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Anthocyanin Interactions with DNA: Intercalation, Topoisomerase I Inhibition and Oxidative Reactions

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Abstract

Anthocyanins and their aglycone anthocyanidins are pigmented flavonoids found in significant amounts in many commonly consumed foods. They exhibit a complex chemistry in aqueous solution, which makes it difficult to study their chemistry under physiological conditions. Here we used a gel electrophoresis assay employing supercoiled DNA plasmid to examine the ability of these compounds (1) to intercalate DNA, (2) to inhibit human topoisomerase I through both inhibition of plasmid relaxation activity (catalytic inhibition) and stabilization of the cleavable DNA-topoisomerase complex (poisoning), and (3) to inhibit or enhance oxidative single-strand DNA nicking. We found no evidence of DNA intercalation by anthocyan(id)ins in the physiological pH range for any of the compounds used in this study—cyanidin chloride, cyanidin 3-O-glucoside, cyanidin 3,5-O-diglucoside, malvidin 3-O-glucoside and luteolinidin chloride. The anthocyanins inhibited topoisomerase relaxation activity only at high concentrations (> 50 μ M) and we could find no evidence of topoisomerase I cleavable complex stabilization by these compounds. However, we observed that all of the anthocyan(id)ins used in this study were capable of inducing significant oxidative DNA strand cleavage (nicking) in the presence of 1 mM DTT (dithiothreitol), while the free radical scavenger, DMSO, at concentrations typically used in similar studies, completely inhibited DNA nicking. Finally, we propose a mechanism to explain the anthocyan(id)in induced oxidative DNA cleavage observed under our experimental conditions.

Keywords

Anthocyanin; topoisomerase I; oxidation; DNA; intercalation; strand-cleavage; DNA nicking

INTRODUCTION

The blue and red colors of most fruits and vegetables are mainly attributable to the pigmented flavonoids—the glycoside anthocyanins and their aglycon anthocyanidins. These compounds (primarily the anthocyanins) may be highly concentrated in plants commonly consumed as foods and human consumption may reach significant levels (USDA, 2006; Wu et al. 2006). A long list of positive therapeutic effects have been attributed to the anthocyan(id)ins (Wang et al. 1997). Fruits and vegetables have also been shown through epidemiological studies to be associated with a number of beneficial health effects, including cancer prevention (Kamei et al. 1995), and it has been speculated that there may

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Practical Applications: This study provides improved understanding of the mechanisms by which anthocyan(id)ins interact with DNA. By characterizing the chemistry and solution properties of these important dietary components, we obtain improved information on how the anthocyan(id)ins might function in living systems.

be a direct relationship between anthocyanin content of fruits and vegetables and their health effects (Katsube et al. 2003; Edenharder et al. 1997). But there have been relatively few studies of the biological effects of anthocyan(id)ins at the biochemical or molecular level. Anthocyan(id)ins have been shown to alter cell cycling, to promote apoptosis, and stimulate cell differentiation, for example, but how these processes are triggered is not clear (Kamei et al. 1995; Koide et al. 1997; Gasiorowski et al. 2001; Katsube et al. 2003). As with other flavonoids, the potent antioxidant properties of anthocyanins as free radical scavengers and metal chelators are often assumed to play a role in their bioactivity (Satué-Gracia et al. 1997; Wang et al. 1997; Russo et al. 2000; Ramirez-Turtosa et al. 2001; Suda et al. 2002; Acquaviva et al. 2003). However, some studies have demonstrated that flavonoids can act as pro-oxidants (Galati et al. 2002) and there may be other mechanisms, such as inhibition of enzymes or receptor interference, by which anthocyan(id)ins elicit their biological effects (Choi et al. 2004; Quiney et al. 2004; Williams et al. 2004). These apparently contradictory results may be due to the fact that many *in vitro* assays have not taken into account solution conditions occurring *in vivo* even though subtle changes in pH, ionic strength, etc. can have significant effects on solution activity of flavonoids and their concomitant biological activity (Webb and Ebeler, 2004; Borkowski et al. 2005).

In plant cells, anthocyan(id)ins are stabilized in the generally red flavylium form by a number of mechanisms that include concentration to near anhydrous conditions, sequestration in acidic vacuoles and substitution with complex, often acylated, sugar moieties (Haslam, 1998). However when dissolved in aqueous solution, anthocyan(id)ins are quite labile and undergo complex chemical transformations depending upon pH (Figure 1), temperature, and the presence of metals and oxygen (Brouillard and Dubois, 1977; Mistry et al. 1991). When diluted in low pH solutions (< pH 2) the flavylium form predominates and is stable (Figure 1). As the pH increases, however, attack by water at the 2 position carbon results in the reversible formation of the colorless hemiacetal (carbinol base) form which then reversibly converts to chalcone forms through ring opening. Further increase in pH above ~pH 4 leads to the reversible conversion of the flavylium form to the blue quinoidal (anhydro base) forms through oxidation of the A or B ring hydroxyl groups. These hydroxyls may also deprotonate at pH above ~7 to anionic forms. The quinoidal and chalcone forms degrade to poorly defined products at rates dependent on solution conditions. Thus, anthocyan(id)ins in aqueous solution will be in equilibrium with several possible forms dependent upon the pH of the solution.

Natural anthocyan(id)ins consumed in the diet can be absorbed and found intact in the blood (Tsuda et al. 2000; Matsumoto et al. 2001) however, little is known of the chemical interactions that occur *in vivo* between anthocyan(id)ins and other biological molecules. At physiological pH, the anthocyan(id)ins are expected to be predominately converted to the carbinol base form and, to a lesser extent, the quinoidal forms will occur, though the equilibria determining these transformations may be shifted in a number of ways (Mazza and Brouillard, 1987). For example, intramolecular self-association of complex anthocyanins, or (at relatively high concentrations) association with self and/or other flavonoids or aromatic ring containing compounds can significantly shift equilibrium concentrations and stabilize the various forms under conditions that would otherwise result in transformation (Brouillard et al. 1991; Mistry et al. 1991; Houbiers et al. 1998). This process, referred to as copigmentation when heterogeneous compounds are involved, appears to involve pi-pi stacking arrangements between the aromatic rings of the associating compounds (Boulton, 2001). A similar process apparently occurs when the flavylium form of some anthocyanins is stabilized by intercalation into DNA and RNA under mildly acidic conditions (Mistry et al. 1991). The binding of anthocyanins to other biological macromolecules such as proteins has not been extensively studied and it is possible that

selective binding of specific anthocyanin forms could provide another means by which those forms are stabilized in aqueous solutions.

In this study, the interactions of several anthocyanidins and anthocyanins (Fig. 2) with plasmid DNA at physiological pH are investigated. Using a topoisomerase inhibition and DNA intercalation assay developed in this laboratory, we will evaluate the ability of these structurally simple and common anthocyan(id)ins to intercalate DNA, to inhibit the human topoisomerase IB enzyme, and to enhance or inhibit DNA oxidation reactions (measured as DNA strand cleavage) (Webb and Ebeler, 2003). The apparent ability of anthocyan(id)ins to intercalate DNA has not been carefully evaluated in the physiological pH range. Intercalation of the DNA molecule by small molecular weight compounds such as these has implications for the proper functioning of a number of DNA maintenance enzymes.

Topoisomerase IB (topo I) and topoisomerase II (topo II) are DNA-maintenance enzymes which regulate the supercoiling of chromosomal DNA and play pivotal roles in chromosome replication, transcription, recombination, segregation, condensation and repair (Wang, 2002). These enzymes facilitate the relaxation of supercoiled DNA, essentially through a mechanism involving the breakage of a phosphodiester bond of either one strand (topo I) or both strands (topo II) of the duplex DNA. The ability of exogenous chemicals to inhibit the topo I and II enzymes may play a role in the induction of apoptosis, inhibition of cell proliferation, and perturbations of cell cycling, possibly by trapping ordinarily transient enzyme-DNA intermediates (cleavable complex stabilization) long enough to block DNA processing by other DNA protein complexes. This stalling of the DNA-topoisomerase intermediate by the anthocyan(id)ins, referred to here as topoisomerase poisoning, is distinguished from 'conventional' inhibition of catalytic activity which occurs as a result of binding of the inhibitor to the active site or alteration of the binding behavior of the enzyme with its substrate. These interactions, particularly the DNA oxidation reactions, will be evaluated and more closely characterized at the chemical level with the goal of understanding how solution conditions may influence anthocyan(id)in reactions *in vivo*.

MATERIALS AND METHODS

Reagents

Supercoiled pGEM plasmid DNA was obtained from Promega Corp., Madison, WI and human type IB topoisomerase (topo I) was obtained from TopoGEN, Columbus, OH. The topo I inhibitor, camptothecin, and catalase (bovine liver) were purchased from Sigma Chemical Co., St. Louis, MO. Dnase-free bovine serum albumin (BSA) was purchased from Amersham Pharmacia Biotech, Inc., Piscataway, NJ, and proteinase K derived from *Tritirachium album* was obtained from USB Corp., Cleveland, OH. The anthocyan(id)ins, cyanidin chloride; cyanidin 3-O-glucoside; cyanidin 3,5-O-diglucoside; and malvidin 3-O-glucoside (all as the pyranoside), were obtained from Polyphenols Laboratories AS, Sandnes, Norway, and luteolinidin chloride was purchased from Extrasynthese, Genay, France.

Relaxation buffer consisted of 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and BSA at 0.3 mg/mL, unless otherwise noted. Loading (solubilizing) buffer consisted of 2% sodium dodecyl sulfate (SDS), 14% Ficoll 400, and 0.1% bromophenol blue. Gel electrophoresis buffer (TBE; Tris/Boric Acid/EDTA) was prepared from Tris (89 mM) pH 8.3, boric acid (89 mM), EDTA (2 mM), and SDS at 0.1%.

Topoisomerase inhibition and DNA intercalation assay

The intercalation and topoisomerase inhibition assay used here has been described elsewhere in detail (Webb and Ebeler, 2003; Webb and Ebeler, 2004). Briefly, a solution of supercoiled

pGEM DNA was prepared by dilution into relaxation buffer to give a concentration of 4.4 – 6 ng/ μ L. Fully relaxed plasmid was prepared from an aliquot of this solution by the addition of the appropriate amount of topo I calculated to give full relaxation when incubated at 37°C for 2 hr. Test compound solutions, control compounds, or blank solutions were added (2 μ L) to 17 μ L of either the relaxed or super-coiled plasmid dilution (75 – 100 ng DNA total). All reactions were carried out in Dnase-free 0.5 mL microcentrifuge tubes. Samples were then incubated, for 10 – 20 minutes at room temperature (23 – 25°C), after which 1 μ L of topo I solution (at 10 times the concentration necessary to give full plasmid relaxation) was added and the samples were immediately incubated in the dark at 37°C for 1 hr with gentle shaking. The reaction was stopped by the addition of 2.5 μ L of a 10% SDS solution followed by 2.5 μ L of proteinase K at 1 mg/mL. Protein digestion was then carried out in total darkness for 1 hr at 45°C after which 6.5 μ L of electrophoresis solubilizing buffer was added. The samples were immediately analyzed by gel electrophoresis as described below.

Test compounds were prepared by dissolving in 0.02 M HCl solution with mild sonication and were used immediately or frozen at –70°C. Prior to use, test and control compounds were diluted to appropriate concentration (~5 – 300 μ M) by addition of 0.02 M HCl solution or 0.02 M HCl solution containing 5 – 20% DMSO. A stock solution of 8 mM camptothecin in DMSO was diluted, immediately prior to use, in 33% DMSO/water.

As part of the assay, an additional dilution series of test compounds was included for determination of inhibition of catalytic relaxation activity. These samples were prepared using the above procedure with the following modifications. Samples containing supercoiled plasmid only (75–100 ng) were treated as above with a dilution series of the test compound but the amount of topo I added was calculated to be 1–2 times the amount of enzyme necessary to fully relax the plasmid (instead of the ten-fold amount used above). From this point the samples were treated exactly as described above.

Samples dissolved in electrophoresis buffer were analyzed by gel electrophoresis using 1% agarose gels containing 0.1% SDS. Electrophoresis was carried out using TBE buffer in a horizontal electrophoresis apparatus (Biorad Sub-cell GT) for, typically, 10 hours at 1.25 V/cm. The gel was then stained in 1 μ g/mL ethidium bromide/TBE solution for 1 hour. Following staining, the gel was reinserted in the electrophoresis apparatus and again electrophoresed for 45 – 60 min at 3 V/cm either in TBE buffer or TBE buffer containing ethidium bromide at 1 μ g/mL. This step ensures a clear separation of nicked, covalently-closed relaxed, partially relaxed to fully supercoiled topoisomers and linearized plasmid bands (Webb and Ebeler, 2003). The gel was then visualized under UV illumination using a ChemiImager 4000 Low light Imaging System (Alpha Innotech Corp, Transilluminator Model TM-26) and photographed. Band fluorescence intensities (reported in arbitrary units) were measured by scanning the sample bands using AlphEase software (v. 3.3d). In no case was DNA detectable in gel sample wells following electrophoresis.

Assay for oxidative nicking of DNA plasmid

In order to evaluate oxidative DNA damage, DNA plasmid nicking (single-strand breakage or scission) was assumed to result solely from oxidative cleavage of a DNA strand and was measured in the absence of topoisomerase using the topoisomerase inhibition and DNA intercalation assay which had been modified as follows. Incubations were carried out as described above using only the supercoiled form of the pGEM plasmid without the addition of topoisomerase. Samples were incubated at 37°C as described but were then prepared for electrophoresis by the addition of solubilizing buffer (no proteinase K digestion was performed). Samples were electrophoresed as described above except that the second electrophoresis step was omitted. The degree of oxidative DNA damage was measured as the amount of nicked plasmid formed relative to the control (untreated) plasmid. All

incubations were carried out in the dark using atmosphere equilibrated solutions. No significant amounts of linearized plasmid were formed in any of the experiments.

Assay reproducibility and statistical analysis

All analyses were replicated a minimum of two times on separate gels and identical trends were observed for all replications. The average relative standard deviation for replicate measurements of nicked band intensity is less than 25%. Care was taken to ensure reproducible sample loading as demonstrated previously (Webb and Ebeler, 2003), however comparison of absolute pixel intensity among gels and between days remains difficult due to inhomogeneity of the UV light source and inconsistencies in the staining process. Means, standard deviations, linear regression, and Student's t-test statistics were calculated on replicate measurements using Excel (Microsoft, Redman, WA).

RESULTS AND DISCUSSION

Intercalation

There was no evidence of DNA intercalation by any of the compounds used in this study (cyanidin chloride, cyanidin 3-O-glucoside, cyanidin 3,5-O-diglucoside, malvidin 3-O-glucoside and luteolinidin chloride) at concentrations up to at least 125 μM (Figure 3; data shown for malvidin 3-O-glucoside only). In this assay, evidence of intercalation would be indicated by the appearance of partially super-coiled bands (topoisomers) extending from the relaxed band toward the fully super-coiled form. The degree of intercalation would be indicated by the degree of topoisomer formation. Lane 3 (Figure 3) shows this effect for the DNA intercalator ethidium bromide at 0.13 $\mu\text{g/mL}$.

It has been reported by others that anthocyanidins are capable of binding DNA and RNA through intercalation between the stacked DNA/RNA bases (Mistry et al. 1991; Sarma and Sharma, 1999; Habermeyer et al., 2005). However, these previous studies have generally examined binding at pH conditions below approximately pH 4 where the anthocyanidins would be expected to be primarily in the carbinol form and in equilibrium with a small pool of the flavylum form (Figure 1). It is most likely the flavylum form that intercalates DNA due to its planar configuration and cationic nature, and this form probably accounts for the binding observed in these previous studies. Our experiments were performed at pH 7.5 where the concentration of the flavylum form would be extremely low, if present at all, and the equilibrium would be shifted toward the quinone forms which are also unlikely to intercalate DNA. We also examined anthocyanidin-DNA interactions spectrophotometrically at pH 7.5 but were unable to demonstrate significant or consistent shifts in λ_{max} or maximum absorbance that would indicate interaction between the two at this pH as has been observed for other flavonoids (Webb and Ebeler, 2004).

Inhibition of catalytic activity

The anthocyan(id)ins inhibited the catalytic relaxation activity of topoisomerase only when present at relatively high concentrations. For cyanidin, inhibition was observed at concentrations above 50 μM while the other compounds tested were only inhibitory at concentrations greater than 125 μM (Figure 4; gels shown for cyanidin and cyanidin-3,5-O-diglucoside only). In this assay topoisomerase relaxation inhibition is indicated by the loss of the ability to convert the supercoiled plasmid substrate to the relaxed form in the presence of the test compound. In Fig. 4, for example, it can be seen that cyanidin chloride at a concentration of 63 nM prevents conversion of the supercoiled plasmid to the relaxed form (lane 4) while cyanidin-3,5-O-diglucoside even at 125 nM has no effect (lane 8). It is unclear if the slightly greater potency of cyanidin is due to the presence of the 3-OH group; further study is needed to evaluate this structure activity relationship.

Cleavable complex stabilization (poisoning)

Under normal assay conditions (no DMSO present), plasmid nicking is associated with topoisomerase poisoning due to cleavage of one of the bonds of the phosphodiester backbone (Webb and Ebeler, 2004) (*e.g.*, see nicked bands in Figure 4). However, in this study we observed that all of the anthocyan(id)ins were able to generate DNA strand breaks (nicking) independent of topoisomerase. For example, digitized nicked band intensities for the test compound cyanidin chloride, shown in Figure 5, indicate that even when no topo I was present, the addition of cyanidin at concentrations greater than 31 μM (Figure 5, samples 10–12) significantly increased (*t*-test, $p < 0.03$) DNA strand breakage compared to the control (Figure 5, sample 3). Similarly cyanidin 3,5-diglucoside, cyanidin 3-O glucoside, and malvidin 3-O-glucoside at concentrations of 31 μM (and in the absence of topo I) also significantly increased nicked band intensities by more than 15%, compared to the control. Therefore, using the assay under these conditions (no DMSO present), it is difficult to distinguish between oxidative DNA strand breaks induced directly by the anthocyan(id)ins and DNA nicking which occurs as a result of anthocyan(id)in poisoning. In fact, the presence of topo I appears to slightly protect against anthocyan(id)in-induced DNA nicking at least at low anthocyan(id)in concentrations but only at the 10X level of topo I, although the effects are not statistically significant (Fig. 5, compare sample 6 with 10 and sample 8 with 11). At the lower levels of topoisomerase used in the catalytic inhibition portion of the assay (1X), the presence of topo I slightly enhanced nicking (Fig. 5, compare samples 10–12 with samples 14–16). Since we observed that the anthocyan(id)ins were only inhibitory at relatively high concentrations (greater than 50 μM for cyanidin and greater than 125 μM for all others, as discussed above), it is not likely that inhibition of the enzyme activity by the anthocyan(id)ins plays a role in any of the effects seen here, although non-specific interactions between the anthocyan(id)ins and the enzyme cannot be completely excluded. It may be that binding of topo I to the plasmid provides protection against strand breaking agents so that at high concentrations of topo I, less nicking can occur. This would not explain, however, the slightly enhanced nicking (relative to topo I independent nicking) seen in the presence of low levels of topoisomerase. Also, if stabilization of the topo I cleavable complex by anthocyan(id)ins is contributing to the strand scission seen here it should be greater in the presence of higher levels of topo I. Finally, no evidence of poisoning was observed when the free radical scavenger, DMSO (at concentrations less than 3% (v/v)) was included in the poisoning assay in order to minimize confounding effects due to oxidative strand nicking (data not shown). For these reasons we do not believe that anthocyan(id)ins are capable of stabilizing the topo I cleavable complex in the absence of DMSO.

Oxidative DNA strand nicking

Based on the above results indicating that the anthocyan(id)ins were able to generate DNA strand breaks (nicking) independent of topoisomerase, we therefore investigated the nature of the anthocyan(id)in induced (topoisomerase independent) nicking in more detail. In this experiment, conditions were simplified by using only supercoiled plasmid (in the absence of DMSO) and by excluding topo I and the final proteinase K incubation so that plasmid nicking could be more easily attributed to oxidative damage alone. It can be seen (Fig. 6) that DNA nicking increases linearly with cyanidin 3-O-glucoside concentration up to about 150 μM ($r^2 = 0.9797$ from 0 – 150 μM). At 300 μM the supercoiled plasmid is at least 33% converted to nicked form (calculated as change in fluorescence intensity of the supercoiled form in the presence and absence of the test compound) after 1 hr at 37°C (with no linearized band detectable). In the absence of DMSO, similar increases in DNA nicking with increasing anthocyanin concentration were obtained for all compounds studied.

In contrast, no evidence of oxidative DNA nicking was observed when 2.0% (v/v) DMSO was added to up to 230 μM of the test compounds cyanidin, cyanidin 3,5-O-diglucoside, and

malvidin 3,5-O-diglucoside (data not shown). In addition, when DMSO was present, these compounds did not induce DNA nicking even when incubations as long as 8 hr at room temperature were used. The ability of DMSO (at 0.5 – 2.0%) to attenuate anthocyanidin induced nicking could be attributed to DMSO acting as a free radical scavenger. At these concentrations, DMSO should be able to block hydroxyl radical attack to a sufficient degree to provide the protection observed here. A similar mechanism has been proposed for DNA strand scission by resveratrol acting with Cu^{2+} (Ahmad et al. 2000). Alternatively, reactive forms of the anthocyanin (quinones or quinone methides, for example) may be able to react directly with DNA generating a strand nick through uncharacterized mechanisms.

To evaluate further whether the observed nicking may be due to reactive oxygen species, incubations were carried out in the presence of various combinations of catalase (65 $\mu\text{g/mL}$) and iron (100 μM FeCl_3) using cyanidin 3-O-glucoside as a test compound (Fig. 7). Cyanidin 3-O-glucoside-induced nicking of plasmid DNA was suppressed by the presence of catalase (sample 2 compared to sample 4; $p < 0.05$, t-test, $n = 4$). On the other hand, Fe^{3+} increased cyanidin 3-O-glucoside induced nicking (sample 2 compared to sample 6; $p < 0.03$, t-test, $n = 4$). We hypothesize that quinones formed from anthocyan(id)ins at pH 7.5 autoxidize to produce hydrogen peroxide (Singleton, 1987). Fe^{3+} will then increase anthocyanin induced cleavage since, when reduced, Fe^{3+} can stimulate the production of hydroxyl radicals from hydrogen peroxide. It is likely that hydroxyl radical is the actual active cleavage agent because DNA is relatively resistant to direct damage by hydrogen peroxide (Sakano et al. 2004). Since catalase actively degrades hydrogen peroxide, the ability of catalase to attenuate anthocyan(id)in induced DNA nicking further suggests this DNA damage is mediated through production of hydrogen peroxide.

In this scenario then, strand cleavage by anthocyan(id)ins could be catalyzed by the presence of trace amounts of contaminating transition metals such as iron which generate hydroxyl radicals from hydrogen peroxide that then cleave a DNA strand through any of several known mechanisms. This process would be catalyzed by oxidized transition metals such as Fe^{3+} only if they can be efficiently reduced by a reductant. Sufficient amounts of reductant would ensure the redox cycling necessary to generate reactive oxygen species in the amounts needed to produce the level of DNA damage demonstrated here. In our system the reductant is likely to be DTT (1 mM) or possibly a form of anthocyan(id)in maintained by the presence of DTT.

The importance of DTT for generating DNA damage in this system was therefore tested by repeating the experiment shown in Fig. 7 in the absence of DTT (Fig. 8). In these experiments, Fe^{3+} was not able to catalyze the anthocyan(id)in induced DNA nicking reaction (lanes 5 and 6 vs. lanes 1 and 2). DTT is a strong reductant and is used experimentally not only for its ability to maintain protein sulfhydryl groups in their reduced forms but for its ability to neutralize reactive oxygen species. However, in the presence of iron and oxygen at critical concentrations, DTT can also act as an oxidant, in effect generating reactive oxygen species (Netto and Stadtman, 1996). In addition, it has been shown that flavonoids are capable of acting as oxidants in the presence of glutathione (GSH) through $\text{GS}^{\bullet}$ radical formation and subsequent generation of superoxide; it is possible that DTT acts in a similar manner in the presence of flavonoids (Galati et al. 2002).

Anthocyan(id)in structure-activity relationships

The fact that all the anthocyan(id)ins studied here were capable of causing DNA strand nicking, despite having few common structural features (Figure 2), is noteworthy. While we did not attempt to quantify the degree of strand nicking caused by the anthocyan(id)ins, they were all potent inducers of nicking in the presence of DTT when no DMSO was present. It seems unlikely then that the B-ring catechol functional groups found in some of these

compounds is primarily responsible for their DNA damaging activity since malvidin is still capable of initiating strand cleavage even though its B-ring 3' and 5' hydroxy groups are blocked by methylation. This would also seem to exclude an important role for metal ion chelation in the strand nicking reaction since most, but not all, models of flavonoid-metal ion chelation involve chelation through the B-ring vicinal hydroxyl groups. The strand nicking reaction also appears to be fully functional in the absence of a 3 carbon hydroxyl (luteolinidin) or a blocked 3 and 5 carbon hydroxyl (cyanidin 3,5-O-diglucoside). While it is still possible for the A and B rings to be stabilized through charge delocalization when the hydroxyl groups at the 3' and 5 carbon positions are deprotonated, there appears to be no simple model to explain how these structures could generate ROS. However, an analogous compound, hydroxylated melatonin, has recently been shown to be capable of inducing DNA oxidation in the presence of Cu^{2+} (Sakano et al. 2004). In this system, Cu^{2+} reduction in the presence of oxygen leads to superoxide and hydrogen peroxide formation and a redox cycle involving a quinone methide form of the hydroxylated melatonin ensures that significant amount of ROS are formed. It is not unreasonable to suggest then that a similar process may be occurring with anthocyan(id)ins that accounts for their prooxidant activity in the system studied here.

Significance of these findings to biological studies

While anthocyan(id)ins have been reported to be potent antioxidants by many investigators, we have shown here that under *in vitro* conditions which mimic physiological pH and redox conditions, these compounds may in fact act as prooxidants inducing DNA strand breakage. Clearly more needs to be known about the dynamics of anthocyan(id)in transformations under various solution conditions before definitive statements about their actual antioxidant properties can be made. Studies have shown that some anthocyan(id)ins are potent inhibitors of cell growth and can induce apoptosis, cell cycle perturbations, and differentiation in a variety of cell models (Kamei et al. 1995; Koide et al. 1997; Gasiorowski et al. 2001; Katsube et al. 2003). Others have reported that anthocyan(id)ins are capable of inducing DNA and chromosomal damage in simple cell systems *ex vivo* (Pool-Zobel et al. 1999; Popp and Schimmer, 1991; Habermeyer et al., 2005). To ascribe all of these biological effects to the antioxidant properties of anthocyan(id)ins seems implausible. Theoretically, at least, it may be easier to ascribe most or all of these biological effects to the consequences of DNA damage through oxidation or to cellular responses to DNA damage and interference with DNA maintenance enzymes.

A model has been proposed here for how anthocyan(id)ins might function as pro-oxidants under certain conditions (*i.e.*, physiological pH, the presence of relatively high concentrations of sulfhydryl-containing or other reductants (*e.g.*, glutathione, ascorbic acid, etc.), trace amounts of transition metals, and the absence of relatively high concentrations of free radical scavenging solvent molecules). Other studies can be found in the literature in which experiments have been conducted using conditions similar to those described but it is not clear that the importance of these conditions for determining experimental outcomes is fully appreciated. There also seems to be little standardization of experimental conditions in the literature so that drawing conclusions from the results of different studies is often problematic. With regard to the anthocyan(id)ins specifically, a better understanding of the factors controlling the transformation of these compounds in solution is needed before we can propose mechanisms for how they might function in living systems. Besides those already mentioned here, there may be other factors (for example, non-intercalative DNA or protein binding of anthocyaninins) involved in anthocyan(id)in (bio)chemical effects that are not known at this time. Since the human diet contains anthocyan(id)ins (sometimes in significant amounts; Wu et al. 2006) it is important that we characterize the chemistry and solution properties of these compounds more fully. These properties can then be used to

design additional studies that will explore the mechanisms responsible for the *in vivo* biological effects elicited by anthocyan(id)ins.

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Figure 1. Effect of pH on structure and color of anthocyanins. Example shown for malvidin-3-O-glucoside.

Compound	Substitution Pattern					
	3	5	7	3'	4'	5'
Luteolinidin	H	OH	OH	OH	OH	H
Cyanidin	OH	OH	OH	OH	OH	H
Cyanidin 3-O-glucoside	Glucose	OH	OH	OH	OH	H
Cyanidin 3,5-O-diglucoside	Glucose	Glucose	OH	OH	OH	H
Malvidin 3-O-glucoside	Glucose	OH	OH	OCH ₃	OH	OCH ₃

Figure 2.
Structure of the anthocyanins and anthocyanidins used in this study.

Figure 3.

Malvidin-3-O-glucoside in the presence of DNA and topoisomerase I does not show significant intercalation. Lane 1, supercoiled pGEM plasmid (6.7 ng/μl) control; lane 2, supercoiled pGEM in the presence of 10X topoisomerase; lane 3, pGEM plasmid + topoisomerase (10X) + ethidium bromide (0.13 μg/ml) showing distribution of topoisomers; lanes 4–8, pGEM plasmid + topoisomerase (10X) + malvidin-3-O-glucoside at 6, 12, 30, 60 and 120 μM, respectively.

Figure 4.

Topoisomerase inhibition by cyanidin chloride and cyanidin 3,5-O-diglucoside. Lane 1, 60 ng supercoiled pGEM DNA; lane 2, 60 ng relaxed pGEM DNA; lane 3, 60 ng supercoiled pGEM DNA + 1X Topo I; lane 4, 60 ng pGEM DNA + 1X Topo I + 63 μ M cyanidin chloride; lane 5, 60 ng pGEM DNA + 1X Topo I + 125 μ M cyanidin chloride; lane 6, 60 ng supercoiled pGEM DNA + 1X Topo I; Lane 7, 60 ng supercoiled pGEM DNA + 1X Topo I + 63 μ M cyanidin 3,5-O-diglucoside; lane 8, 60 ng pGEM DNA + 1X Topo I + 125 μ M cyanidin 3,5-O-diglucoside. Reaction conditions are as described in methods section.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
pGEM	rel	rel	SC	SC	rel	rel	rel	rel	rel	SC	SC	SC	SC	SC	SC	SC
Topo I	-	10	-	10	10	10	10	10	10	-	-	-	1X	1X	1X	1X
Cyanidin	-	-	-	-	-	31	63	125	310	31	125	310	-	31	125	310
Campt	-	-	-	-	10	-	-	-	-	-	-	-	-	-	-	-

Figure 5.

Topoisomerase I dependent and independent plasmid nicking by cyanidin chloride. Relaxed (rel) or supercoiled (SC) pGEM plasmid (6.25 ng/μL) in relaxation buffer containing 1 mM DTT was incubated in the dark with the indicated concentration (μM) of cyanidin chloride for 10 min at room temperature and then for 1 hour at 37°C with either no topo I, a 10X concentration of topo I, or a 1X concentration of topo I. The incubation was stopped by the addition of SDS and proteinase K, incubated a second time and then run by gel electrophoresis as described in the text. The topo I poisoning compound camptothecin (campt) was also included (10μM) in the incubations as both a reference and a control. Values represent averaged duplicate samples; experiments replicated on separate days show similar trends.

Figure 6.

The effect of cyanidin 3-O-glucoside on pGEM plasmid nicking. Supercoiled pGEM plasmid (6.67 ng/ μ L) in reaction buffer containing 1 mM DTT was incubated with increasing concentrations of cyanidin 3-O-glucoside for 1 hour at 37°C in the dark, electrophoresis buffer was added and the samples were run by gel electrophoresis as described in the text. Nicked band fluorescence intensity was measured after ethidium bromide staining and is shown plotted in arbitrary units; experiments replicated on separate days show similar trends.

Figure 7.

The effect of transition metals and catalase on cyanidin 3-O-glucoside induced plasmid nicking in the presence of DTT. Supercoiled pGEM plasmid (6.67ng/μL) in reaction buffer containing 1 mM DTT was incubated for 1.5 h at 37°C in the dark with either blank solution, 100 μM cyanidin 3-O-glucoside, 100 μM FeCl₃, and catalase at 67 μg/ml or various combinations of these. DMSO was not present. Samples were run by gel electrophoresis immediately after addition of electrophoresis buffer and nicked band fluorescence intensity was measured after ethidium bromide staining. Values represent averaged duplicate samples; experiments replicated on separate days show similar trends.

Cyan 3-gluc	-	+	-	+	-	+
Catalase	-	-	+	+	-	-
Fe (III)	-	-	-	-	+	+

Figure 8.

The effect of transition metals and catalase on cyanidin 3-O-glucoside induced plasmid nicking in the absence of DTT. Supercoiled pGEM plasmid (6.67ng/μL) in reaction buffer containing 1 mM DTT was incubated for 1 h at 37°C in the dark with either blank solution, 100 μM cyanidin 3-O-glucoside, 100 μM FeCl₃, and catalase at 67 μg/ml or various combinations of these. DMSO was not present. Samples were run by gel electrophoresis immediately after addition of electrophoresis buffer and nicked band fluorescence intensity was measured after ethidium bromide staining. Values represent averaged duplicate samples; experiments replicated on separate days show similar trends. Absolute values for nicking cannot be directly compared to those in Figure 7 due to variability in staining between gels.