Anthocyanins suppress the cleavable complex formation by irinotecan and diminish its DNA-strand-breaking activity in the colon of Wistar rats

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In the present study, the question was addressed whether anthocyanins interfere with the topoisomerase I poison irinotecan in vivo. In vivo complexes of enzyme to DNA bioassay was used to detect irinotecan-induced stabilization of topoisomerase I/DNA complexes and single cell gel electrophoresis to determine DNA-strand-break induction in the colon of male Wistar rats. Furthermore, analysis of anthocyanin concentrations in rat plasma and rat colon was included in the testing, demonstrating that anthocyanins reach the colon and the concentrations do not differ between rats that only received anthocyanins and the anthocyanin/irinotecan group. Blackberry extract was found to significantly reduce irinotecan-mediated topoisomerase I/ DNA cleavable complex formation. Overall, anthocyanins did not notably increase cleavable complex formation. However, a significant increase of DNA damage was shown after a single dose of irinotecan as well as the single compounds cyanidin (cy) and cyanidin-3-glucoside (cy-3-g). Furthermore, a significant reduction of irinotecan-induced DNA-strand breaks after a pretreatment with cy, cy-3-g and blackberry extract was observed. Thus, the question arises whether anthocyanin-rich preparations might interfere with chemotherapy or whether, due to low systemic bioavailability, the preparations might provide protective potential in the gastrointestinal tract.

Introduction

There is strong evidence that diets rich in plant-based foods are associated with health benefits, particularly with respect to the cardiovascular system, cancer or type II diabetes mellitus (1–5). Anthocyanins represent a class of colored plant constituents occurring in many fruits and vegetables of our daily diet. They are prominent in berries, reach a concentration of irinotecan-induced stabilization of topoisomerase I/DNA complexes and single cell gel electrophoresis to determine DNA-strand-break induction in the colon of male Wistar rats. Furthermore, analysis of anthocyanin concentrations in rat plasma and rat colon was included in the testing, demonstrating that anthocyanins reach the colon and the concentrations do not differ between rats that only received anthocyanins and the anthocyanin/irinotecan group. Blackberry extract was found to significantly reduce irinotecan-mediated topoisomerase I/DNA cleavable complex formation. Overall, anthocyanins did not notably increase cleavable complex formation. However, a significant increase of DNA damage was shown after a single dose of irinotecan as well as the single compounds cyanidin (cy) and cyanidin-3-glucoside (cy-3-g). Furthermore, a significant reduction of irinotecan-induced DNA-strand breaks after a pretreatment with cy, cy-3-g and blackberry extract was observed. Thus, the question arises whether anthocyanin-rich preparations might interfere with chemotherapy or whether, due to low systemic bioavailability, the preparations might provide protective potential in the gastrointestinal tract.

Introduction

There is strong evidence that diets rich in plant-based foods are associated with health benefits, particularly with respect to the cardiovascular system, cancer or type II diabetes mellitus (1–5). Anthocyanins represent a class of colored plant constituents occurring in many fruits and vegetables of our daily diet. They are prominent in berries, reaching concentrations in excess of 10 g/kg in some cultivars. Intake of anthocyanins may exceed 200 mg/day (6, 7).

Berry anthocyanins have been reported to modify cancer biomarkers in vitro, e.g. reducing DNA damage or inhibiting tumor cell growth (8–14). Several overlapping mechanisms of action have been reviewed in detail, including the inhibition of receptor tyrosine kinases, induction of apoptosis and the interference with human topoisomerase (14–22). Progress has been made in understanding the mechanism of berry anthocyanins as well as the free aglycons, the so-called anthocyanins, as topoisomerase inhibitors in vitro. Delphinidin, cyanidin (cy) and several anthocyanin-rich extracts have been characterized as catalytic topoisomerase inhibitors in human colon carcinoma cells.

Abbreviations: bw, body weight; cy, cyanidin; cy-3-g, cyanidin-3-glucoside; HPLC, high-performance liquid chromatography; i.p., intraperitoneal, TFA, trifluoroacetic acid.

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used here is grossly comparable. Calculating the human equivalent dose using allometric factors [conversion based on body surface area (36)], 100 mg/kg bw cy in rats correspond to 16 mg/kg bw in humans, which can easily be consumed with the diet. For example, a human weighing 70 kg has to drink 720 ml of an anthocyanin-rich fruit juice (1.5 g/l) to consume the dose equivalent to that in humans.

All animals (mean experimental bw 363.86 ± 31.97 g) underwent the following experimental schedule: after a 14 h fasting period, the animal received the respective control or monosubstance or the extract by intra-gastric gavage.

Two hours after oral gavage, the animals received a single i.p. injection of either the irinotecan hydrochloride or sodium chloride and 4 h after i.p. injection, animals were killed by decapitation. Biological samples (plasma and colon) were obtained and processed as detailed below for the respective methods.

Analytical methods

Sample clean-up plasma. Before extraction, the internal standards cy (1 nmol) and pelargonidin (0.5 nmol) were added to the samples. Plasma samples were loaded onto a preconditioned (2 ml of methanol and 2 ml of water containing 10% formic acid) Strata-X cartridges (30 mg/ml, Phenomenex, Aschaffenburg, Germany). The anthocyanins/anthocyanidins were eluted after washing (1 ml of water) with 2 ml of methanol containing 10% formic acid, and then 25 µl trifluoroacetic acid (TFA) was added to the eluate. The extract was evaporated under a stream of nitrogen gas before the remaining aqueous residue was loaded on a preconditioned (4 ml of methanol as well as 4 ml of bidistilled water containing 10% formic acid) Strata-X cartridge (100 mg/6 ml, Phenomenex). The cartridge was rinsed with 1 ml of bidistilled water and the anthocyanins were eluted with 2 ml of methanol containing 10% formic acid. Furthermore, 25 µl TFA was added to the eluate. After evaporating the extract to dryness, the residue was dissolved in 100 µl aqueous TFA (5%, v/v) and an aliquot was subjected to HPLC analysis.

HPLC analysis. Quantification of anthocyanins was performed by HPLC using a high-pressure gradient system from Shimadzu (Duisburg, Germany) equipped with an autoinjector and a photodiode array detector. Separation was carried out on a Luna C18 (150 × 4.6 mm, particle size 3 µm) reversed phase column (Phenomenex). Solvent A consisted of 0.1% formic acid in water (pH 3) and solvent B of acetonitrile. The following gradient was used: from 100% solvent A in 5 min, from 90 to 91% solvent A in 5 min, from 83 to 65% solvent A in 10 min and from 65 to 0% solvent A in 5 min. The flow rate was set to 1 ml/min and the eluent was recorded at 520 nm for the anthocyanins. Observed peaks were scanned between 200 and 600 nm. Quantification of anthocyanins was performed by external calibration. Calibration curves for cy, cy-3-g, peonidin and peonidin-3-glucoside were constructed in the range of 0.1–100 µM, in which the linearity of the response was given. The limit of detection has been estimated between 4 and 6 pmol on column based on the lowest level of the calibration curve. Quantification of those anthocyanins that are not commercially available was based on the calibration of cy-3-g (groups cy-3-g and blackberry extract) and cy (groups cyanidin), respectively. The recovery ranged between 54 and 148% obtained after spiking the tissue samples with the corresponding standards.

Identification of blackberry anthocyanins and anthocyanin metabolites in the rat colon was carried out by HPLC/MS/mass spectrometry analysis. This analysis was performed on a JJP 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, a binary HPLC pump, an autoinjector and a column oven. The HPLC system was directly coupled to a hybrid triple quadruple linear ion trap mass spectrometer (3200 QTrap; Applied Biosystems, Darmstadt, Germany), equipped with a TurboSpray source. For HPLC, the same conditions as described above were used. The analytes were detected in the positive ion mode at a vaporizer temperature of 650°C and an ion spray voltage of 5.5 kV. Nitrogen was used for both, curtain gas and collision gas, at 10 psi and medium, respectively. The collision energy and declustering potential were set to 10 V. Data acquisition was performed in a full scan mode (enhanced mass spectrometry mode) and in multiple reaction monitoring mode by monitoring the transition of parent and product ions specific for each compound using a dwell time of 50 ms.

In vivo complexes of enzyme to DNA bioassay

Immediately after decapitation of the animals (n = 9 per group), the distal part of the colon was rapidly removed and transferred into a 50 ml tube filled with phosphate-buffered saline. The organs were immediately cut into small pieces with a scalpel and homogenized thoroughly in 10 ml N-laurylsarcosine-containing (1%, w/v) TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid]. The highly viscous DNA-containing lysates were sheared with an 18-gauge needle and stored at −80°C. The in vivo complexes of enzyme to DNA bioassay followed a recently published protocol adapted to in vivo derived samples (35). Cell lysates were layered onto a cesium chloride gradient in polyallomer tubes (14 ml, SW40, Beckman Coulter GmbH, Krefeld, Germany). Cesium chloride solutions with densities of 1.82, 1.72, 1.5 and 1.37 were prepared in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid). The gradient consisted of four layers (2 ml/layer) of cesium chloride with a decreasing density from the bottom to the top. The tubes were centrifuged at 100 000 rpm for 24 h at 20°C. Twenty fractions (30 µl) were collected from the bottom of the tubes. The DNA concentration in each fraction was measured at 260 nm using a NanoDrop spectrophotometer (Peglab Biotechnologie GmbH, Erlangen, Germany).

All fractions were diluted with the equivalent volume of 25 mM sodium phosphate buffer (pH 6.5) and blotted onto a nitrocellulose membrane using a slot blot apparatus (Miniifold II, Whatman®/Schleicher & Schuell, Dassel, Germany). After blocking the membrane with TBST buffer [20 mM Tris/HCl, pH 7.5, 137 mM NaCl and 0.1% (v/v) Tween-20], the membrane was incubated with a primary antibody, which was directed against the c-kit receptor (1:1000, Santa Cruz Biotechnology) followed by a secondary antibody (1:2000, Santa Cruz Biotechnology) and a chemiluminescent detection system (Amersham Pharmacia). Cell fractions from the middle of the gradient (3–6) were pooled for each group. DNA content was incorporated in the calculation of the test over colon control (% values), DNA concentration was incorporated.

Statistical analysis

All data are presented as the means ± SD. Statistical calculations in the in vivo complexes of enzyme to DNA assay were performed with the Microcal Origin program (OriginLab Corporation, Northampton, MA). Normal distribution of the results was tested by Shapiro–Wilks test. Statistical analysis was performed by Mann–Whitney U-test. For comet assay data, two-way analysis of variance was performed to analyze treatment effect (solvent/anthocyanin/anthocyanins and irinotecan/NaCl), using SigmaPlot for Windows, Version 11 (Systat Software, San Jose, CA). When treatment effects were significantly different (P ≤ 0.05), means were tested by Tukey’s test.

Results

Anthocyanins in rat plasma and rat colon

Table I lists the plasma concentrations of the administered anthocyanin/anthocyanins (cy, cy-3-g and cy-3-g as the main compound of the blackberry extract) as well as of total anthocyanins (administered compounds plus identified metabolites) 6 h postdose. No statistically significant differences in the concentrations of anthocyanins were found between the NaCl and irinotecan groups treated with the three anthocyanins. We identified cy monogluturonic acid as metabolite in cy-treated groups. In the cy-3-g groups, we specified the metabolites cy, cy monogluturonic acid, peonidin glucoside and isopeonidin glucoside. Besides cy-3-g, we were not able to distinguish between further blackberry anthocyanins and metabolites in the blackberry groups.
Anthocyanins as topoisomerase inhibitors in vivo

Table I. Concentration (nmol/l) of anthocyanins in the rat plasma after administration of cy, cy-3-g and the blackberry extract 6h postdose

<table>
<thead>
<tr>
<th></th>
<th>Administered anthocyanins</th>
<th>Total anthocyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy NaCl</td>
<td>16.8 ± 11.9</td>
<td>23.6 ± 16.2</td>
</tr>
<tr>
<td>Cy irinotecan</td>
<td>13.3 ± 12.9</td>
<td>14.5 ± 15.3</td>
</tr>
<tr>
<td>Cy-3-g NaCl</td>
<td>14.2 ± 18.8</td>
<td>19.5 ± 22.2</td>
</tr>
<tr>
<td>Cy-3-g irinotecan</td>
<td>11.4 ± 10.3</td>
<td>14.9 ± 12.0</td>
</tr>
<tr>
<td>Blackberry extract NaCl</td>
<td>22.9 ± 18.2 a,b</td>
<td>24.9 ± 19.2</td>
</tr>
<tr>
<td>Blackberry extract irinotecan</td>
<td>38.5 ± 28.3 a,b</td>
<td>43.6 ± 32.3</td>
</tr>
</tbody>
</table>

Data presented as means ± SD (n = 9).

Table II. Concentration (nmol/g tissue) of anthocyanins in the rat colon after administration of cy, cy-3-g and the blackberry extract 6h postdose

<table>
<thead>
<tr>
<th></th>
<th>Administered anthocyanins</th>
<th>Total anthocyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy NaCl</td>
<td>1.5 ± 2.6</td>
<td>1.6 ± 2.7</td>
</tr>
<tr>
<td>Cy irinotecan</td>
<td>1.3 ± 1.6</td>
<td>1.7 ± 1.8</td>
</tr>
<tr>
<td>Cy-3-g NaCl</td>
<td>9.8 ± 11.3</td>
<td>12.0 ± 12.2</td>
</tr>
<tr>
<td>Cy-3-g irinotecan</td>
<td>15.2 ± 31.1</td>
<td>18.4 ± 33.8</td>
</tr>
<tr>
<td>Blackberry extract NaCl</td>
<td>2.1 ± 3.5 a,b</td>
<td>2.9 ± 4.4</td>
</tr>
<tr>
<td>Blackberry extract irinotecan</td>
<td>4.5 ± 4.0 a,b</td>
<td>6.6 ± 5.6</td>
</tr>
</tbody>
</table>

Data presented as means ± SD (n = 9).

Anthocyanins modulate the DNA-strand-breaking activity of irinotecan

In control rats receiving solvent for anthocyanin/anthocyanins (0.1% citric acid orally) and i.p. NaCl, the DNA damage in colon mucosa cells was low, as evaluated by the comet assay (Table III). Intervention with blackberry extract (blackberry extract/NaCl) had no effect. Both cy and cy-3-g alone (cy/NaCl, cy-3-g/NaCl) caused a slight but statistically significant increase in DNA damage as compared with the solvent/NaCl group. Treatment with irinotecan caused a significant (about 3.5-fold versus NaCl group) increase in DNA-strand breaks. All three tested anthocyanidin/anthocyanin preparations significantly diminished the levels of DNA-strand breaks induced by irinotecan (cy/irinotecan versus solvent/irinotecan; cy-3-g/irinotecan versus solvent/irinotecan; blackberry extract/irinotecan versus solvent/irinotecan) (Table III).

Discussion

We reported previously that the aglycons delphinidin and cy as well as anthocyanin-rich berry extracts act as catalytic topoisomerase inhibitors in cell culture. Furthermore, an interference with classic topoisomerase poisons, e.g. camptothecin or doxorubicin, has been shown to result in a decreased formation of covalent enzyme/DNA intermediates and reduced DNA damage (21–24). Protection against topoisomerase poisoning may be beneficial for healthy individuals but might be counterproductive for cancer patients receiving standard cytostatic drugs acting on topoisomerases. The chemotherapeutic irinotecan is used for treating tumors of several organs, for instance pancreas, lung or cervix. In combination with 5-fluorouracil, irinotecan has been adopted worldwide for the treatment of colorectal cancers (25,39,40). Gastrointestinal toxicity, in particular unpredictable severe diarrhea, has been reported as one dose-limiting factor of irinotecan (40,41). The systemic bioavailability of anthocyanins was found to be low (42–44). Therefore, the probability of a systemic interaction of topoisomerase poisons with anthocyanins might be restricted. However, preventive effects of food constituents directly in the gut lumen to ameliorate diarrhea could be expected.

Overall, the knowledge of in vivo concentrations of anthocyanins and their metabolites in the gut and plasma are quite limited so far; therefore, we included analytical methods and data in our study. Furthermore, we addressed the question whether irinotecan affect anthocyanin absorption and metabolism. At our target site colon and in plasma, no differences between the NaCl and irinotecan groups for the three anthocyanin formulations (cy, cy-3-g and blackberry extract) were observed (Tables I and II).

As the major metabolite of cy, the respective cy monoglucuronide was detected in the plasma. In the plasma of rats treated with cy-3-g or blackberry extract, the metabolites cy, cy monoglucuronide, peonidin glucoside and isopeonidin glucoside were identified. In addition to other studies, we also detected the aglycon cy in the plasma (45–48). In rat colon, the methylated form peonidin was found after treatment with the aglycon cy. Cy-3-g was metabolized to cy, cy monoglucuronide, peonidin, peonidin glucoside and isopeonidin glucoside. After rat treatment with blackberry extract, the metabolites cy-3-g, cy-dioxalyl-glucoside, the cy pentoside with exception of cy rutinoside and methylated and glucuronated forms of the cy derivatives were detected.
Talavera et al. (47) reported that anthocyanins are efficiently absorbed from the small intestine influenced by the aglycon and the glycosidic moiety. In contrast to our findings, they identified only anthocyanin glycosides and their methylated/glucuronidated derivatives in bile and urine. Several authors postulated that intestinal β-glycosidases play an important role in flavonoid absorption (49,50). This might be an explanation for the characterized aglycons after rat treatment with cy-3-g or blackberry extract. The observed in vivo methylation of cy-3-g resulting in respective peonidin metabolites has been reported previously (44–47). But information about the mechanism of anthocyanin glucuronidation is limited so far. One postulated pathway is that cy-3-g would be absorbed intact and then partly methylated in the liver. Subsequently, both forms cyt-3-g and peonidin-3-glucoside may represent a substrate for uridine 5′-diphospho-glucuronosyltransferase (UGT). An alternative hypothesis is based on deglycosylation to the respective aglycon in the intestine. The resulting aglycons could be directly glucuronidated at the target site (44,45,47,48).

Overall, a low bioavailability of anthocyanins was observed in the plasma and in colon tissue, in line with various reports (46, 49). Furthermore, one might expect that high amounts of the absorbed anthocyanins are metabolized to non-colored chalcones or respective degradation products, as has been well described in literature (50–52), leading to a lack of quantification under the chosen analytic conditions.

In this study, we observed that oral treatment of male Wistar rats with blackberry anthocyanins, its main compound cy-3-g or its respective aglycon cy did not statistically enhance topoisomerase I/DNA intermediates in the colon compared with the control group (Figure 1). These results are in line with our previous in vitro studies characterizing the aglycons delphinidin and cy as well as various anthocyanin-rich plant extracts as topoisomerase I inhibitors (21–24). However, administration of the single compounds cy and cy-3-g resulted in a slight but significant increase in DNA-strand-break rate in colon mucosal cells of the Wistar rats (Table III). We postulate that a reduced activity of topoisomerase I may induce torsional stress in cells resulting in a higher level of DNA-strand breaks (53,54).
This study shows that the oral uptake of an anthocyanin-rich blackberry extract prior to irinotecan treatment caused a significant decrease of cleavable complex formation in the colon of rats in comparison with the irinotecan group receiving solvent instead of anthocyanin formulation, accompanied by a reduction of DNA damage. So far, only Lin et al. (55) reported a pharmacokinetic interaction of irinotecan with another secondary plant polyphenol, the green tea catechin (–)-epigallocatechin-3-gallate. (–)-Epigallocatechin-3-gallate was mentioned as a modulator of ATPase activity of P-glycoprotein resulting in an inhibition of the irinotecan transport into the biliary tract. The half-life of irinotecan in plasma could, thereby, be substantially extended by reduction of hepatobiliary excretion (55). One detoxification pathway for irinotecan is the formation of an inactive SN-38 glucuronide by UGT1A1 in the liver. Catechins have been reported to inhibit UGT1A1-dependent glucuronidation of SN-38, which might be of clinical relevance (56). However, to our knowledge, the direct interference of food constituents with topoisomerase poisons in vivo has not been reported so far.

It has to be noted that the doses of irinotecan administered to Wistar rats in this study were higher than the doses applied to cancer patients during chemotherapy. Moreover, the concentrations of anthocyanins used in this study exceed the usual daily intake by far, reflecting the pharmaceutical rather than a nutritional study design. Nevertheless, this model system shows that in the colon of rats, interference of anthocyanins with the chemotherapeutic irinotecan results in a significant modulation of clinically relevant endpoints. These findings may be of great importance for cancer patients receiving chemotherapy and additionally consuming food supplements rich in anthocyanins. So far, little is known about how food supplements may influence the efficacy of a chemotherapy based on topoisomerase poisons. Our results show the importance of assessing the respective consumption habits of food or herbal supplements by patients undergoing chemotherapy. Moreover, further studies are necessary to investigate the interactions between these food constituents and irinotecan at lower concentration ranges.

In conclusion, we showed that anthocyanins and their respective metabolites were absorbed and reached the target organ, which is a cation pathway for irinotecan is the formation of an inactive SN-38 glucuronide by UGT1A1 in the liver. Catechins have been reported to inhibit UGT1A1-dependent glucuronidation of SN-38, which might be of clinical relevance (56). However, to our knowledge, the direct interference of food constituents with topoisomerase poisons in vivo has not been reported so far.

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Conflict of Interest Statement: None declared.

References