the mean ± SD of at least three independent experiments, each performed in quadruplicate.

EC, a constituent of the apple juice extract, exhibited only marginal inhibitory properties in this test system. In order to characterize structural elements crucial for the target interaction, a series of related catechins were included into the testing. Of the catechins tested, Cat showed the lowest inhibitory properties *versus* the protein tyrosine kinase (PTK) activity of the EGFR (11% inhibition at 300 µM concentration), comparable to that of the stereoisomer EC (24% inhibition at 300 µM). EGC showed a concentration-dependent inhibition of the PTK activity at concentrations ≥ 100 µM, achieving inhibition of 50% of the enzyme activity only at about 300 µM (Table 2). In contrast, PB1 and PB2 were found to be more effective, without significant differences between the two epimers. EGCG and ECG exhibited by far the highest PTK-inhibitory properties, representing highly potent inhibitors (Fig. 3A, Table 2). Within the class of catechins the potency for the inhibition of the PTK activity of the EGFR can be summarized as EGCG > ECG >> PB2 ≈ PB1 >> EGC > EC ≈ Cat (Fig. 3A, Table 2). The dihydrochalcon glycoside phloridzin showed only weak inhibitory properties in this test system (IC_{50} = 267 ± 50 µM), whereas the respective aglycon phloretin was clearly more potent (IC_{50} = 43.4 ± 5.1 µM) (Fig. 3B).

In the class of flavonols, quercetin was found to represent a potent inhibitor of the PTK activity of the EGFR (0.9 ± 0.4 µM). The presence of a galactose or a glucose residue at position 3 (q-3-gal, q-3-glc) of the flavonol ring system resulted in a clear decline of inhibitory properties, but still preserved effective PTK inhibition (Fig. 3C, Table 2). A rhamnose residue in position 3 (q-3-rha) strongly diminished the PTK inhibitory properties (Fig. 3C, Table 2). The presence of a rutinose resulted even in a further loss of PTK inhibition (32% inhibition at 300 µM). Within the class of flavonols the effectiveness for the inhibition of the PTK activity of the EGFR can be summarized as quercetin > q-3-glc ≈ q-3-gal >> q-3-rha > q-3-rut. Caffeic acid, chloro-

genic acid, and 4-coumaroylquinic acid exhibited only marginal inhibitory properties (Table 2).

An artificial mixture of these apple constituents (mixture A) showed no effect at all in this test system up to 53 μM PE (200 μg/mL). Even at the highest concentration tested (66 μM PE = 250 μg/mL), only a marginal inhibition of the PTK activity (<10%) was detected. Addition of PB1 and PB2 to the polyphenol mixture (mixture B, Table 1) slightly enhanced the inhibitory properties (Fig. 3D). In contrast, the polyphenol-rich apple juice extract (AE02) was found to potently inhibit EGFR kinase activity (10 ± 5 μg/mL = 0.6 ± 0.3 μM PE) (Fig. 3 D).

3.3 Inhibition of the MAPK cascade activity

We furthermore addressed the question if the inhibitory effect of the apple juice extract AE02 is limited to the isolated EGFR, as used in the ELISA, or is of relevance for the activity of the subsequent MAPK cascade. We therefore investigated the effect of AE02 on the phosphorylation status of the MAP kinases ERK1/ERK2 (p42/p44) as a measure for the activity of the upstream kinase cascade. Phosphorylated ERK1 and ERK2 was determined by Western blotting using respective antiphospho-ERK1/2 antibodies. HT29 cells were growth-arrested by serum-deprivation (see Section 2). Thereafter, the cells were stimulated with EGF in the presence or absence of AE02. Already at 0.6 nM PE, a significant decrease of phosphorylated p44 was observed (Fig. 4). The content of phosphorylated p42 and p44 reached a minimum at 0.006 μM PE. At concentrations ≥ 0.6 μM PE a significant recurrence of phosphorylated p42/p44 was observed, being most pronounced for the p44 isoform (Fig. 4).

We further addressed the question if the effects on the activity of the MAPK cascade, measured as changes in the phosphorylation status of ERK1/2, might be of relevance further downstream on the transcriptional level, affecting the regulation of gene expression. The activity of the MAP kinases ERK1/ERK2 were measured as phosphorylation of the transcription factor Elk-1 in a reporter gene assay. A431 cells, known to express substantial amounts of functional EGFR [6], were transiently cotransfected with a plasmid encoding a fusion protein consisting of the DNA binding domain (dbd) of GAL4 and ELK-1 together with a plasmid containing the upstream activating domain (UAS) of GAL4 and the luciferase gene as a reporter. Activation of the MAPK cascade results in the phosphorylation not only of native ELK-1 but also of the fusion protein. The phosphorylation of the fusion protein enables its binding to the UAS of GAL4, resulting in the expression of luciferase (Fig. 5A). Treatment of these transiently transfected cells with EGF induced an increase in luciferase expression of

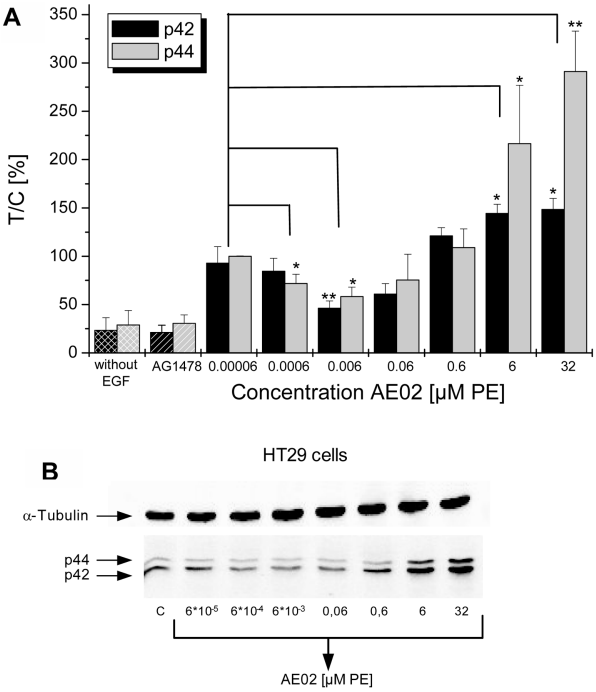


Figure 4. Western blot analysis of phosphorylated p42/p44 (ERK1/ERK2) in HT29 cells stimulated by EGF (100 ng/mL) after 5 h treatment with AE02. α-Tubulin was included in the test as a loading control. (A) The data are presented as % of the solvent control stimulated by EGF. The plotted data are the mean ± SD of three independent experiments with similar outcome (* = $p < 0.05$; ** = $p < 0.01$). (B) Western blot of a representative experiment. (C) Solvent control stimulated by EGF.

2.6 ± 0.6-fold. In the presence of the dihydrochalcon phloretin the expression of luciferase was diminished in a concentration-dependent manner (IC_{50} = 26 ± 1 μM) (Fig. 5B). Quercetin potently inhibited luciferase expression at concentrations ≥ 1 μM (IC_{50} = 16 ± 2 μM).

The apple juice extract AE02 was found to potently suppress luciferase expression (Fig. 5C). Already at 6 nM PE about 50% of the luciferase expression was inhibited. The effect was even enhanced at 60 nM PE. However, a further increase in the concentration failed to fortify the inhibitory effect, reaching an apparent minimum level. A further raise in extract concentration even resulted in the recurrence of luciferase expression at concentrations ≥6 μM PE. In comparison to the original extract AE02, the artificial polyphenol mixtures A and B showed a significant decrease in luciferase expression at clearly higher concentrations (Fig. 5C). However, despite the fact that the effective concentrations were higher (minimum mixture A: 13 μM PE, mixture B: 2.4 μM PE), the shape of the curves appeared to be comparable, showing an initial decrease followed by a recurrence of luciferase expression at higher concentrations.

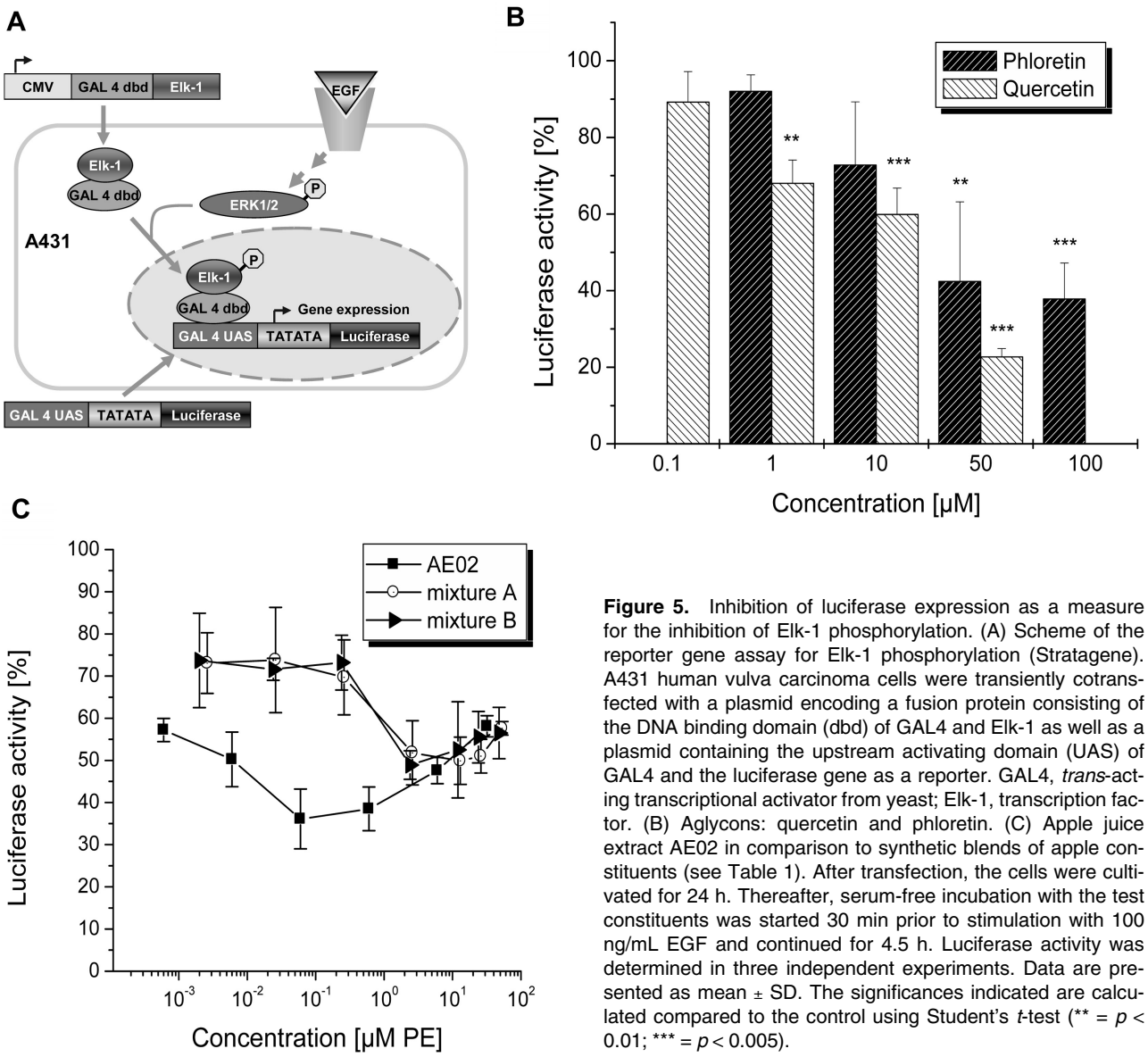


Figure 5. Inhibition of luciferase expression as a measure for the inhibition of Elk-1 phosphorylation. (A) Scheme of the reporter gene assay for Elk-1 phosphorylation (Stratagene). A431 human vulva carcinoma cells were transiently cotransfected with a plasmid encoding a fusion protein consisting of the DNA binding domain (dbd) of GAL4 and Elk-1 as well as a plasmid containing the upstream activating domain (UAS) of GAL4 and the luciferase gene as a reporter. GAL4, *trans*-acting transcriptional activator from yeast; Elk-1, transcription factor. (B) Aglycons: quercetin and phloretin. (C) Apple juice extract AE02 in comparison to synthetic blends of apple constituents (see Table 1). After transfection, the cells were cultivated for 24 h. Thereafter, serum-free incubation with the test constituents was started 30 min prior to stimulation with 100 ng/mL EGF and continued for 4.5 h. Luciferase activity was determined in three independent experiments. Data are presented as mean ± SD. The significances indicated are calculated compared to the control using Student's *t*-test (** = $p < 0.01$; *** = $p < 0.005$).

4 Discussion

In the present study we show that polyphenol-rich apple juice extract effectively inhibits the growth of human colon carcinoma cells (HT29) *in vitro* (Fig. 2A). Substantial growth inhibition (IC_{50} = 134 ± 18 µg/mL) is achieved at concentrations clearly below the concentration of these polyphenols in consumer-relevant apple juice (about 500 µg/mL). Apple extracts have been reported earlier to inhibit the proliferation of colon carcinoma cells *in vitro*, an effect which is mainly attributed to the comprised polyphenols and flavonoids [10, 11]. Amongst the apple constituents identified and available so far several compounds were found to possess antiproliferative properties, with caffeic acid and EC being the most potent (Fig. 2B, Table 2). The observed growth inhibition induced by caffeic acid is within the range of earlier reports on growth-inhibitory effects, amongst others, in colon carcinoma cells [12, 13]. EC is known to possess some antiproliferative properties, however, being one of the least potent within the class of catechins [14 – 16]. The presence of a gallate residue in position 3, characteristic for green tea catechins (*e.g.*, ECG, EGCG), clearly enhances the inhibitory effect on tumor cell growth *in vitro* [14, 15, 17, 18] as also shown in Table 2. We found that the dimeric catechins, PB1 and PB2, possess only limited growth inhibitory properties *in vitro*, comparable in the extent to the effect of chlorogenic acid (Fig. 2B, Table 2).

The apple characteristic dihydrochalcon glucoside phloridzin as well as the identified quercetin glycosides (q-3-glc, q-3-gal, q-3-rha, q-3-rut) were found to be ineffective with respect to growth inhibition. In contrast, the much more effective free aglycons phloretin and quercetin (Table 2), known to inhibit tumor cell growth *in vitro* [19 – 24], were not detected so far in the original apple juice or the respective extract.

Calculating the proportion of the different apple constituents, found to possess antiproliferative activity, in the polyphenol-rich extract and considering the inhibitory potency of the single compounds, these constituents were expected to administer only a minor contribution to the total inhibitory effect of the complex mixture. The extrapolation of the prospective growth inhibitory properties to be mediated by a mixture of the available apple constituents let anticipate only less than 1% inhibition of cell growth in a concentration resulting in 50% inhibition with the original extract AE02 (9 ± 1 μM PE). Indeed, an artificial mixture of the identified constituents available so far (mixture A) was found to be clearly less effective with respect to growth inhibition than the original extract AE02 (Fig. 2A). However, the concentration of mixture A inducing 50% growth inhibition (34 ± 8 μM PE) was expected to achieve only about 20% growth inhibition, extrapolating from the results with the single compounds in an additive manner. These results indicate that the effect of the respective polyphenols in mixture on tumor cell growth is not simply additive, but at least overadditive or even synergistic. But the results also clearly show that we successfully identified apple constituents which are likely to contribute to the growth inhibitory properties of the apple juice extract, however, so far not identified constituents apparently account for a substantial proportion of the growth inhibitory effect of the original extract.

The EGFR and its subsequent signaling cascade is known to play an important role in the regulation of cell proliferation in HT29 cells [25]. Several colon carcinoma cell lines have been shown to express substantial amounts of functional EGFR [26 – 28]. Considering the role of the EGFR and subsequent signalling in the regulation of cell proliferation, effective inhibitors might not only be of relevance in chemotherapy, but might also be of interest with respect to chemoprevention. Several flavonoids of different classes have been shown to inhibit the PTK activity of the EGFR [3, 25, 29, 30]. In the present study we showed that a polyphenol-rich apple juice extract (AE02) effectively inhibits the PTK activity of the EGFR (10 ± 5 μg/mL = 0.6 ± 0.3 μM PE) at a concentration clearly below the concentration of these polyphenols in the original apple juice (about 500 μg/mL).

Of the apple constituents identified and available so far, q-3-gal and q-3-glc were identified as the most potent inhibitors of the PTK activity of the EGFR, followed by PB1 and PB2. Interestingly, the catechin dimers, PB1 and PB2, were found to represent quite effective inhibitors, whereas the monomeric EC was not active. However, despite the more potent inhibition of the EGFR activity, PB1 and PB2 were found to be less effective with respect to growth inhibition compared to EC. As one possibility, differences in cellular bioavailability have to be considered.

The structure-activity studies indicate that in contrast to other flavonoids classes vicinal hydroxy groups at the B-ring are not sufficient for potent EGFR inhibition within the class of catechins. A third hydroxy group at the B-ring (EGC) was found to implicate only a marginal enhancement of inhibitory properties (Fig. 3A, Table 2). However, substantial improvement of EGFR inhibition was, comparable to the results of the SRB assay, associated with the presence of a gallate residue at position 3 (Fig. 3A, Table 2). EGCG, a characteristic green tea catechin, is known to represent a highly potent inhibitor of the EGFR [3, 18, 31 – 34]. Potent inhibition of the EGFR activity was also found for the flavonol quercetin in accordance with earlier reports [22, 35, 36]. However, so far only various quercetin glycosides, but not the free aglycon, have been detected in the original apple juice extract. The presence of glucose or galactose in the 3-position of quercetin significantly diminished EGFR inhibition compared to the free aglycon, however, still potent PTK inhibition was observed (Fig. 3C, Table 2). In contrast, the glycosylation with a rhamnose or rutinose resulted in a nearly complete loss of inhibitory potency. These data show that the glycosylation is not necessarily connected with a direct loss of inhibitory properties, but depends strongly on the nature of the sugar residue. However, despite the potent inhibition of the EGFR activity by q-3-glc and q-3-gal, both compounds failed to inhibit tumor cell growth in the SRB assay, which might be due to limited cellular uptake of the glycosides. The apple characteristic dihydrochalcon phloridzin was found to possess only marginal EGFR inhibitory properties, in contrast to the more potent free aglycon phloretin (Fig. 3B, Table 2), a known inhibitor of the EGFR [37]. However, similar to quercetin, the free aglycon phloretin has not been found so far to be present in the apple juice extract.

Calculating the proportion of the apple constituents in the original extract, found to possess EGFR-inhibitory properties, and considering the inhibitory potency of the single compounds, the effect to be expected of an equimolar mixture compared to AE02 was extrapolated. At a concentration of 0.6 μM PE (IC_{50} of AE02 = 0.6 ± 0.3 μM PE) the equimolar mixture of all constituents available so far, including PB1 and PB2 (mixture B), let expect an inhibition of less than 5% (Fig. 3D). In the experiments mixture B induced an inhibition of 10 ± 4.5%, indicating a slight overadditive effect of the constituents in mixture (Fig. 3D). The differ-

ence in effectiveness between mixture A and B indicates that PB1 and PB2 contribute to some extent to the EGFR-inhibitory properties of the original extract. However, the data also clearly show that yet unknown apple constituents contribute substantially to the EGFR inhibition.

With respect to the concentration range a difference of one order of magnitude was observed between the inhibition of cell growth in the SRB assay (IC_{50} = 9 ± 1 μM PE) and the inhibition of the PTK activity of the EGFR in the ELISA (IC_{50} = 0.6 ± 0.3 μM PE). This discrepancy was not limited to the complex original extract, but was observed for all the tested compounds found to exhibit EGFR inhibitory properties (Table 2). The discrepancy in effectiveness might result from differences between the possible direct interaction of compounds with the isolated receptor in the ELISA, and cellular pharmacokinetics, such as cellular uptake, subcellular distribution, *etc.*, which have to be considered incubating whole cells in the SRB assay. A comparable discrepancy in effectiveness was reported earlier for the anthocyanidins delphinidin and cyanidin [3].

Effective inhibition of the activity of the EGFR is connected with a suppression of the subsequent MAPK cascade, interfering with the regulation of cell growth. In HT29 cells, we found that treatment with the apple juice extract results in a decrease in the phosphorylation of the MAP kinases ERK1 and ERK2, suggesting a reduction of the activity of the upstream signalling cascade (Fig. 4). However, a minimum of the phosphorylation level of ERK1/ERK2 was already observed at 0.006 μM PE (Fig. 4), clearly below growth-inhibitory concentrations in the SRB assay (Fig. 2A). At higher concentrations the recurrence of phosphorylated ERK1/ERK2 was apparent, even up to a significant induction at concentration ≥6 μM. This U-shaped curve of effectiveness was also found one step further downstream on the level of the phosphorylation of the transcription factor ELK-1, as measured in the reporter gene assay (Fig. 5). However, although the U-shaped curve occurred in both test systems, no complete overlay was observed. In the reporter gene assay an inhibition of ELK-1 phosphorylation was measured over a broader concentration range, including growth-inhibitory concentrations. Whereas a complete recurrence of phosphorylated ERK1/ERK2 was observed in HT29 cells at 0.6 μM PE in the Western blot, in A431 cells (reporter gene assay) the phosphorylation of ELK-1 was maintained still at a minimal level. At 6 μM PE even an induction of phosphorylated ERK1/ERK2 was found in HT29 cells, whereas in A431 cells the inhibition of ELK-1 phosphorylation was still about 50%. This apparent discrepancy might be due to differences between the two cell lines of different tissue origin. Both cell lines are known to overexpress the EGFR and are sensitive to specific EGFR inhibitors, such as tyrphostin AG1478 [3, 6, 25]. The sensitivity of both cell lines *versus* the growth-inhibitory properties of tyrphostin AG1478 are comparable (IC_{50} in the SRB-assay, 72 h incubation: HT29, 0.9 ± 0.2 μM; A431, 0.4 ± 0.1 μM).

The reporter gene assay was originally established in A431 cells due to the effectiveness, stability, and reproducibility of transfection with the plasmids and the activity of the respective pathway. The results clearly show that depending on the concentration range, the MAP kinase cascade is affected by the apple juice extract in A431 cells. In accordance with the reporter gene assay in A431 cells, the Western blot analysis of phospho-ERK1/ERK2 in HT29 cells show that treatment with the apple juice extract affects the activity of the MAPK cascade. However, beside the probable EGFR inhibition additional effects appear to compensate the downstream inhibitory effect at higher concentrations, resulting in the recurrence and even induction of ERK1/ERK2 phosphorylation. Interestingly, the modulation of ERK1/ERK2 phosphorylation in HT29 cells by the apple juice extract was not directly reflected by a respective change in cell proliferation. These data indicate that in addition other signalling components involved in the regulation of cell growth are affected, completely compensating the potential proproliferative effect of ERK1/ERK2 phosphorylation at higher concentrations. Potential additional cellular targets of the apple juice extract are currently under investigation.

In summary, we found that the polyphenol-rich extract of a consumer-relevant apple juice extract potently inhibited the growth of human colon carcinoma cells (HT29) *in vitro*. The extract effectively blocked the activity of isolated EGFR, an effect which might be interesting with respect to chemoprevention. We succeeded to identify apple juice constituents with EGFR-inhibitory properties. However, the results show that so far unknown apple constituents apparently contribute substantially to the EGFR-inhibitory properties and also antiproliferative effects of the extract. Downstream of the EGFR the MAP kinase cascade was found to be affected in its activity indicating that the effect on the EGFR is not limited to the isolated receptor preparation, but is also mediated in downstream cascades. However, the powerful EGFR inhibition appears to be compensated at higher concentrations. The fact that this compensation is not reflected concomitantly by an increase in cell proliferation let assume that, in addition to the effects on the EGFR, other cellular targets involved in the regulation of cell growth must be affected. In conclusion, polyphenol-rich apple juice extract appears to possess several bioactive properties, which affect cellular signalling cascades regulating cell proliferation.

The study was performed as a part of the NutritionNet, funded by the German Ministry of Research and Education (01EA0101).

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