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Oxidative stress-based cytotoxicity of delphinidin and cyanidin in colon cancer cells

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ABSTRACT

Colorectal cancer is the second most frequent cause of cancer death in the western world. Although the prognosis has improved after the introduction of newer anticancer drugs, the treatment of metastatic colorectal cancer still remains a challenge due to a high percentage of drug-resistant tumor forms. We aimed at testing whether anthocyanidins exerted cytotoxicity in primary (Caco-2) and metastatic (LoVo and LoVo/ADR) colorectal cancer cell lines. Both cyanidin and delphinidin, though neither pelargonidin nor malvidin, were cytotoxic in metastatic cells only. The cell line most sensitive to anthocyanidins was the drug-resistant LoVo/ADR. There, cellular ROS accumulation, inhibition of glutathione reductase, and depletion of glutathione could be observed. This suggests that anthocyanidins may be used as sensitizing agents in metastatic colorectal cancer therapy.

Introduction

Colorectal cancer is the second leading cause of cancer-related death in the 'developed' world [1]. About 50% of patients who undergo potentially curative surgery alone ultimately experience disease recurrence [2] and is therefore treated with combination chemotherapy. Drug resistance develops however in nearly all patients [3], calling for finding novel agents capable of killing drug-resistant colorectal cancer cells.

Anthocyanidins, a sub-class of flavonoids, have been suggested as useful agents for chemoprevention [4]. Recent studies show that they can trigger apoptosis in human leukemia cell lines through induction of oxidative stress [5,6], and their potential role in cancer therapy has been evaluated [7,8].

With regard to that, the aim of this study was to evaluate whether anthocyanidins are cytotoxic in different colorectal carcinoma cell lines, including drug-resistant cells.

Anthocyanidins were selected for this study, because, in the colon, they can be released from dietary anthocyanins by the action of glycosidases of the colonic microflora [9]; though they are less stable than their glycosylated precursors at neutral pH yielding phenolic aldehydes and phenolic acids [10], they seem to mimic *in vivo* conditions.

Indeed, we found that delphinidin and cyanidin were cytotoxic in the metastatic human colorectal cancer cell lines, LoVo and LoVo/ADR, where they inactivated the glutathione antioxidant system and promoted oxidative stress.

Materials and methods

Chemicals

All components for cell culture, camptothecin, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP[2]), Hank's buffered salt solution (HBSS), glutathione reductase (GR), NADPH, glutathione (GSH), glutathione disulfide (GSSG) and 4′,6-diamidino-2-phenylindole (DAPI) dilactate were from Sigma–Aldrich (Milano, Italy). Stock solutions of delphinidin, cyanidin, malvidin and pelargonidin chloride, (Extrasynthèse, Genay, France) were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. The final content of DMSO in all experiments was

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[2] *Abbreviations used:* ABAP, 2,2′-azobis (2-amidinopropane) dihydrochloride; CAA, cellular antioxidant activity; DAPI, 4′,6-diamidino-2-phenylindole; DCFH-DA: 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; HBSS, Hank's buffered salt solution; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PI, propidium iodide.

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kept under 0.2%. Annexin V was from Immunostep (Salamanca, Spain).

Cell lines

The human colorectal carcinoma cell line, Caco-2, was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy. Cells were used between passages 35–45 and cultured in Minimum Essential Medium Eagle containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin and sodium pyruvate (1 mM final concentration). LoVo cells, isolated from human colorectal adenocarcinoma metastasis, and LoVo/ADR cells, a doxorubicin-resistant metastatic human colorectal adenocarcinoma cell line, were kindly provided by Dr. C. Gambacorti, Milan, Italy. LoVo cells were routinely grown in RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin. LoVo/ADR cells were maintained in RPMI-1640 supplemented with 10% FBS, 1% penicillin–streptomycin, and doxorubicin (200 ng/ml). Seven days before each experiment doxorubicin was removed from the medium. Cell cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

MTT assay for cellular viability

Cells (4×10^3) were seeded in 96-well plates in complete culture medium and treated with camptothecin (0.001–50 μM) or anthocyanidins (0.78–100 μM), for 68 h at 37 °C in a humidified 5% CO_2 atmosphere. Each experiment was performed using eight replicate wells for each drug concentration. Control wells contained cells without drug and, in some cases with 0.2% of DMSO (v/v). After incubation for specified times, 20 μl of sterile MTT (5 mg/ml) were added in each well for additional 4 h at 37 °C [11]. The culture medium was then removed and formazan crystals were dissolved with 200 μl of DMSO. Absorbance was recorded on an automated microplate reader EL 311s (Bio-Tek Instruments, Winooski, VT) at 540 nm with a reference wavelength of 630 nm. The IC_{50} (concentration required for 50% inhibition of cell viability) was defined as the drug concentration required to reduce the optical density in each test to 50% of controls. All IC_{50} values were determined from three independent experiments.

Determination of apoptosis

Morphological analysis

LoVo and LoVo/ADR cells were seeded at a density of 15×10^4 cells/well in a six-well plate onto sterilized microscope glasses, left to attach for few hours after which they were treated with delphinidin or cyanidin (100 μM) for 24 h. The control wells contained only medium. After the indicated period, DAPI staining was performed as reported. The medium was aspirated and the glasses were washed with phosphate buffered saline (PBS); the cells were fixed with 2% paraformaldehyde (diluted in PBS) at room temperature for 30 min, and then permeabilized with 0.2% Triton X-100 (diluted in PBS). After few new washes, the cells were incubated with 5 μg/ml DAPI, for 5–7 min in the dark at room temperature, and washed again. Finally, they were dehydrated with 70%, 90%, 100% ethanol, coverslipped and observed under a fluorescence microscope (Leica Stereoscan 430i).

Flow cytometric analysis

Cells were treated with anthocyanidins in the same manner as described above. Apoptosis was measured using FITC-labeled recombinant human Annexin V assay, according to the manufacturer's instructions. Briefly, after washing with PBS, the cells were resuspended in binding buffer with 5 μl of Annexin V FITC and 5 μl of propidium iodide (PI) (1 mg/ml) for 15 min at room temperature, washed with PBS and resuspended in 200 μl of binding buffer. For each measurement 50,000 events were acquired with a standard FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer and analysis of data were performed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cellular antioxidant activity (CAA) assay

The intracellular formation of peroxyl radical was detected by the method of Wolfe and Liu [12]. Caco-2, LoVo and LoVo/ADR cells were seeded at a density of 1×10^4 cells/well on a 96-well microplate in 100 μl of growth medium/well. The outside wells of the plate were not used. Twenty-four hours after seeding, the growth medium was removed, the wells were washed with PBS, and the cells were treated for 1 h with delphinidin or cyanidin (25 μM, 50 μM, 100 μM) plus 25 μM DCFH-DA dissolved in treatment medium. After the indicated period, cells were washed and 600 μM ABAP dissolved in HBSS was added to the cells. Fluorescence was measured (λ_{ex} = 485 nm, λ_{em} = 538 nm) every 5 min for 1 h at 37 °C on a microplate reader (Plate Chameleon, HIDEX). Each concentration of each substance was repeated in six wells. Each plate also included six control and six blank wells: control wells contained cells treated with the dye (DCFH-DA) and the oxidant (ABAP); blank wells contained cells treated with DCFH-DA and HBSS without the oxidant.

Quantification of CAA

After blank subtraction from fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the CAA value at each concentration of delphinidin and cyanidin as follows: CAA unit = $100 - (\int SA/\int CA) \times 100$, where $\int SA$ is the integrated area under the sample fluorescence versus time curve and $\int CA$ is the integrated area from the control curve [12].

Glutathione reductase (GR) (EC.1.8.1.7) activity

The cells were seeded on a six-well plate at a density of 2.5×10^5/well. Upon reaching confluence, the growth medium was removed, the wells were washed with PBS, and cells were treated for 1 h with delphinidin or cyandin (25 μM, 50 μM, 100 μM), washed again, scraped with a rubber policeman, centrifuged, resuspended in 1 ml PBS, counted and sonicated. After these steps, samples were assayed spectrophotometrically (λ = 340 nm) for GR activity. The assay mix contained: 0.3 ml of the sample; 0.1 M phosphate buffer, pH 7.0; 1 mM EDTA; 0.26% Triton X-100 ("peroxide free"); 0.15 mM NADPH, and H_2O to 1.5 ml, at t = 37 °C. The baseline was recorded for 5 min, then 2 mM GSSG was added and the signal was recorded for further 10 min. GR activity was calculated from the net change of A_{340} and expressed as nmol NADPH $min^{-1} \times 10^{-6}$ cells.

Quantitative determination of GSH and GSSG levels

Cells were treated with anthocyanidins in the same manner as for the determination of GR activity, harvested, counted, centrifuged, and resuspended in 1 ml of ice-cold extraction buffer (0.1% Triton X-100 and 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5). The cell suspension was sonicated and centrifuged. The supernatant (0.180 ml) was put into a 96-well plate. Then, 0.020 ml 5 mM DTNB were added and A_{412} was recorded from a microplate reader (Plate Chameleon, HIDEX). The rate of formation of the chromophore TNB is proportional to the concentration of GSH in the sample [13], calculated on the basis of a GSH calibration curve. To assess cellular GSSG levels, the cell extracts were first treated with 2-vinylpyri-