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ARTICLE

Gut Metabolites of Anthocyanins, Gallic Acid, 3-*O*-Methylgallic Acid, and 2,4,6-Trihydroxybenzaldehyde, Inhibit Cell Proliferation of Caco-2 Cells

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Gut microflora metabolize anthocyanins to phenolic acids and aldehydes. These metabolites may explain the relationship between anthocyanin consumption and reduced incidence of colon cancer. Here, all six major metabolites, along with a Cabernet Sauvignon anthocyanin extract, were incubated with Caco-2 cells at concentrations of 0−1000 μM over 72 h to determine effects on cell proliferation and for 24 h to assess cytotoxicity effects and at 140 μM for 24 h to measure induction of apoptosis. These measurements were based on colorimetric methods. Gallic acid and 3-*O*-methylgallic acid inhibited cell proliferation and lacked cytotoxicity at low concentrations. The aldehyde metabolite and anthocyanin extract also inhibited cell proliferation at low concentrations and had low cytotoxicity at a wide range of concentrations. Of the four substances that effectively reduced cell proliferation, the aldehyde was the best inducer of apoptosis. In addition, these same four treatments degraded quickly in growth media, suggesting the involvement of subsequent oxidation products in the reduction of cell viability. These results indicate that the anthocyanin microfloral metabolites gallic acid, 3-*O*-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde reduce cell proliferation in Caco-2 cells more effectively than anthocyanins and may offer protection against colon cancer after their formation in the gut.

KEYWORDS: Apoptosis; phenolic acids; proliferation; colon cancer

INTRODUCTION

Evidence exists for an inverse relationship between moderate intake of wine and incidence of colon cancer (*1*, *2*). Anthocyanins are polyphenols that provide color in red wine. Anthocyanins can inhibit cell proliferation of colon cancer cells (*3*−*5*) and reduce tumor load in colon cancer animal models (*6*, *7*), thereby possessing chemotherapeutic properties. Consumption of these compounds could be the basis for the inverse relationship between wine and colon cancer. In vivo effects, however, may not be due directly to anthocyanins, because they are poorly absorbed into the bloodstream (*8*). Instead, anthocyanins are metabolized by gut microflora via glycosylation and ring fission of the C-ring to produce phenolic acids and aldehydes (*9*, *10*). Bacteria that reside in the large intestine are primarily responsible for these transformations (*11*), which could include *Bacteroides*, *Clostridium*, *Eubacterium*, *Ruminococcus*, and *Eggertheilla* genera (*12*).

One recently identified anthocyanin metabolite is 3-*O*-methylgallic acid, which is presumably the metabolite of petunidin-3-glucoside, but possibly also a demethylation product of malvidin-3-glucoside (*13*). When human subjects consume red wine, gallic acid, 4-*O*-methylgallic acid, and 3-*O*-methylgallic acid are detected in plasma (*14*), yet it is not clear how these compounds are formed. Also, gallic acid consumed from black tea can be methylated to form urine metabolites including 3-*O*-methylgallic acid (*15*). Absorption of these metabolites from the gut is likely different depending upon their chemical structures. Gallic acid is much less absorbed into the bloodstream of rats than *p*-coumaric acid (*16*), which could be due to a difference in relative polarity. It may also be due to gallic acid not being transported by the monocarboxylic acid transporter (MCT) in cells, but instead by paracellular diffusion (*17*).

Protocatechuic acid is another widely reported anthocyanin metabolite and is formed specifically from cyanidin-3-glucoside. This metabolite is abundant in the feces (*18*) and in the bloodstream when cyanidin-3-glucoside is fed to humans, making it more bioavailable than the parent compound (*18*, *19*). Protocatechuic acid has been investigated as a potential agent against colon cancer and has been able to both prevent and treat colon carcinogenesis in F344 rats (*20*), although the compound is generally more effective in reducing tumor incidence after disease initiation, especially at higher concentrations of protocatechuic acid. Other anthocyanin metabolites have been identified, which may contribute to the benefits associated with anthocyanin consumption. Syringic and vanillic acid can be well absorbed by Caco-2 cells (*21*), which would allow them to pass through the bloodstream barrier. A study involving the feeding of a *Trichilia emetica* extract, rich in phenolic acids, to rats found that the phenolic acids including protocatechuic acid, vanillic acid, and gallic acid, among others,

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were detected in plasma in unconjugated and glucuronidated forms (*22*).

Due to the extent of their formation and chemotherapeutic properties, we have chosen to investigate the effects of all known major anthocyanin metabolites on cell proliferation of colon cancer cells, to support the hypothesis that the metabolites are primarily responsible for the chemotherapeutic effects of anthocyanins. The effects of these anthocyanin metabolites on cell proliferation have not yet been compared. Cancer cell lines provide a systematic way to compare cell proliferation. The Caco-2 cell line was chosen for this study, not only for its use as a colon cancer model but also because it is commonly used for phenolic absorption experiments (*17*, *23*, *24*). This can be examined in the future on the basis of our results. Six anthocyanin metabolites were incubated with Caco-2 cells, to assess effects on cell proliferation, cytotoxicity, and apoptosis. Metabolites chosen for this experiment were gallic acid, 3-*O*-methylgallic acid, syringic acid, protocatechuic acid, vanillic acid, and 2,4,6-trihydroxybenzaldehyde (*10*, *13*); results from these treatments were compared with results from a Cabernet Sauvignon anthocyanin grape extract. The cytotoxicity and apoptosis assays provide basic information on how the compounds affect cell viability. If the compounds are able to inhibit cell proliferation but are highly cytotoxic, they would not be desirable treatment compounds. If they are found to induce apoptosis, then they would be candidates for further investigation as colon cancer treatments. A lack of cytotoxicity or ability to induce apoptosis would indicate that other mechanisms for reducing cell proliferation need to be considered.

MATERIALS AND METHODS

Chemicals. Syringic acid was purchased from Eastman Organic Chemicals (Kingsport, TN). Gallic acid, protocatechuic acid, and vanillic acid were purchased from Sigma (St. Louis, MO), and 2,4,6-trihydroxybenzaldehyde was purchased from Alfa Aesar (Ward Hill, MA). Cell culture grade dimethyl sulfoxide (DMSO) was purchased from ATCC (American Type Culture Collection, Manassas, VA). Trypsin/EDTA solution (0.25%) was produced by Gibco (Carlsbad, CA). Trypan blue and Coomassie Brilliant Blue G-250 were purchased from MP Biomedicals, LLC (Solon, OH). The anthocyanin extract and 3-*O*-methylgallic acid were prepared according to the method of Forester and Waterhouse (*13*). An extra step in the previously described anthocyanin extract preparation was taken, where the anthocyanin aqueous fraction was freeze-dried so that it could be weighed out on an analytical balance.

Cell Culture. Caco-2 cells were obtained from ATCC (passage 18). The cells were cultured in an incubator at 37 °C and 5% CO_2 in air (Sanyo Gallenkamp, Loughborough, Leicestershire, U.K.). The growth medium consisted of Minimum Essential Medium (MEM) and 20% fetal bovine serum, supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin antibiotics, all produced by Gibco. Cells were further passaged in T-75 cell culture flasks when they reached roughly 80% confluence. Trypsin/EDTA (0.25%) was used to detach cells, which were subcultured into five new T-75 flasks. Contamination was monitored by observing cells through an inverted phase contrast microscope. All solutions and conditions utilized for cell culture were sterile.

Treatment Incubation with Caco-2 Cells. Prior to addition of treatments, the cells were seeded in 96-well and 6-well cell culture plates for 24 h. Cells were added at a level of 10,000 cells per well (100 μL) in the 96-well plates and at 1,000,000 cells per well (10 mL) in the 6-well plates. Counting of the cells was performed by staining cells with trypan blue and using a hemacytometer to count under a normal phase microscope. After 24 h, the media were aspirated and replaced with treatment media. Each treatment was added in triplicate. Treatment blanks were included for the cytotoxicity assay, for each treatment and concentration variable. Anthocyanin metabolite treatments included 3-*O*-methylgallic acid, gallic acid, syringic acid, protocatechuic acid, vanillic acid, and 2,4,6-trihydroxybenzaldehyde. The Cabernet Sauvignon anthocyanin extract contained delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside (5.2, 6.9, 15.0, and 73.0%, respectively, expressed as equivalent moles of malvidin-3-glucoside). Structures for all treatments are found in **Figure 1**. Treatment concentrations were 10, 70, 140, 500, and 1000 μM for the cell proliferation and cytotoxicity assays. More concentrations were used for 3-*O*-methylgallic acid and gallic acid, which included 5, 10, 25, 50, 70, 75, 100, 140, 200, 300, 400, 500, and 1000 μM. The concentration of treatments for the apoptosis assay was 140 μM. All treatments were weighed on an analytical balance, added to freshly prepared media, and vortexed briefly. DMSO was added to solubilize syringic acid, protocatechuic acid, and 2,4,6-trihydroxybenzaldehyde, but never exceeded 0.1%. Controls were either media containing no treatment or media containing 0.1% DMSO. All treatment media was then sterile filtered with a 0.22 μm filter and subsequently diluted with sterile media to obtain a range of concentrations. For the cell proliferation assay, cells were incubated for 72 h, replacing with fresh treatment media each day. For the cytotoxicity and apoptosis assays, cells were incubated for 24 h with treatment media.

Cell Proliferation, Cytotoxicity, and Apoptosis Assays. Cell proliferation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) and measuring response at 490 nm. Briefly, this colorimetric assay involved the reduction of a tetrazolium compound to a formazan product by live cells. All treatment and control media were aspirated and replaced with fresh media just prior to performance of the assay. The standard curve ranging from 0 to 100,000 cells/well (100 μL) was generated by staining cells with trypan blue and counting them with a hemacytometer under a normal phase microscope. A serial dilution of the cell suspension was performed, and wells containing different concentrations of cells were measured with the assay kit.

Cytotoxicity was measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega), and response was measured at 490 nm. The LDH enzyme level was measured in the supernatant of each well after 24 h of incubation with treatments. All supernatant absorbances were corrected for background absorbances of the treatments. LDH enzyme level in the attached cells was measured by adding a lysis solution and placing the plates in a −80 °C freezer overnight. The plates were thawed the next day, and LDH level was measured immediately in the lysates. Due to high background readings of LDH after 30 min of incubation with the substrate mixture, the incubation time was changed to 15 min. Final cytotoxicity values were calculated as the ratios of absorbance in the supernatant to the total absorbance (supernatant plus attached cells) relative to controls.

Apoptosis was measured with the CaspACE Assay System, Colorimetric kit (Promega), and measuring a response at 405 nm. Cells were harvested and lysed after 24 h of incubation with treatments according to procedures described in the kit. Briefly, cells were washed in ice-cold phosphate-buffered saline (PBS). The cells were then scraped with a rubber policeman in 1 mL of ice-cold PBS. The cells were transferred to 1.5 mL microfuge tubes and centrifuged at 450*g* and 4 °C for 10 min (Eppendorf Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany). The cells were then lysed by freeze−thawing in a cell lysis buffer. Lysates were then centrifuged at 4500*g* and 4 °C for 20 min. The supernatants were used for the apoptosis assay. All absorbance measurements were performed in triplicate with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA).

Apoptosis values were normalized for protein content of each repetition lysate supernatant (including inhibition readings). Protein determinations were done according to the Bradford assay (*25*), measuring absorbance at 595 nm. Protein readings were taken on an Agilent 8453 (Santa Clara, CA) spectrophotometer, and concentrations were calculated on the basis of a bovine albumin standard curve.

Persistence of Treatment Compounds in Media. Treatment compounds were added at final concentrations of 1000 μM in complete growth media. The treated medium was then added in 100 μL aliquots to 96-well tissue culture plates and placed in a CO_2 incubator. Concentrations of compounds were then measured at times 0, 0.5, 1, 3, 6, 12, and 24 h by high-performance liquid chromatography. Three wells were analyzed for each treatment at a given time point. The anthocyanins and metabolite compounds were quantified as previously described (*13*).

Statistical Analysis. PRISM statistical analysis software package version 5 (GraphPad, La Jolla, CA) was used to generate IC_{50} values and dose response curves. Treatment concentrations were logarithmically

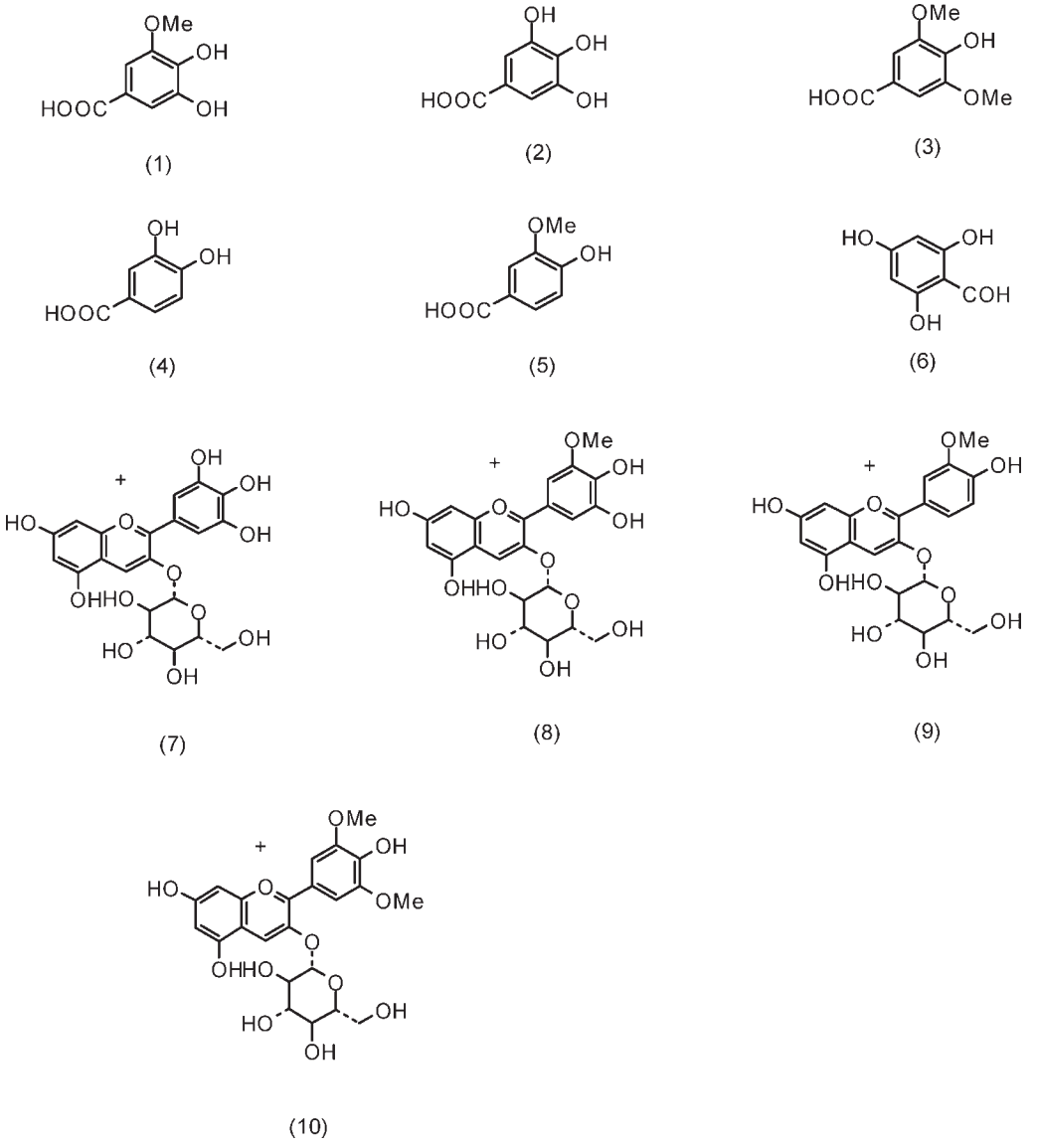

Figure 1. Treatment structures: (1) 3-*O*-methylgallic acid; (2) gallic acid; (3) syringic acid; (4) protocatechuic acid; (5) vanillic acid; (6) 2,4,6-trihydroxybenzaldehyde; (7) delphinidin-3-glucoside; (8) petunidin-3-glucoside; (9) peonidin-3-glucoside; (10) malvidin-3-glucoside.

transformed, and a nonlinear regression (curve fit) analysis was performed with log(inhibitor) versus response, variable slope fit. The fitting method used a least-squares fit. IC_{50} values are reported with 95% confidence intervals. All other values are reported as means ± SEM ($n = 3$). Persistence, cytotoxicity, and apoptosis graphs were prepared in Microsoft (Redmond, WA) Excel 2003 software. Two-tailed Student's t tests were performed on apoptosis data. Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Persistence of Treatment Compounds in Media. The recovery of treatment compounds over 24 h of incubation was determined in this study. We decided to quantify the persistence of treatment compounds in media without cells to check their stability under our experimental conditions. The percent recoveries of the compounds at various time points are given in **Figure 2**. Gallic acid, 3-*O*-methylgallic acid, 2,4,6-trihydroxybenzaldehyde, and the anthocyanins were all subject to heavy loss over time. Gallic acid had the lowest persistence, with only 3.9% ± 0.1 detected after 0.5 h and none detected after 1 h. Protocatechuic acid displayed some loss over time (65.2% ± 1.0 after 24 h), whereas syringic acid and vanillic acid did not. It is assumed that any loss of treatment compounds was due to oxidation.

Cell Proliferation, Cytotoxicity, and Apoptosis Assays. Dose response curves for each treatment can be found in **Figure 3**. Gallic acid and 3-*O*-methylgallic acid inhibited Caco-2 cell proliferation at low concentrations, with IC_{50} values of 68.7 and 24.1 μM, respectively. Cytotoxicity values were low at these concentrations (**Figure 4A**). The aldehyde and anthocyanin extract also inhibited proliferation, with IC_{50} values of 76.7 and 204.9 μM, respectively, and have low cytotoxicity at even wider concentration ranges (**Figure 4B**). The aldehyde had the lowest cytotoxicity of these four treatments, which never exceeded 19.8%. This compound is cytotoxic toward other cancer cell lines (*26*), emphasizing the importance in studying this compound in a range of cell models before testing in animals or humans.

The three other metabolite treatments did not appear to inhibit cell proliferation below 50%. Vanillic acid actually increased cell proliferation at the lowest concentration. There is no explanation for this observation, yet it is clear that vanillic acid has a protective effect in Caco-2 cells at low concentrations. Protocatechuic and syringic acid behaved similarly, and there appears to be a slight protective effect or simulation in cell proliferation at the highest treatment concentration (1000 μM). Syringic acid has no apparent cytotoxicity toward Caco-2 cells. Protocatechuic acid is

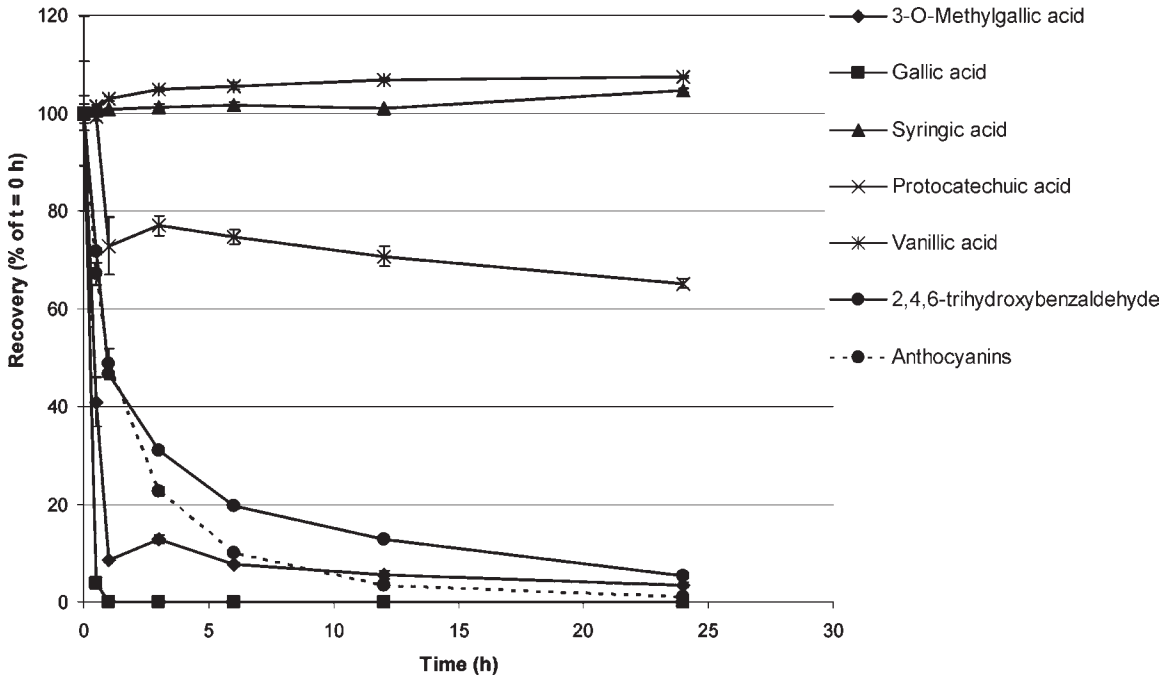

Figure 2. Persistence of treatment compounds in complete media. All treatments were added at a final concentration of 1000 μM. Gallic acid could not be detected after 1 h. Values are means ± SEM, $n = 3$ ($n = 2$ for syringic acid at $t = 0$, 12, and 24 h; and for anthocyanins at $t = 24$ h).

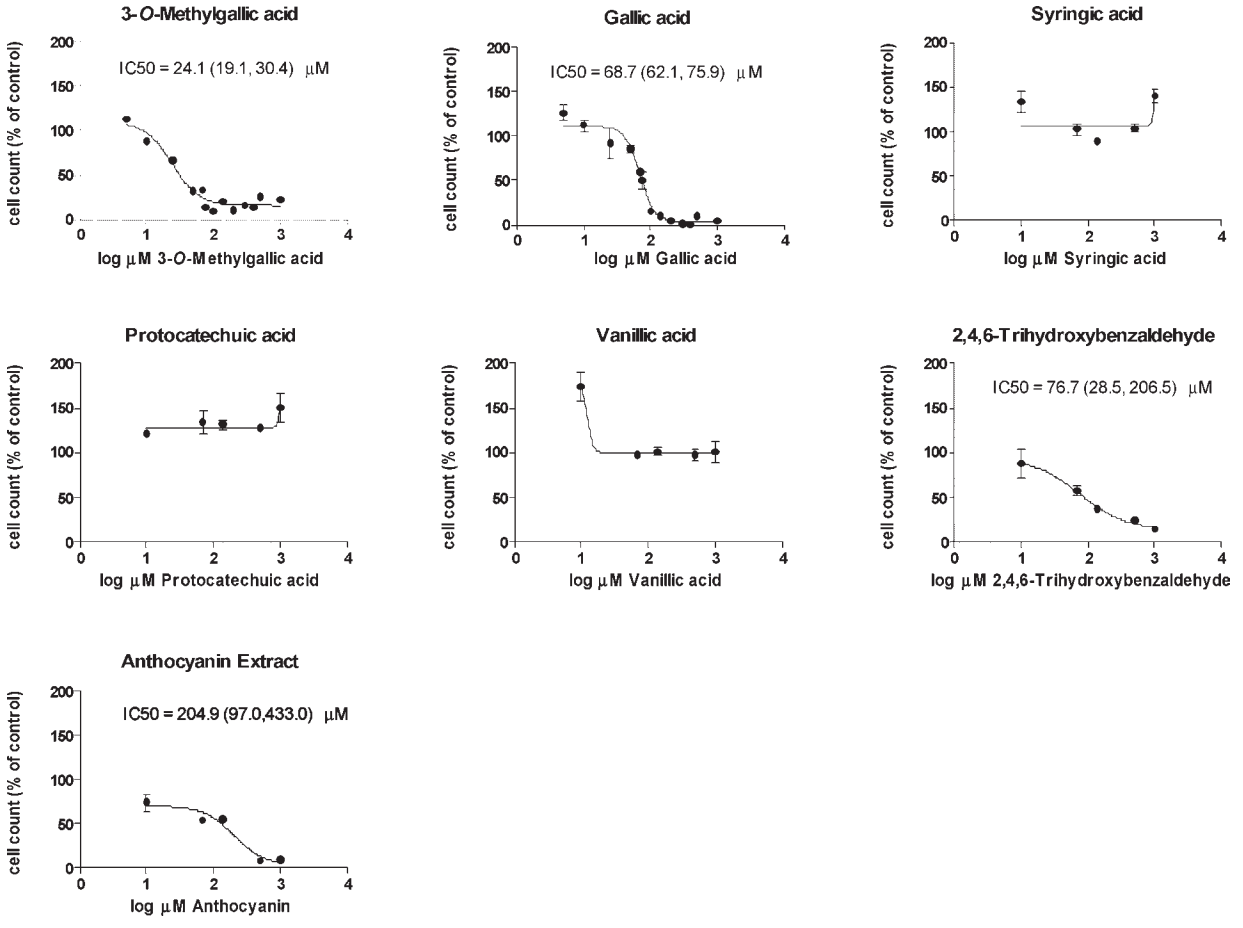

Figure 3. Cell proliferation curves of Caco-2 cells after 72 h of incubation with various treatments. Values on curves are means ± SEM, $n = 3$ ($n = 2$ for vanillic acid at 10 μM). IC_{50} values are shown as averages with 95% confidence intervals in parentheses. Syringic acid, protocatechuic acid, and vanillic acid did not inhibit cell proliferation below 50% within the treatment concentrations used in this experiment.

also not cytotoxic, except at a concentration of 500 μM, at which it is 47.5% cytotoxic. Concentration appears to be important regarding the cell proliferation effects of these antioxidant compounds and needs to be closely considered in future experiments.

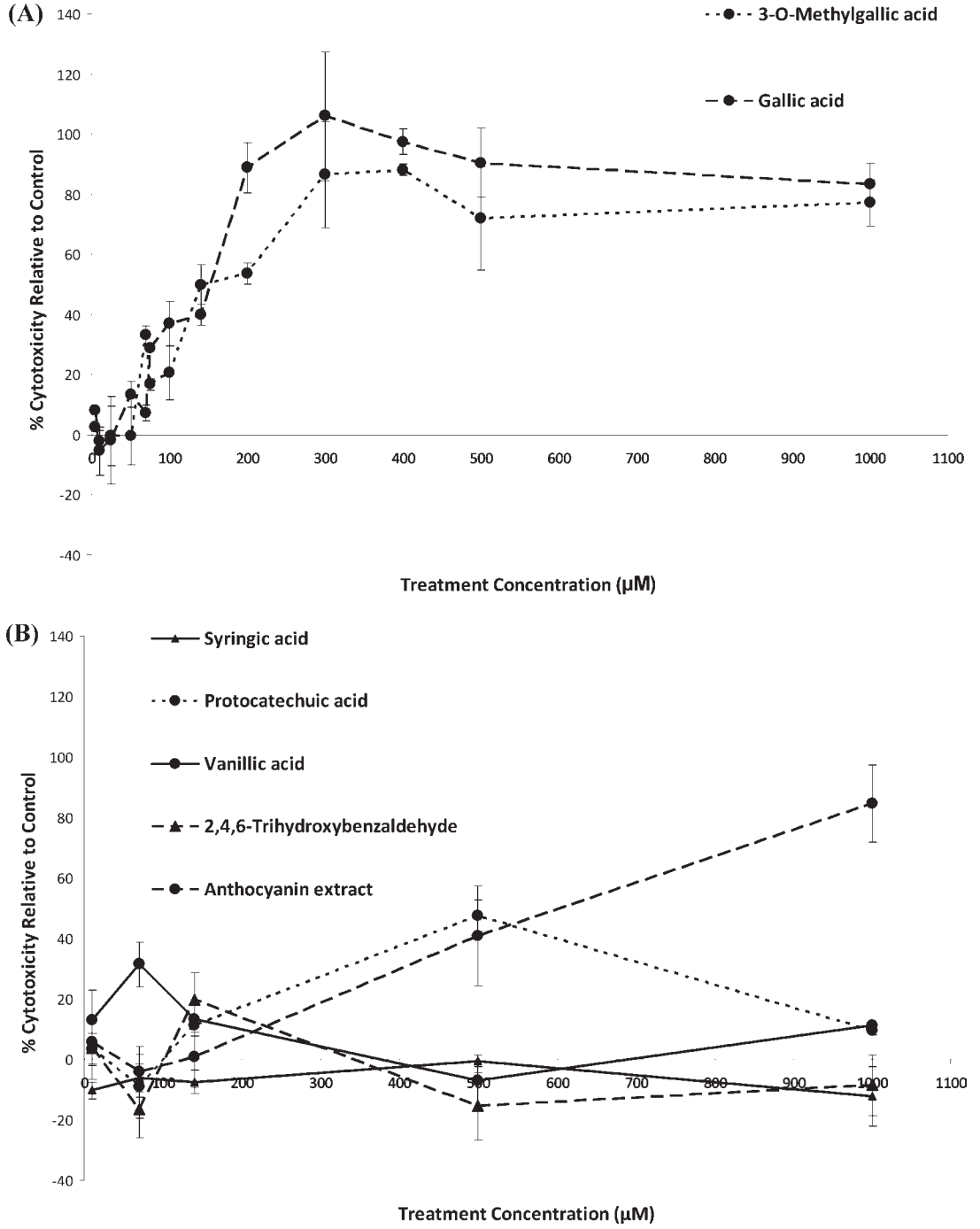

Figure 4. Cytotoxicity of treatments after 24 h of incubation with Caco-2 cells: (**A**) 3-*O*-methylgallic acid and gallic acid cytotoxicity; (**B**) syringic acid, protocatechuic acid, vanillic acid, 2,4,6-trihydroxybenzaldehyde, and anthocyanin extract cytotoxicity. Cytotoxocity values are means ± SEM, $n = 3$.

The concentration of treatments for the apoptosis assay was 140 μM, which was chosen on the basis of the range of IC_{50} values for the most effective compounds. An increase of caspase-3 specific activity in cells treated with gallic acid, 3-*O*-methylgallic acid, 2,4,6-trihydroxybenzaldehyde, and the anthocyanin extract was therefore expected at this concentration. This is also a realistic phenolic acid concentration found in human fecal water samples (*27*). Of the four most effective treatments in inhibiting cell proliferation (gallic acid, 3-*O*-methylgallic acid, 2,4,6-trihydroxybenzaldehyde, and the anthocyanin extract), the aldehyde appears to be the best inducer of apoptosis (**Figure 5**). It induced a caspase-3 specific activity of 6.2 ± 0.4 pmol of p-nitroaniline (pNA) liberated/h/μg of protein. Because 2,4,6-trihydroxybenzaldehyde had low cytotoxicity over the concentration range tested, its ability to induce apoptosis in Caco-2 cells may be responsible for the dose response effect seen in **Figure 2**. Gallic acid and 3-*O*-methylgallic acid induced caspase-3 specific activities of 1.9 ± 0.1 and 1.7 ± 0.2 pmol of pNA liberated/h/μg of protein, respectively, making them the poorest inducers of caspase-3 activity. The anthocyanin extract was a better inducer of apoptosis with a caspase-3 specific activity of 4.0 ± 0.6 pmol of pNA liberated/h/μg of protein (**Figure 5**). The other anthocyanin metabolites, including syringic acid, vanillic acid, and protocatechuic acid, had similar effects on induction of apoptosis as compared with 2,4,6-trihydroxybenzaldehyde (5.9 ± 0.4, 5.7 ± 0.5, and 5.8 ± 0.7 pmol of pNA liberated/h/μg of protein, respectively).

It is apparent that the anthocyanin gut metabolites gallic acid, 3-*O*-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde can inhibit

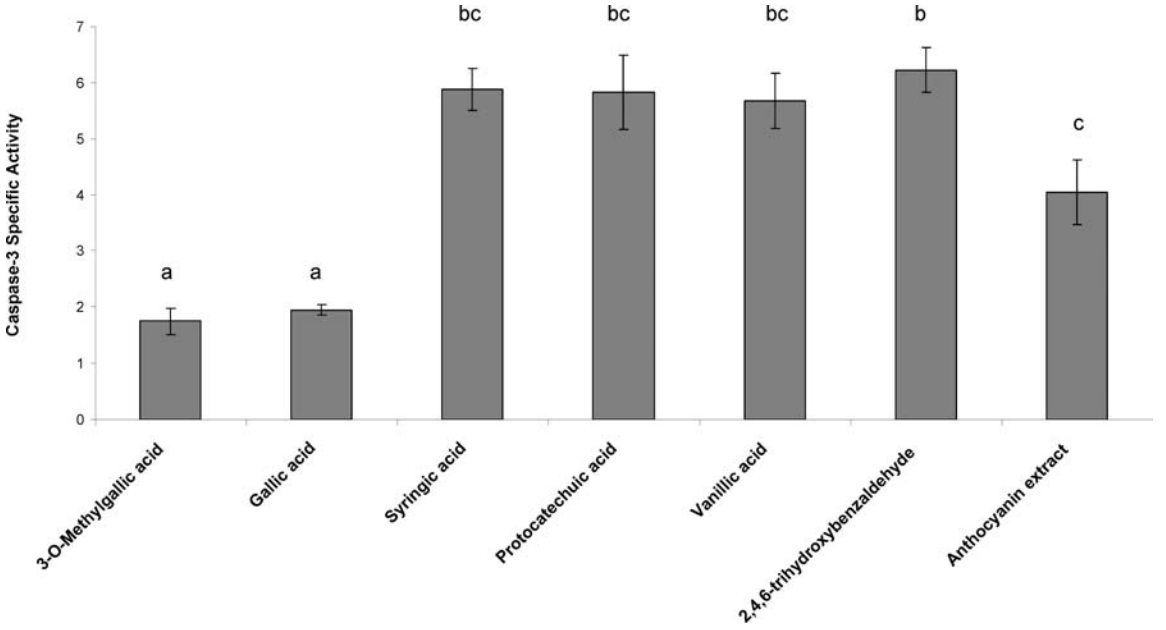

Figure 5. Induction of apoptosis in Caco-2 cells after 24 h of incubation with treatments. Compounds were added to growth media at a final concentration of 140 μM. Caspase-3 specific activity is expressed as picomoles of pNA liberated per hour per microgram of protein. Control samples were used to calculate caspase-3 activity for each treatment. Values are means $\pm$ SEM, $n = 3$. Treatments with a common letter designation are not significantly different from each other but are significantly different from treatments with no letter in common, $P < 0.05$.

in vitro proliferation of Caco-2 cells. These compounds dramatically reduced cell viability without having any cytotoxic effects at low concentrations. This result does not reveal a mechanism, but these results are an indication that these compounds could inhibit growth of colon cancer cells in vivo. It is notable that these compounds were more effective than the anthocyanin extract, supporting the hypothesis that metabolites are responsible for the chemotherapeutic properties of anthocyanin consumption.

Prior work has shown that gallic acid reduces cell viability of colon cancer cells (*28*−*31*) including Caco-2 cells (*32*), and it affects proliferation in Caco-2 cells by altering the percentage of cells in each cell cycle phase (*32*). Gallic acid can also induce apoptosis in some cancer cells, including Colo 205 cells (*33*), and not in normal cells (*29*); yet gallic acid and 3-*O*-methylgallic acid do not appear to be strong inducers of apoptosis in Caco-2 cells compared with the other treatments tested in our experiment. Protocatechuic acid was previously reported to have little effect on cell proliferation and apoptosis in colo320 cells (*34*). This agrees with our cell proliferation assay data, whereas our caspase-based assay showed a strong effect. These compounds need to be examined further before conclusions can be made regarding apoptosis.

Although future studies will be necessary to probe the mechanisms of the effects on cell viability, the high concentration toxicity of gallic acid has been linked to the generation of hydrogen peroxide (*31*). Whereas phenolic compounds are good radical scavengers (*35*), they can also act as pro-oxidants, being a critical hydrogen donor in converting O_2 to hydrogen peroxide, which subsequently yields the damaging hydroxyl radical. Thus, their effect may depend upon induction of reactive oxygen species (ROS). This could also be true of 3-*O*-methylgallic acid and the anthocyanin extract, because their cytotoxic profiles are similar to that of gallic acid.

The substances that had the greatest effect in decreasing cell viability (3-*O*-methyl gallic acid, gallic acid, 2,4,6-trihydroxybenzaldehyde, and the anthocyanin extract) were, surprisingly, also those found to have the lowest persistence in the growth media. This suggests that a reduction in cell population may be associated with oxidative reactions caused by treatment compounds, resulting in a cytotoxic effect by hydrogen peroxide production (and subsequent hydroxyl radical formation). The instability of gallic acid in growth media has been shown and linked to the production of hydrogen peroxide. The production of hydrogen peroxide by gallic acid did not completely explain the reduction of cell viability in HT29 cells (*28*, *30*), yet it did have some effect. Lee et al. performed a similar study involving Caco-2 cells, in which the effect of hydrogen peroxide generation on cell viability was substantial (*31*). Similar experiments need to be conducted with 3-*O*-methylgallic acid and 2,4,6-trihydroxybenzaldehyde, so that the impact of our persistence results on Caco-2 cell viability can be better understood.

The connection between substances with a short lifetime and reduced cell viability suggests that the generation of ROS, at low concentrations, could be desirable when treating colon cancer, because Caco-2 cells will undergo apoptosis if exposed to peroxyl radicals (*36*). It is also possible that oxidative products, which need to be identified, could be inhibiting proliferation.

It has been shown that gallic acid is quickly absorbed by Caco-2 cells and disappears after 24 h (*32*). The disappearance may be due either to cell metabolism or oxidation of gallic acid. It is possible that our cells were absorbing/metabolizing gallic acid, 3-*O*-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde, causing further depletion in the media. It is hoped that future investigations will directly address the connection between treatment persistence, fate of the phenolics, and effects on cell viability while monitoring the absorption/metabolism of active compounds by Caco-2 cells. Caco-2 cells are able to perform glucuronidation, sulfation, and methylation reactions (*37*), which provides a starting point for targeted indentification of cell metabolites.

Although it is useful to test the effects of compounds on cancer cells in vitro, factors such as inflammation, pH, oxidative status of the gut, and diet in a colon cancer patient could alter the overall effect on cancer cells. Unlike in vitro results, the effect of these compounds on colon cancer cells in vivo may not be fully dependent on the presence of ROS, as oxidative reactions would behave differently in gut physiological conditions. As it is important to

measure the generation of hydrogen peroxide in in vitro experiments, it would be necessary to test the formation of hydrogen peroxide in vivo. This is now possible by using chemiluminescent nanoparticles that can be imaged in the body for hydrogen peroxide detection (*38*).

This is the first study to show that the anthocyanin metabolites 3-*O*-methylgallic acid and 2,4,6-trihydroxybenzaldehyde could be responsible for the chemotherapeutic effects of anthocyanins in the gut. The mechanism by which these compounds exhibit their effect is becoming clear, and future studies will address the production of ROS as well as monitor cell cycle and apoptosis regulating proteins such as CDKs, PARP, Bcl-2, caspase-9, and NF-κB. The poor persistence of the active compounds suggests that their oxidative degradation pathways in media could be linked to their activity. Thus, in vitro oxidative and cell metabolism products should be identified in the future and tested in vivo. These results suggest that future human studies should address the bioavailability of the other anthocyanin (and other flavonoid) gut metabolites, including levels in the colon, to establish whether such metabolites could play a part in altering disease development.

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