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On the bioavailability of flavanols and anthocyanins: Flavanol–anthocyanin dimers

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ABSTRACT

The bioavailability of flavanols, anthocyanins and anthocyanin-derived pigments like flavanol–anthocyanin dimers already reported to occur in food products is a major unsolved issue. The absorption of the flavanol–anthocyanin dimer (+)-catechin-(4,8)-malvidin-3-*O*-glucoside (Cat–Mv3glc) through Caco-2 cells was assessed by performing transepithelial transport assays. The ability of Cat–Mv3glc to cross Caco-2 cells was compared with that of malvidin-3-glucoside (Mv3glc), (+)-catechin (Cat) and procyanidin B3 (Cat–Cat), in order to evaluate the influence of some structural features on the transport efficiency. The flavanol–anthocyanin dimer was absorbed in this intestinal model although with a lower efficiency than the monomers Cat and Mv3glc. On the other hand, Cat–Mv3glc was found to cross the intestinal barrier model more significantly than Cat–Cat. This feature may be related to the presence of the glucose moiety in its structure. Overall, this study brings more insights into the bioavailability of anthocyanins and flavanols and represents the first report on the bioavailability of flavanol–anthocyanins.

1. Introduction

Flavanols and anthocyanins constitute two major classes of flavonoids that occur in nature arising from plant secondary metabolism. The daily intake of flavanols including catechins, proanthocyanidin dimers and trimers has been estimated to be 18–50 mg/d, with the main sources being tea, chocolate, apples, pears, grapes, and red wine (Arts, van de Putte, & Hollman, 2000a, 2000b). Flavanols are biologically active molecules that have a wide range of effects and are known as very strong antioxidants that can scavenge various forms of free radicals (Ricardo da Silva, Darmon, Fernandez, & Mitjavila, 1991; Saint-Cricq de Gaulejac, Provost, & Vivas, 1999). They may be involved in the prevention of cardiovascular diseases, probably through their ability to inhibit oxidation of low density lipoprotein (LDL), to lower the plasma cholesterol level, and to prevent platelet aggregation (Jeong & Kong, 2004). In addition, there is increasing evidence of the cancer chemopreventive properties of catechins and procyanidins (Jeong & Kong, 2004; Santos-Buelga & Scalbert, 2000).

Anthocyanins constitute the largest group of water-soluble pigments in the plant kingdom, being responsible for the colours displayed by many flowers, fruits and leaves. These pigments are usually present in red fruits but they also occur in vegetables, roots, legumes and cereals (Clifford, 2000). Pure anthocyanins or anthocyanin-rich extracts have been found to play a role in the prevention of cardiovascular diseases and to be involved in several different events, such as the prevention of DNA damage, oestrogenic activity, enzymatic inhibition, anti-inflammation response and lipid peroxidation inhibition (Liu, Lee, Shih, Chyau, & Wang, 2008). In recent years, more attention has been paid to the putative anti-tumoral properties of anthocyanins and anthocyanin extracts (Faria et al., 2010; Fernandes et al., 2010; Li et al., 2009).

Due to their high chemical reactivity, anthocyanins may also be ingested as anthocyanin-derived pigments. Direct reactions between anthocyanins and flavanols have already been demonstrated in model solutions (Dueñas, Fulcrand, & Cheynier, 2006; Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 1999) and food matrices, especially red wine, where these compounds play an important role in their colour (Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000). These pigments are usually associated with reactions taking place during processing and storage of plant-derived foods and beverages. Indeed, these compounds have already been identified through chromatographic procedures in strawberries (Fossen, Rayyan, & Andersen, 2004), pomegranate juice (Sentandreu, Navarro, & Sendra, 2010), beans (González-Paramás et al., 2006), grape skins (González-Paramás et al., 2006) and purple corn (González-Paramás et al., 2006). The detection of these pigments in plant extracts may suggest that they are natural pigments and not products exclusively formed during storage and ageing of processed foods and beverages, as previously

Abbreviations: Mv3glc, malvidin-3-glucoside; Cat, (+)-catechin; Cat–Cat, (+)-catechin-(4α → 8)-(+)-catechin; Cat–Mv3glc, (+)-catechin-(4,8)-malvidin-3-*O*-glucoside.

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assumed. Being part of the human diet, these pigments could contribute to increase both anthocyanin and flavanol intake. The bioavailability of anthocyanins and flavanols is a major issue regarding their biological effects.

Studies involving individual anthocyanins revealed that their amount in plasma is generally only 1% of consumed quantities, due to limited intestinal absorption, although additional factors contributing to anthocyanin bioavailability may have been overlooked (Vanzo et al., 2008). On the other hand, there are only a few *in vitro* studies assessing anthocyanin bioavailability (Pacheco-Palencia, Mertens-Talcott, & Talcott, 2010; Steinert, Ditscheid, Netzel, & Jahreis, 2008; Yi, Akoh, Fischer, & Krewer, 2006).

Concerning the bioavailability of the main flavanols present in the diet, catechin and epicatechin are readily detected in plasma after their consumption from food or beverages but are rapidly eliminated from the organism (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). As to proanthocyanidins, their polymeric nature and high molecular weight are likely to limit their absorption through the gut barrier. Absorption studies with flavanols and procyanidins using Caco-2 cells are scarce (Deprez, Mila, Huneau, Tome, & Scalbert, 2001; Vaidyanathan & Walle, 2001). A high proportion of the *in vivo* studies performed so far suggests that only procyanidin dimers can be absorbed (Baba, Osakabe, Natsume, & Terao, 2002; Prasain, Peng, Dai, Moore, Arabshahi, Wilson, et al., 2009; Shoji et al., 2006; Tsang et al., 2005).

The present work aimed to study the transepithelial transport through Caco-2 cells of a flavanol–anthocyanin (Cat–Mv3glc) dimer as well as three other compounds (Mv3glc, Cat and Cat–Cat), in order to evaluate the influence of some structural features, such as the anthocyanin and flavanol moieties, in the transport efficiency of these compounds. Based on the literature data of structurally related compounds, it was already anticipated that the Cat–Mv3glc pigment would be absorbed in a much lower percentage than the monomers composing it. However, the remaining question would be whether its transport efficiency would be increased in comparison with Cat–Cat.

2. Materials and methods

2.1. Reagents

Malvidin-3-glucoside was purchased from Extrasynthèse SA (Genay, France). (+)-Catechin, Hanks' medium (HBSS), MEME, foetal bovine serum (FBS), 0.25% trypsin–EDTA, PBS and antibiotic/antimycotic solution (100x) were supplied by Sigma–Aldrich (Madrid, Spain). Tissue culture supports were supplied by TPP (Trasadingen, Switzerland).

2.2. Synthesis of procyanidin B3 ((+)-catechin-(4α → 8)-(+)-catechin) and (+)-catechin-(4,8)-malvidin-3-O-glucoside

Procyanidin B3 and (+)-catechin-(4,8)-malvidin-3-*O*-glucoside were synthesised and purified as described elsewhere (Bras et al., 2010; Nave, Teixeira, Mateus, & de Freitas, 2010). The purity of the final material obtained was confirmed by HPLC–MS and NMR.

2.3. Stability assays

All compounds (100.0 μM) were incubated in phosphate buffer (adjusted to pH 2.0 with 6 M HCl) for 3 h at 37 °C. The monomer and dimer stability was followed using a reverse phase HPLC protocol every 60 min, as described below.

2.4. Cell culture conditions

Caco-2 cells were grown in a humidified atmosphere of 5% CO_2/95% air, in minimum essential medium Eagle (Sigma, St. Louis, MO) supplemented with 15% FBS, 25 mM HEPES, 100 units/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 1 min, 37 °C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; TPP Techno Plastic Products AG, Trasadingen, Switzerland). For transport experiments, Caco-2 cells were seeded on transwell inserts (polycarbonate membrane, 0.4 μm pore size, 24 mm diameter; Corning Costar, Corning, New York, NY). Cells were allowed to grow and differentiate to confluent monolayers for 21 days after the initial seeding. The cell medium was free of foetal calf serum for 24 h before the experiments.

2.5. Transport studies

Transepithelial electrical resistance of cells grown in the transwell was measured using MILLICELL-ERS epithelial voltohmmeter (Millipore Co., Bedford, MA) with "chopstick" electrodes. Experiments were conducted only in cell monolayers that showed a transepithelial electrical resistance >230 Ω.Medium was removed and cells were washed with Hanks' medium, pH 7.4. Compound solution in Hanks, with a final concentration of each compound in the media of 100.0 μM, was added to the apical side of the cells and Hanks containing 2% FBS was added to the basolateral compartment. Transepithelial transport was followed as a function of time. One hundred and fifty microlitres were taken from the basolateral side and replaced by fresh medium (Hanks containing 2% FBS) at 30, 60 and 120 min of incubation. Each time sample (150 μL) was acidified with HCl to a final concentration of 0.06 M. All samples, from both apical and basolateral sides, were acidified and frozen (−18 °C) to ensure that the cumulative amounts of each compound that were later quantified were not altered during sample delay until HPLC analysis. Compounds were quantified using the respective calibration curves. Transport efficiency percentages were calculated based on: ((compound concentrations at the basolateral side overtime)/(compound concentrations at the apical side at the zero hours)) × 100.

2.6. HPLC-DAD analysis

All samples were analysed by HPLC (Elite Lachrom system (L-2130) on a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt, Germany); detection was carried out using a diode array detector (L-2455). The solvents were A: H_2O/HCOOH (9:1), and B: H_2O/HCOOH/CH_3CN (6:1:3). The gradient consisted of 20–52.5% B for 35 min at a flow rate of 1.0 mL/min. The column was washed with 100% B for 15 min and then stabilised at the initial conditions for another 15 min. For Cat–Cat the gradient started with 0–52% B.

2.7. HPLC–MS analysis

HPLC–MS analysis of metabolites was performed on a liquid chromatograph (Hewlett–Packard 1100 series) equipped with an Aqua (Phenomenex, Torrance, CA) reversed-phase column (150 × 4.6 mm, 5 μm, C18), thermostatted at 35 °C. Solvents were A: H_2O/HCOOH (9.9:0.1) and B: H_2O/CH_3CN/HCOOH (6.9:3:0.1). The HPLC gradient used was the same as that reported above for the HPLC analysis. The mass detector was a Finnigan LCQ (Finnigan Corporation, San Jose, CA) equipped with an API source, using an electrospray ionisation (ESI) interface. Both the auxiliary and the