Neurotensin- and EGF-Induced Metabolic Activation of Colon Carcinoma Cells Is Diminished by Dietary Flavonoid Cyanidin but Not by Its Glycosides

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Abstract: Dietary polyphenols, including anthocyanidins and their glycosides anthocyanins, are suggested to be involved in the protective effects of fruits and vegetables against cancer. Very few data are available concerning the effects of anthocyanidins/anthocyanins on cellular processes induced by growth factors such as neurotensin and epidermal growth factor (EGF), which are implicated in the pathophysiology of colon cancer. Here, we show that neurotensin and EGF caused an increase in the extracellular acidification rate, which could reflect the activity of cellular metabolism, in the human carcinoma cell line HT29 clone 19A. Neurotensin and EGF also caused a strong rise in the intracellular Ca2+ concentration, induced phosphorylation of extracellular signal-regulated kinases (ERK1 and ERK2), and stimulated growth of human carcinoma cells. Cyanidin (10 µM), but not its glycosides cyanin and idaein, was able to inhibit the neurotensin- and EGF-induced increased rate of extracellular acidification. In contrast to N-ethyl-N-isopropyl amiloride, an inhibitor of Na+/H+ exchange, cyanidin did not alter the rate of intracellular pH recovery of cells loaded by NH3/NH4+ i, indicating that cyanidin inhibits cellular metabolism, rather than directly altering Na+/H+ exchange. Cyanidin, but not cyanin and idaein, was able to inhibit an increase in intracellular Ca2+ concentration induced by neurotensin. Neurotensin- and EGF-induced phosphorylation of ERKs was not affected by cyanidin, cyanin, and idaein at ≤100 µM. Only cyanidin (100 µM), but not cyanin and idaein, was able to inhibit cellular growth induced by EGF. Thus these findings suggest that a dietary polyphenol cyanidin, but not its glycosides, is a potent inhibitor of mitogen-induced metabolic activity, increase in free intracellular Ca2+, and cellular growth of cultured colon carcinoma cells.

Introduction

Colorectal cancer is one of the leading causes of cancer-related deaths, and dietary factors appear to be among the most important determinants of colorectal cancer risk (1). Dietary polyphenols such as anthocyanidins/anthocyanins, the major sources of red, violet, and blue pigmentation in berries and grapes, are thought to be involved in the protective effects of fruits and vegetables against cancer. The intake of anthocyanins in humans has been reported to be 180–215 mg/day in the United States (2). Cyanidin (Fig. 1) is one of the most widespread anthocyanidins in the plant kingdom (3) and occurs in plants mostly in the glycoside form. It has been reported that cyanidin is able to inhibit growth of cultured tumor cells (4), and cyanin (cyanidin-3,5-diglucoside; Fig. 1) had an antitumor effect in an in vivo study (5).

Our previous findings have shown that cyanidin and its glycosides cyanin and idaein are powerful antioxidants that may provide extracellular protection against H2O2-induced DNA damage in the differentiated human colon cell line HT29 clone 19A (6). In contrast, endogenous generation of oxidized DNA bases was not prevented by any of the investigated compounds. This finding raises questions with respect to the cancer-preventive potential of anthocyanins in specific tissues, such as the colon, especially since the molecular mechanisms of action of these phytochemicals are poorly understood. It may very well be possible that these flavonoids do not exert activities solely by antioxidative mechanisms but also by their growth-arresting effects in human colon cells. To obtain more information on potential intracellular effects of these important plant ingredients, we have analyzed them for molecular mechanisms of cell metabolism and hormone-induced signal transduction in HT29 clone 19A cells. Specifically, we have investigated the effects of cyanidin and its glycosides cyanin and idaein on cellular metabolism, determined by measuring proton extrusion. Tumor cells are known to exhibit high metabolic (glycolytic) activity and correspondingly increased production of protons. It is proposed that proton extrusion is involved in the regulation of cell growth induced by mitogens (7). Also, intracellular signaling pathways such as extracellular signal-regulated kinases (ERK1 and ERK2) and changes in intracellular Ca2+ concentration ([Ca2+]i) were determined. Moreover, we investigated the anthocyanin-regulated modulation of these effects induced by physiological growth pep-
tides, such as neurotensin, which is able to promote colon carcinogenesis in animal experiments (8). Neurotensin receptors are exclusively present in transformed colon cells, and thus specific inhibition of neurotensin-induced cellular effects may affect tumor progression.

**Materials and Methods**

**Chemicals**

Unless otherwise stated, all chemicals were purchased from Merck (Darmstadt, Germany). Cyanidin chloride, cyanin chloride (cyanidin chloride-3,5-diglucoside), and idaein chloride (cyanidin chloride-3 galactoside) were purchased from Carl Roth (Karlsruhe, Germany); fura 2-acetoxymethyl ester, fura 2 pentapotassium salt, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), ethylisopropylamiloride (EIPA), and pluronic acid from Molecular Probes (Eugene, OR); Dulbecco’s modified Eagle’s minimum essential medium (DMEM), low-buffering HCO₃⁻-free DMEM, glutamine, penicillin, streptomycin, and phosphate-buffered saline without Mg²⁺ and Ca²⁺ from Life Technologies (Eggenstein, Germany); fetal calf serum (FCS) and 3-[4,5-di-methyl-thiazol-2-yl]-2,5-diphenylietrazolium bromide (MTT) from Roche (Mannheim, Germany); protein assay from Bio-Rad (Munich, Germany); hydroxyethylpiperazine-N²'-2-ethanesulfonic acid (HEPES) and tris(hydroxymethyl)-aminomethane (Tris) from Serva (Heidelberg, Germany); and sodium dodecyl sulfate (SDS) from Sigma Chemical (St. Louis, MO).

**Cell Culture**

Human colon carcinoma cell line HT29 clone 19A was a gift from L. Laboisse (9). Cells (Passages 20–40) were plated on glass coverslips (23 mm²) in 10-cm² culture wells (Greiner, Frickenhausen, Germany) or Transwell inserts (1-cm² Costar, Badhoevedorp, The Netherlands). The culture medium consisted of DMEM (with 4.5 g/l glucose), supplemented with 2 mM glutamine, 25 IU/ml penicillin, 25 μg/ml streptomycin, and 10% (vol/vol) FCS. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Culture medium was replaced three times a week, and cells were used 1 day after change of the culture medium.

**Measurement of Proton Extrusion**

Proton extrusion was measured by determination of the rate of extracellular acidification using a pH-sensitive silicon-based sensor system (Cytosensor Microphysiometer, Molecular Devices, Munich, Germany) (10). Briefly, HT29 clone 19A cells were seeded on polycarbonate Transwell filters (1-cm² inserts, Costar) at a density of 3 × 10⁵ cells/filter. After the cells were cultured for 48 h in culture medium, the filters were transferred to the sensor chamber. Cells in the chamber were perfused with low-buffering HCO₃⁻-free DMEM at a rate of 100 μl/min. The chamber temperature was adjusted to 37°C. Every 2 min the flow was stopped for 30 s to measure the extracellular acidification. Basal acidification rates were allowed to stabilize for ≥30 min and subsequently normalized to 100%. Cells were exposed to 10 nM neurotensin and 1 nM epidermal growth factor (EGF) for 2 and 20 min, respectively, and additionally with cyanidin, cyanin, or idaein (each 10 μM) for 20–60 min. In control experiments, the effects of subsequent addition of neurotensin and EGF as well as the effects of repetitive addition of each individual agonist were investigated. The effects of cyanidin, cyanin, and idaein were compared with addition of vehicle [2% ethanol (vol/vol)].

**Measurement of Intracellular pH**

Cells grown on glass coverslips for 2–3 days were loaded with BCECF by incubation with 5 μM BCECF-acetoxymethyl ester, 0.1% (vol/vol) dimethylsulfoxide, and 0.025% (wt/vol) pluronic acid in a HEPES-buffered saline solution (138 mM NaCl, 5 mM KCl, 0.8 mM NaH₂PO₄, 4.2 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM D-glucose, and 10 mM HEPES) for 1 h at 37°C in the dark. Subsequently, the coverslips were mounted in a thermostated chamber (Temperature Controller Type II, Luigs and Neumann, Ratingen, Germany) placed on the stage of an inverted Diaphot microscope (TMD-EF, Nikon, Düsseldorf, Germany). Cells were superfused with HEPES-buffered saline solution at a rate of 1 ml/min, and the temperature of the chamber was set to 37°C. The microscope was connected to a digital video imaging system consisting of an amplified charged-coupled device camera (Hamamatsu Photonics, Herrsching, Germany) and a computer-controlled image processor (VideoProbe, ETM Systems, Irvine, CA) and filter wheel (Lambda 10, Sutter Instrument, Novato, CA). BCECF fluorescence was measured at 440- and 490-nm excitation wavelengths with fluorescence emission measured at >515 nm. The fluorescent intensities at each excitation wavelength were collected for 1 s at a rate of six pairs of images per minute. The background fluorescence of unloaded cells was subtracted. Na⁺/H⁺ exchange activity was studied.
by measuring cellular BCECF fluorescence ratios during superfusion with Na+ containing HCO₃⁻-free medium (HEPES-buffered saline solution) and during the recovery from an NH₄/H⁺-induced intracellular acid load (11,12). For these experiments, cells were superfused with HEPES-buffered saline solution, of which 30 mM NaCl had been replaced with 30 mM NH₄Cl. Cells were exposed to NH₄/H⁺ for 5 min at a perfusion rate of 3 ml/min. The subsequent recovery of intracellular pH (pHᵢ) was determined in the absence or presence of 10 µM compounds tested. The Na⁺/H⁺ exchange inhibitor EIPA (10 µM) served as a positive control.

Measurement of Intracellular Ca²⁺

Cells grown on glass coverslips for 2–3 days were loaded with fura 2 by incubation with 5 µM fura 2-acetoxyethyl ester, 0.1% (vol/vol) dimethylsulfoxide, and 0.025% (wt/vol) pluronic acid in low-buffering HCO₃⁻-free DMEM for 1 h at 37°C. Subsequently, the coverslips were mounted in the thermostated chamber placed on the stage of the inverted microscope and digital imaging system described above. Fura 2 fluorescence was measured at 350- and 380-nm excitation wavelengths with fluorescence emission measured at 510 nm. The fluorescent intensities at each excitation wavelength were collected for 1 s at a rate of 12 image pairs per minute. Background fluorescence of unloaded cells was subtracted. The corrected 350-nm-to-380-nm (350:380) intensity ratio was calibrated to [Ca²⁺]ᵢ, using the equation published by Grynkiewicz et al. (13). Maximum and minimum ratios and scale factor (Sₒ) were determined in vitro using fura 2 and a calibration kit. The dissociation constant was determined in vivo using the following protocol. Fura 2-loaded cells were washed three times with phosphate-buffered saline without Mg²⁺ and Ca²⁺ supplemented with 1 mM CaCl₂ and 2 mM EGTA. The free Ca²⁺ concentration was measured using the Ca²⁺ calibration kit and was determined to be 180 ± 20 nM. Subsequently, the cells were transferred to the fluorescence imaging system and the 350:380 ratio was determined after addition of 10 µM ionomycin or bromo-A23187. The dissociation constant of fura 2 in the cells was obtained from the 350:380 ratio measured under these conditions, with the assumption that [Ca²⁺]ᵢ equals extracellular Ca²⁺ concentration and from the minimum and maximum ratios and Sₒ of the in vitro calibration. Changes in [Ca²⁺]ᵢ were measured during superfusion with low-buffering HCO₃⁻-free DMEM at a flow rate of 1.6 ml/min and a constant temperature of 37°C in the perfusion chamber. Cells were exposed to neurotensin and anthocyanidin or anthocyannins by changing the perfusate from low-buffering HCO₃⁻-free medium gel electrophoresis lysis buffer [125 mM Tris, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 100 mM dithiothreitol, and 0.2% (wt/vol) bromphenol blue, pH 6.8]. The lysates were heated at 95°C for 5 min and were used for SDS-polyacrylamide gel electrophoresis or were frozen until use. Samples (25 µl) were subjected to gel electrophoresis on 12% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech Europe, Freiburg, Germany). Immunodetection of phosphorylated ERK1/2 and total ERK1/2 was with phospho-mitogen-activated protein kinase (MAPK; New England Biolabs, Schwabach, Germany) and polyclonal MAPK (New England Biolabs) antibodies, respectively, using the enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech Europe).

Cell Proliferation Assay

For the determination of cellular proliferation, cells were seeded into microtiter plates (24 wells) at a concentration of 6 × 10⁴ cells/well and incubated for 48 h with serum-free culture medium in the presence and absence of mitogenic factors and cyanidin, cyanin, or idaein. Living and metabolically active cells were determined via the reduction of MTT to form a blue-colored formazan. For this purpose, the assay was performed according to the manufacturer’s protocol. Cells were incubated with MTT for 2 h, and the formed formazan was measured using a microplate reader (SpectraFluor Plus, Tecan Deutschland, Crailsheim, Germany) set to read the difference between the absorption at 560- and 690-nm wavelengths.

Statistics

Values are means ± SD or SE. Data were analyzed using GraphPad Prism software (San Diego, CA). When two experimental groups were compared, an unpaired Student’s t-test was used. In the case of more than two experimental groups, analysis of variance followed by contrast analysis according to Tukey when overall effects were significantly different (P < 0.05), was used.

Results

Extracellular Acidification

Proton extrusion serves a number of functions, including a housekeeping function to preserve pHᵢ, and reflects metabolic activity of cells. It has also been proposed that proton excretion is involved in the regulation of cell growth induced by mitogens (7). Here, we show that neurotensin caused a concentration-dependent increase in the rate of extracellular acidification with a half-maximal activation at 1.1 ± 0.5 (SE) nM
The time course of the increase in extracellular acidification rate in HT29 clone 19A cells induced by 10 nM neurotensin is shown in Fig. 2A. The first exposure to 10 nM neurotensin induced a fast and pronounced increase in the rate of extracellular acidification of 212 ± 13% (mean ± SE, n = 6). The second addition of neurotensin (~30 min later) caused an increase of 185 ± 25% (mean ± SE, n = 7). EGF (1 nM) induced a slight increase in steady-state proton extrusion of 140 ± 2% (mean ± SE, n = 3) for ≥30 min (Fig. 2B). EIPA (10 µM) completely blocked proton secretion, indicating the involvement of Na+/H+ exchange in the increase in the extracellular acidification rate (data not shown).

Cyanidin at 10 µM significantly (P < 0.05) inhibited the extracellular acidification rate induced by neurotensin (114 ± 20%, mean ± SE) and EGF (107 ± 5%, mean ± SE; Fig. 2). In contrast, the cyanidin glycosides cyanin and idaein had no effect (data not shown). To elucidate whether cyanidin inhibits extracellular acidification rates as a result of direct blocking of proton transport across the cell membrane or reduction of cellular metabolism, the effect of cyanidin on the recovery of pH_i from the acid load by NH₃/NH₄⁺ was investigated. As shown in Fig. 3, cyanidin did not influence steady-

Figure 2. Attenuation of neurotensin- and epidermal growth factor (EGF)-induced changes in extracellular acidification rate of HT29 cells by cyanidin. A: cells were exposed to 10 nM neurotensin. Exposure to neurotensin was repeated in the presence of 10 µM cyanidin (NT; NT + cyanidin) or 0.2% (vol/vol) ethanol (NT; NT). Values are means ± SE of 6–7 experiments. DMEM, Dulbecco’s modified Eagle’s medium. B: cells were exposed to 1 nM EGF alone, EGF and 0.2% (vol/vol) ethanol (control), or EGF and 10 µM cyanidin. Values are means ± SE of 3 experiments.

Figure 3. Effects of cyanidin and ethylisopropylamiloride (EIPA) on steady-state intracellular pH (pH_i) in HT29 clone 19A cells and recovery after an intracellular acid load through NH₄Cl (30 mM) prepulsing. Cellular pH was measured in single cells using pH-sensitive fluorescent dye 2’7’-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Changes in fluorescence excitation intensity ratio (490-nm wavelength/440-nm wavelength) were measured in cells cultured for 2–3 days. For steady-state pH measurements, cells were exposed to vehicle [0.02% (vol/vol) ethanol], 10 µM cyanidin, or 10 µM EIPA in HCO₃⁻-free buffer. Effects on pH_i recovery from an acid load were studied on exposure of cells to 0.02% (vol/vol) ethanol, 10 µM cyanidin, or 10 µM EIPA after 5 min of exposure to 30 mM NH₄⁺/NH₃. Values are means ± SE of 3–5 experiments.
state pH, in HT29 clone 19A cells during superfusion with HCO₃⁻-free HEPES-buffered saline solution, whereas under the same conditions the inhibitor of Na⁺/H⁺ exchange, EIPA, caused a small decrease in pH. Cyanidin (10 µM) did not influence the recovery of pH, whereas EIPA completely blocked the pH recovery of cells from the acid load (Fig. 3). This shows that cyanidin does not directly alter cellular proton efflux but inhibits cellular metabolism induced by neurotensin and EGF.

**Intracellular Free Ca²⁺**

Intracellular Ca²⁺ plays an important role in the regulation of a number of cellular processes, including proton extrusion. Neurotensin induces a fast and profound increase in [Ca²⁺], (Fig. 4). The peak value of the intracellular Ca²⁺ release depends on the neurotensin concentration. The concentration of half-maximal increase in [Ca²⁺] for neurotensin was 5 ± 2 (SE) nM (n = 4). This value, as well as the time course, is similar to that observed for the increase in the rate of extracellular acidification caused by neurotensin, indicating that [Ca²⁺] is an important intermediate in the activation of proton extrusion.

The first exposure to 10 nM neurotensin caused an increase in [Ca²⁺], to ~1,000 nM. A subsequent superfusion of cells with cyanidin, but not its glycosides cyanin and idaein, caused a slow and small increase in [Ca²⁺]. The second addition of neurotensin resulted in an increase in [Ca²⁺], to 680 ± 45, 770 ± 95, and 419 ± 56 nM for the control cells and cells that had been superfused with cyanin or cyanidin, respectively (means ± SE of 7, 4, and 5 experiments, respectively). The second exposure of control cells to neurotensin caused a significantly lower increase in [Ca²⁺], than the first. Furthermore, the neurotensin-induced increase in [Ca²⁺], is significantly lower in cells preincubated with cyanidin than in control and cyanin experiments (Fig. 4). Thus cyanidin influences the [Ca²⁺], of HT29 clone 19A cells and reduces the neurotensin-induced increase in [Ca²⁺].

![Figure 4](image-url). Effects of cyanidin and cyanin on intracellular Ca²⁺ concentration ([Ca²⁺]) and neurotensin-induced [Ca²⁺], in HT29 clone 19A cells. Cellular Ca²⁺ was measured in single cells using Ca²⁺-sensitive dye fura 2. Cells were exposed to 10 nM neurotensin and 10 µM cyanidin or cyanin. Data were collected at a rate of 12 image pairs/min during exposure of cells to neurotensin and 1 image pair/min during exposure to vehicle or compounds tested. Ethanol (0.02%, vol/vol) was used as vehicle. Values are means ± SE of 3–7 experiments.
Phosphorylation of ERKs

The importance of ERK1 and ERK2 in mitogenesis and regulation of a number of cell physiological processes has been shown in many different systems. Neurotensin (Fig. 5A) and EGF (Fig. 5B) were able to increase the phosphorylation of ERKs by about three times.

Figure 5, A and B, shows that cyanidin, cyanin, and idaein at 10 µM had no effect on phosphorylation of ERKs induced by neurotensin or EGF and also did not affect phosphorylation of ERKs in the absence of mitogens (Fig. 5B).

Even at 100 µM, the compounds tested did not affect phosphorylation of ERKs (data not shown). In control experiments, we found that an inhibitor of the ERK kinase (MEK) PD-98059 abolished neurotensin- and EGF-induced ERK phosphorylation (Fig. 5C).

Proliferation of the Human Colon Carcinoma Cell Line HT29 Clone 19A

Intracellular signaling pathways investigated here are proposed to be important for cellular growth. Therefore, we...
tested whether suppression of increases in cellular metabolism and intracellular Ca^{2+} plays a role in the proliferation of colon carcinoma cells stimulated by EGF. Figure 6 shows that EGF stimulated cell growth compared with cells that have been cultured in serum-free medium. At 100 µM aglycone cyanidin, but not cyanin and idaein, strongly inhibited EGF-induced cell growth (Fig. 6). This is similar to the effects on intracellular signaling pathways tested here.

**Discussion**

**Molecular Mechanism of Action of Anthocyanidins/Anthocyanins**

Growth factors such as neurotensin and EGF bind to their cellular receptors and activate a number of intracellular signaling pathways. Interaction of neurotensin with its receptor, a G protein-coupled receptor, results in the activation of phospholipase C and its downstream signaling cascades, including an increase in [Ca^{2+}], that is known to mediate stimulation of cellular metabolism (glycolysis) in a number of cell types (14,15). The EGF receptor, a tyrosine kinase, mediates the intracellular signals via two robustly activated pathways: phospholipase C and the ras-raf-MEK-ERKs axis.

Here, we show that neurotensin causes an increase in [Ca^{2+}], and activates the cellular metabolism, which appears to be mediated by phospholipase C-dependent signaling pathways. Furthermore, neurotensin is also able to activate ERKs by an unknown mechanism. Cyanidin alters neurotensin-induced [Ca^{2+}] increase and cellular metabolism but has no effect on phosphorylation of ERKs. Similarly, cyanidin inhibits EGF-induced cellular metabolism and again has no effect on phosphorylation of ERKs. Thus cyanidin does not affect the direct interaction of neurotensin and EGF with their respective receptors but modulates intracellular signaling between receptors and [Ca^{2+}]. This conclusion is also in line with our observations that cyanidin alone (in the absence of neurotensin and EGF) neither alters cellular metabolism nor influences the pH, recovery of cells from the acid load, indicating that cyanidin does not directly inhibit enzymes involved in cellular metabolism or proton extrusion.

It appears that some higher concentration of cyanidin is necessary to inhibit cellular growth than to inhibit cellular metabolism induced by EGF. It could be because a number of different mechanisms are involved in EGF-induced cellular growth, e.g., activation of ERKs.

**Anticarcinogenic Significance**

Anthocyanidins and their glycosides are widespread natural food ingredients in berries such as black and red currants, elderberries, strawberries, and cranberries and in grapes and, thus, red wine. The inhibitory influence of these dietary components on cancer development is of interest. Most of the anthocyanidins present in plants are in the glycosidic form. Glucosidase, found in intestinal bacteria, could hydrolyze anthocyanins, yielding aglycones that, in contrast to their glycosides, are able to inhibit [Ca^{2+}], and cellular metabolism induced by neurotensin and EGF. Thus whether dietary anthocyanins affect cellular metabolism can depend on β-glucosidase activity of the intestinal flora. There is little information about metabolism of cyanidin and cyanidin glycosides in the literature. It has been shown that cyanidin glycosides are absorbed in their unchanged form, and neither glucuronates nor sulfates of anthocyanins were detected in the plasma or urine samples (16). Furthermore, it appears that anthocyanidins can act selectively in the intestinal tract because of their low bioavailability; <0.1% of anthocyanins was found to be absorbed in humans when an elderberry extract was ingested.

Figure 6. Effect of cyanidin, cyanin, and idaein on proliferation of colon carcinoma cell line HT29 clone 19A induced by EGF. Cell proliferation was induced by 1 nM EGF and determined on Day 2 (48 h) after incubation of cells with respective additives (10 or 100 µM). Control experiments were done in absence of EGF and tested compounds, and control is set equal to 100%. Values are means ± SD (n > 6).
(1.5 g anthocyanins) was consumed (17), and 2 h after a single oral administration of anthocyanins (100 mg/kg) to rats, ~92% of anthocyanins was found in the intestine (18). Thus, mainly the intestinal tract could be exposed to these compounds at relatively high concentrations. Fruits and vegetables such as blueberries, elderberries, strawberries, currants, grapes, and cherries are rich in anthocyanins. For example, black currants contain ~250 mg of cyanidin glycosides per 100 g of fresh fruit and sour cherries contain 35–82 mg/100 g (19). The concentration of cyanidin glycosides in an aronia juice was estimated to be ~600 µM (21 mg/100 ml; unpublished data).

Even when accounting for a physiological dilution of the drink after ingestion (e.g., by the intestinal contents or by water secreted by various gastrointestinal organs), the concentration of anthocyanidins/anthocyanins in the intestinal tract could easily be as high as 10–100 µM, which was the test concentration used in this study. Cyanidin at micromolar concentrations or complex fruit extracts containing equivalent amounts of anthocyanins and anthocyanidins were shown to be efficient antioxidants and to protect HT29 clone 19A cells against colon cancer promoted by mitogens.

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