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Transepithelial Permeability Studies of Flavan-3-ol-*C*-glucosides and Procyanidin Dimers and Trimers across the Caco-2 Cell Monolayer

Sarah Hemmersbach,[†] Susanne S. Brauer,[†] Sabine Hüwel,[‡] Hans-Joachim Galla,[‡] and Hans-Ulrich Humpf*[,†]

[†]Institute of Food Chemistry, Westfälische Wilhelms-Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany

[‡]Institute of Biochemistry, Westfälische Wilhelms-Universität Münster, Wilhelm-Klemm-Strasse 2, D-48149 Münster, Germany

Supporting Information

ABSTRACT: In this study the permeability of two flavanol-*C*-glucosides (FCglcs) and five dimeric and trimeric flavan-3-ols, namely, procyanidins (PCs), was investigated with the human colon carcinoma cell line (Caco-2) model. These compounds can be found especially in cocoa, and they are of great interest due to their assumed beneficial health effects. Transepithelial electrical resistance (TEER) and capacitance were measured online with a CellZscope device prior to and during the whole experiment to guarantee the maintenance of the barrier properties. The transport experiments with pure, single compounds (50−300 μM) from apical to basolateral side showed slight permeation of PCs A2, B2, and B5 and cinnamtannin B1 (CB1) as well as (−)-catechin-6-*C*-glucoside (C6Cglc) and (−)-catechin-8-*C*-glucoside (C8Cglc) of about 0.02−0.2% after 24 h. Transport of PC C1 could not be detected. Inhibition of P-glycoprotein (Pgp) increased the permeation of PC B2 and CB1 to the basolateral side, which indicates that Pgp counteracts the transport of these compounds. Metabolites (epicatechin, 3′- and 4′-*O*-epicatechin) in very small amounts were detectable only for PC B2. These are the first data concerning the permeability of flavan-3-ol-*C*-glucosides across the Caco-2 cell monolayer.

KEYWORDS: *flavan-3-ol-C-glucosides, procyanidin A2, procyanidin B2, procyanidin B5, procyanidin C1, cinnamtannin B1, Caco-2 cells, transport, permeability, TEER, LC-MS/MS*

INTRODUCTION

Procyanidins (PCs) and flavan-3-ol-*C*-glucosides (FCglc) (see Figure 1) are flavanols belonging to the large group of polyphenols.[1] Flavanols exist as monomeric flavan-3-ols and oligomers or polymers named proanthocyanidins.[1] PCs as the largest group within the proanthocyanidins are oligomeric flavan-3-ols comprising only catechin/epicatechin as building blocks[2−4] and occur in several plant-derived foods such as fruits, berries, wine, and cocoa in amounts up to several hundred milligrams per 100 g fresh weight.[5,6]

Flavonoids also often occur in a glycosylated form.[1] Most common is an *O*-linked sugar moiety, but *C*-glucosides are also present, for example, in rooibos tea, cocoa, and rhubarb.[7−9] The flavan-3-ol-*C*-glucosides investigated in this study can be found, for example, in cocoa and rhubarb.[8,9] In contrast to rhubarb, those in cocoa are not present in the untreated cocoa but are formed in the alkalization process during manufacturing.[8]

Polyphenols in general are well-known for their assumed positive health effects, which are related to their antioxidative and anti-inflammatory properties through altering gene expression.[10−13] Polyphenols are discussed to play a role in the prevention of cancer, cardiovascular diseases, inflammatory diseases, diabetes, and metabolic disorders.[12,13] For this reason, knowledge about their bioavailability and metabolism is essential to evaluate any health impact of these compounds in humans. Several studies describe the in vitro and in vivo gastrointestinal absorption of PCs and glycosylated flavonoids (e.g., the reviews of Manach et al.[1] and Aron and Kennedy[10]).

In in vitro experiments with Caco-2 cells, a slight uptake of A-type PC dimers, trimers, and tetramers in a mixture as well as of pure PCs A2 and B2 is reported by Ou et al.[14] In another study Zumdick et al.[15] described poor permeability of PCs B2 and C1 (<0.8 and <0.5% after 2 h of incubation, respectively) in Caco-2 transport studies. After the application of purified cocoa extract to a Caco-2 model system, small amounts of PC B2 and monomeric flavan-3-ols could be detected on the basolateral (serosal) side.[16]

Appeldoorn et al.[17] used an in situ rat small intestinal perfusion model to study the absorption and metabolism of the PCs A1, A2, and B2 as well as the flavan-3-ol monomer epicatechin. They observed slight absorption of unmodified PCs, which was about 5−10% lower than the absorption of epicatechin. However, the absorption of A-type PCs was higher compared to B-type PCs. In vivo experiments with pigs, which have a gastrointestinal tract similar to that of humans, showed a urinary excretion of about 0.01−0.02% of the administered dimeric PCs (B1, B2, B3, and B4) and only 0.004% excretion of PC C1.[18]

As far as we know, data concerning the bioavailability of some PCs are still lacking, in particular for cinnamtannin B1 (an A-type PC trimer) and pure PC B5.

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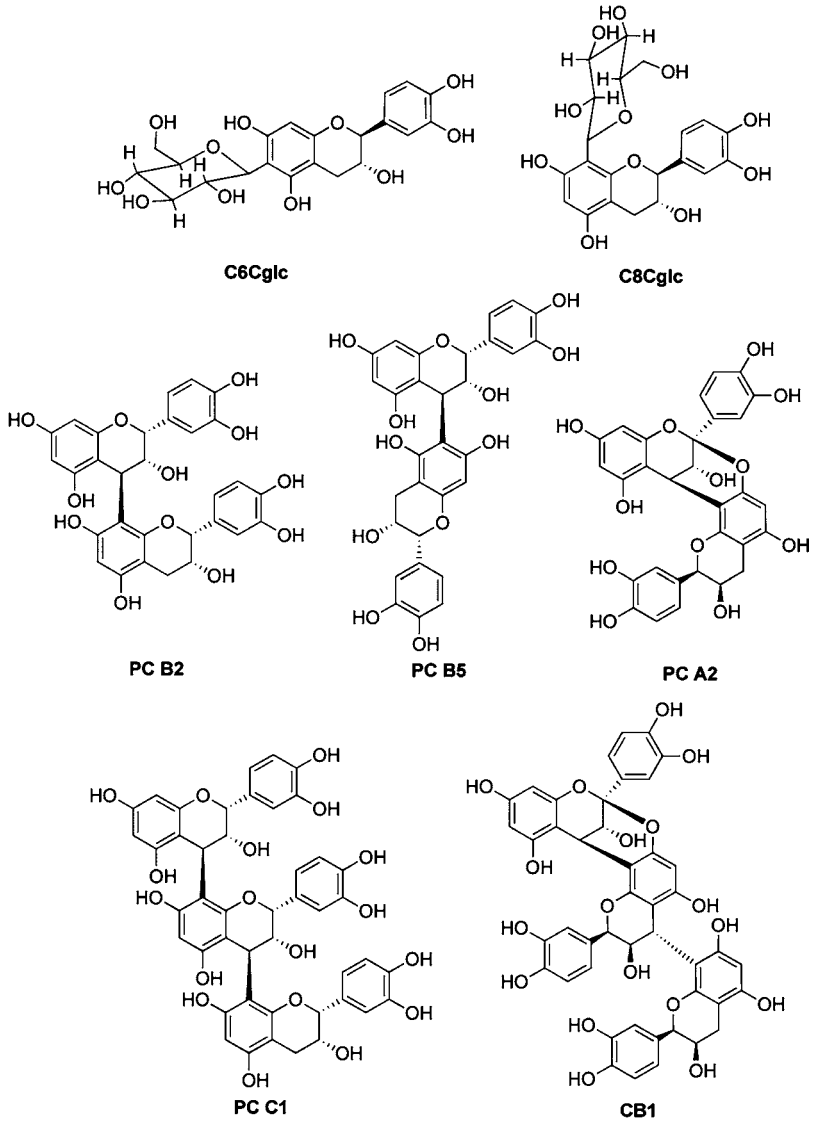

Figure 1. Chemical structures of model compounds investigated in this study: (−)-catechin-6-*C*-glucoside (C6Cglc), (−)-catechin-8-*C*-glucoside (C8Cglc), and procyanidins (PCs) A2, B2, B5, C1, and cinnamtannin B1 (CB1).

Several studies have also described the absorption of glycosylated flavonoids. Quercetin glucoside, which is a flavonol and exhibits one or two *O*-linked glucose moieties, is absorbed in higher amounts than its aglycon in the human small intestine.[19] Other studies describe the hydrolysis of *O*-glucosides prior to absorption.[20−22] However, data about the absorption of *C*-glucosides are rare. It is assumed that *C*-glucosides are more stable against acidic, alkaline, and enzymatic hydrolysis than *O*-glucosides.[23] Hasslauer et al.[24] identified a long-lasting presence of flavan-3-ol-*C*-glucosides in the gastrointestinal tract after in vitro and ex vivo incubation experiments with human saliva, simulated gastric juice, ileostomy fluid, and colostomy fluid. The intestinal stability of these compounds raised the question whether intestinal absorption can be observed. Stalmach et al.[25] showed that flavanone-*C*-glucosides in rooibos tea are absorbed as unmodified molecules.

The aim of our study was to determine the permeability of five PCs (A2, B2, B5, C1, and cinnamtannin B1 (CB1)) and two flavan-3-ol-*C*-glucosides ((−)-catechin-6-*C*-glucoside (C6Cglc) and (−)-catechin-8-*C*-glucoside (C8Cglc)) by performing transport studies with pure, single compounds using the Caco-2 monolayer model. The PCs were selected to verify differences in the intestinal uptake of PCs consisting only of epicatechin units in different amounts and constitutions. Furthermore, it was of particular interest to find out whether the *C*-linked sugar moiety has any influence on the intestinal absorption of flavan-3-ols. To investigate whether an active efflux transport is involved in the transport of model compounds, the influence of P-glycoprotein (Pgp) on the permeability was tested. In addition, the inhibitory effect of the model compounds on the glucose uptake was investigated.

MATERIALS AND METHODS

Chemicals and Reagents. Propranolol, verapamil hydrochloride, and fluorescein isothiocyanate−dextran 4000 (FD4) were obtained from Sigma-Aldrich (Steinheim, Germany), rhodamine 123 was obtained from Merck (Darmstadt, Germany), and ^{14}C-glucose ([1-^{14}C]-D-glucose (50−60 mCi)) and [U-^{14}C]-sucrose were purchased from Hartmann Analytic GmbH (Braunschweig, Germany).

Cell culture medium (minimum essential medium Eagle (MEM)), cell culture chemicals, and cell culture supplies were purchased from

Biochrom AG (Berlin, Germany) and PAA Laboratories (Pasching, Austria).

Solvents used for HPLC and all other chemicals were obtained from Sigma-Aldrich, Gruessing (Filsum, Germany), Carl Roth (Karlsruhe, Germany), and Biochrom AG (Berlin, Germany), unless specified otherwise. Water was purified with a Milli-Q Gradient A10 system from Millipore (Schwalbach, Germany).

Preparation of Model Compounds. Because flavan-3-ol-*C*-glucosides are not commercially available, (−)-catechin-6-*C*-glucoside and (−)-catechin-8-*C*-glucoside (Figure 1) were synthesized, isolated, and purified following the protocol published by Stark and Hofmann[8] with slight modifications during purification: The purification was carried out as described by van't Slot et al.[26] by RP-18 column chromatography (LiChroprep 25−40 μm, Merck) followed by preparative HPLC-UV. Structures were confirmed by proton nuclear magnetic resonance spectroscopy (Bruker DPX 400, Bruker, Rheinstetten, Germany), determination of accurate mass by Fourier transform mass spectrometry (LTQ Orbitrap XL, Thermo Fisher Scientific, Bremen, Germany), and circular dichroism spectroscopy (Jasco J-600 spectropolarimeter, Jasco, Gross-Umstadt, Germany). The data are in accordance with the literature.[8] Procyanidins A2, B2, B5, C1, and CB1 (Figure 1) were isolated and purified from cocoa and litchi pericarp in our laboratory, according to the method of Rzeppa et al.[5]

Stock Solutions and Compound Stability. Stock solutions of FCglcs and PCs were prepared in concentrations of 1, 3, or 5 mM in methanol (MeOH)/autoclaved water (5:95; v/v) except for PC A2, which was dissolved in MeOH/autoclaved water (9:91; v/v). Stock solutions were diluted 1:10 with MEM transport medium as described below. The incubation was performed in a 24-well plate (TPP; Biochrom AG) under the same conditions and concentrations (50, 100, and 300 μM) as in the transport experiments. The plate was incubated for 24 h under humid aerobic conditions at 37 °C. Samples of 20 μL each were taken after 0, 1, 2, 4, 6, 8, and 24 h. To keep the compounds stable during storage and analysis, samples were further diluted with MeOH/aqueous formic acid (FA), 0.1%, 5:95, (v/v). Identification and quantitation was performed by HPLC-MS/MS (API 5500 QTRAP), as described below.

Cell Culture Experiments. *Cultivation of Caco-2 Cells.* The human intestinal Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Caco-2 cells in passage 61 or 62 were used for all experiments. Every experiment was repeated on at least three different days each in at least three independent experiments, unless specified otherwise. The cells were cultured in tissue flasks (75 cm^2) in MEM with Earle's salts and 2.2 g/L $NaHCO_3$ (Biochrom AG) supplemented with 10% (v/v) fetal bovine serum (Biochrom AG), 1% (v/v) nonessential amino acids (Biochrom AG), 2 mM L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (PAA Laboratories) in a humidified atmosphere of 5% CO_2 at 37 °C.

When a microscopic confluence of 70−80% was reached, the cells were subcultured in a ratio of 1:3 using a trypsin/EDTA buffer (0.2 g/L EDTA (disodium salt), 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, g/L trypsin dissolved in autoclaved purified water). At the third or fourth subcultivation (passage 61 or 62), the trypsinized cells were counted by CASY-TT (Innovatis, Reutlingen, Germany) and seeded.

For the permeability studies, 50000 cells/cm^2 (65000 cells per filter insert with a cell growth area of 1.12 cm^2) were seeded onto Transwell Permeable Supports (12 mm Transwell with a 0.4 μm pore polycarbonate membrane insert; Corning Inc., New York, USA). The cells were cultured with 700 μL of MEM in the apical (ap) and 1500 μL of MEM in the basolateral (bl) compartment.

For the cytotoxicity assay (CCK-8 assay), the cells were seeded with the same density as for the permeability studies (15500 cells on 0.31 cm^2) in a volume of 100 μL of MEM each in 96-well plates.

After seeding, the first change of medium was performed after 16 h. Then media changes of the confluent cultures were carried out every 2−3 days. Compounds were applied and transport experiments were carried out 21 days after seeding, when it was assumed that the cells had become completely differentiated.[27]

Establishment and Validation of the Caco-2 Model System. To verify the monolayer integrity, several positive and negative controls (propranolol, FD4, rhodamine 123, ^{14}C-glucose, and [U-^{14}C]-sucrose) were applied to the Caco-2 cell monolayers on the Transwell filter inserts (for details, see the Supporting Information).

Cytotoxicity of Model Compounds (CCK-8 Assay). The cytotoxicity of model compounds was elucidated with the CCK-8 assay. The method for this assay is described in the Supporting Information.

Transepithelial Permeability Studies. For transport experiments, Caco-2 cells were seeded onto 12-well Transwell inserts as described above. Twenty days after seeding, the Caco-2 cells in MEM on Transwell filter inserts were transferred into a CellZscope module (NanoAnalytics GmbH, Muenster, Germany) at 5% CO_2 and 37 °C. To verify the monolayer integrity prior to and during the experiment, long-term monitoring of the transepithelial electrical impedance (represented as transepithelial electrical resistance (TEER) and capacitance (C_{cl})) of the cell monolayers was initiated. Only monolayers showing a TEER value of >3000 Ω·cm^2 and a capacitance of about 3.5 μF/cm^2 during the whole experiment were used.

After about 16 h (on the 21st day post seeding), MEM was replaced with MEM transport medium pH 6.5 or 7.4 (MEM with Earle's salts without $NaHCO_3$ and without phenol red, supplemented with 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 20 mM PIPES, and adjusted to a pH of 6.5 or 7.4 with sodium hydroxide (1 M) or hydrochloric acid (1 M)). The compartments were filled with either MEM pH 6.5 or MEM pH 6.5 on the apical side and MEM pH 7.4 on the basolateral side, creating a pH gradient. The culture conditions were changed to humidified air at 37 °C (without further CO_2 supplementation, to obtain stable pH values in medium). Online monitoring of the TEER (ohmic resistance) and capacitance (C_{cl}) was continued.

After 4−5 h (when TEER values had recovered), model compounds and control compounds were applied. The compounds were applied in the apical, the basolateral, or both compartments by replacing $^1/_{10}$ of the transport medium by compound solution. The resulting compound concentration in the compartments was between 50 and 300 μM for model compounds and between 20 and 200 μM for control compounds. After compound application, the measurement of TEER values and capacitance continued.

After 0, 1, 2, 4, 6, 8, and 24 h of incubation, samples were taken from each compartment (20 μL apical and 40 μL basolateral). To keep the compounds in the samples stable during storage and analysis, C6Cglc and C8Cglc samples were diluted with MeOH/0.1% (v/v) aqueous FA, 5:95 (v/v). PCs A2, B2, B5, C1, and CB1 were diluted with acetonitrile (ACN)/0.1% (v/v) aqueous FA, 10:90 (v/v). Identification and quantitation were performed by HPLC-MS/MS (API 5500 QTRAP).

Permeability Coefficient. The permeability coefficient (P_{app}) was calculated for the permeability experiments using the following equation: $P_{app} = (\delta C/\delta t) \times (V/(A \cdot C_0))$.[28] The P_{app} value is expressed in cm/s. V is the volume of the solution in the acceptor compartment (mL), A is the membrane surface area (cm^2), C_0 is the initial concentration in the donor compartment (μM), and $\delta C/\delta t$ is the change in the compound concentration in the acceptor compartment over time (μM/s).

P-Glycoprotein (Pgp) Inhibition. The Pgp inhibitor verapamil (100 μM; Sigma-Aldrich) was used in these studies. On the day of the permeability studies (21 days after seeding), the Caco-2 cell monolayers were preincubated with 50 μM verapamil in the apical and basolateral compartments, respectively. After 1 h, model compounds were applied on the apical side, and permeability studies were performed.

^{14}C-Glucose Permeability in the Presence of Flavan-3-ol-C-glucosides or Procyanidins. This study was performed in combination with the ^{14}C-glucose and ^{14}C-sucrose experiments within the validation of the Caco-2 model system, and this method is available in the Supporting Information.

Analytical Methods. *HPLC-MS/MS (API 5500 QTRAP).* A QTRAP 5500 MS system (AB Sciex, Darmstadt, Germany) coupled to a LaChrom Ultra HPLC system (VWR-Hitachi, Darmstadt, Germany) was used to detect and quantitate the FCglcs and PCs in multiple reaction monitoring mode (MRM) using negative polarity. PC A2 was quantified as the sum of A-type dimers because of a rearrangement of this PC under transport conditions. Data acquisition was performed with Analyst 1.5.2 software.

Chromatography was carried out on a 150 × 2 mm Nucleodur ISIS RP-18 column, 5 μm (Macherey-Nagel, Dueren, Germany) with a flow rate of 350 μL/min. The injection volume was 25 μL, and the column was kept at 40 °C. The separation of C6Cglc and C8Cglc was performed with a mixture of MeOH (solvent A) and 0.1% (v/v) aqueous FA (solvent B). Initial conditions were 5% A and 95% B (v/v). For C6Cglc, these were maintained for 2 min (for C8Cglc, 1 min) and then increased to 20% A for 2 min (for C8Cglc, to 16% A for 1 min and 45% A for 2 min). Within the next 0.2 min, solvent A was raised to 100% and maintained for 2.4 min.

The PCs (A2, B2, B5, C1, and CB1) were analyzed with the RP-18 column described above with the same flow rate. ACN (solvent A) and 0.1% (v/v) aqueous FA (solvent B) were used as mobile phases. The gradient started with 12% A and 88% B (v/v) for 2 min. Solvent A was increased to 41% for 3.7 min and then directly (0.2 min) to 100%, where it remained for 2.5 min.

At the beginning of each run, polar cell culture components were removed by a diverter valve before entering the ion source. The valve was switched from waste to ion source after 0.5 min for the analysis of catechin-*C*-glucosides (CCglcs) or after 1.5 min for PCs.

Declustering potential (DP), entrance potential (EP), MRM transition, collision energy (CE), and cell exit potential (CXP) were set as follows:

Epicatechin (EC): DP, −100 V; EP, −10 V; 288.9−245.0 (CE, −22 V; CXP, −9 V), 288.9−109.0 (CE, −32 V; CXP, −5 V). PC B2 and PC B5: DP, −130 V; EP, −10 V; 576.8−289.0 (CE, −32 V; CXP, −9 V), 576.8−406.9 (CE, −32 V; CXP, −15 V). PC A2: DP, −95 V; EP, −5 V; 575.0−284.9 (CE, −38 V; CXP, −20 V), 575.0−124.8 (CE, −52 V; CXP: 0 V). PC C1: DP, −180 V; EP, −10 V; 864.9−576.8 (CE, −34 V; CXP, −31 V), 864.9−406.8 (CE, −46 V; CXP, −5 V). CB1: DP, −180 V; EP, −10 V; 862.9−410.9 (CE, −44 V; CXP, −15 V), 862.9−288.8 (CE, −46 V; CXP, −21 V). Monomethylflavan-3-ols: DP, −130 V; EP, −10 V; 302.9−136.9 (CE, −22 V; CXP, −9 V), 302.9−164.8 (CE, −24 V; CXP, −9 V). Monomethylflavan-3-ol-dimers: DP, −130 V; EP, −10 V; 590.8−288.9 (CE, −32 V; CXP, −15 V). Flavan-3-ol-glucuronide: DP, −100 V; EP, −10 V; 465.1−288.9 (CE, −22 V; CXP, −11 V). C6Cglc: DP, −170 V; EP, −10 V; 450.9−331.0 (CE, −22 V; CXP, −1 V), 450.9−361.1 (CE, −22 V; CXP, −5 V), 450.9−122.8 (CE, −52 V; CXP, −9 V). C8Cglc: DP, −80 V; EP, −10 V; 450.9−331.0 (CE, −28 V; CXP, −19 V), 450.9−361.1 (CE, −26 V; CXP, −13 V), 450.9−122.8 (CE, −50 V; CXP, −7 V).

Nitrogen was used as curtain and collision gas. Curtain gas was set at 30 and 35 psi for the PCs and the CCglcs, respectively. Collision gas was set to high for the PCs and to medium for the CCglcs. Ion spray voltage (ISV) was kept at −4500 V. Zero grade air was used as nebulizer gas (35 psi) and turbo gas (45 psi) for solvent drying at a temperature of 350 °C. Q1 and Q3 were maintained at unit resolution. Transition reactions were each monitored for 15 ms for the PCs and at 100 ms for the CCglcs.

HPLC-MS/MS (API 3200). An API 3200 LC-MS/MS system (Applied Biosystems, Darmstadt, Germany) linked to an Agilent 1200 series HPLC (Agilent Technologies, Boeblingen, Germany) was used for quantitation of control compound propranolol in MRM mode with positive ionization. Data acquisition was performed with Analyst 1.4.2 software.

Separation was carried out on a Halo C8 column (2.1 × 50 mm, particle size = 2.7 μm) using a mixture of ACN supplemented with 0.1% FA (solvent A) and 5 mM ammonium formate ($HCOONH_4$) in aqueous FA, 0.1% (solvent B). The column was kept at 40 °C. The flow rate was set at 0.3 mL/min, and the injection volume was 20 μL. The gradient started with 10% solvent A and was kept constant for 0.5 min. Solvent A was increased to 90% within 2.5 min. This condition was maintained for 1 min, and then the solvent A content was decreased to starting conditions within 0.5 min. The column was equilibrated at 10% solvent A for another 4.5 min.

DP, EP, MRM transition, CE, and CXP were set as follows. Propranolol: DP, 36 V; EP, 4.5 V; 260.1−116.1 (CE, 23 V; CXP, 4 V), 260.1−183.3 (CE, 23 V; CXP, 4 V), 260.1−155.2 (CE, 35 V; CXP, 4 V).

Curtain gas and collision gas (nitrogen) were set at 20 psi and 10×10^{-5} Torr, respectively. ISV was kept at 5500 V. Zero grade air was used as nebulizer gas (35 psi) and turbo gas (45 psi). Temperature was set to 350 °C. Q1 and Q3 were maintained at unit resolution. Dwell time was set to 100 ms. Peaks were integrated and quantified using Analyst 1.5.2.

Matrix-Matched Calibration. Due to the lack of internal standards, all model compounds and formed metabolites were quantified by external calibration using matrix-matched standards. Compound instability was taken into account for the quantitation of transport experiments by using the stability control samples (see Stock Solutions and Compound Stability). The samples that were taken during the transport experiments after 24 h were quantified with a calibration curve based on the 24 h stability control stock solution. A calibration curve based on this stock solution was produced with at least five calibration points ranging from 5 to 100 nM (C8Cglc and C6Cglc) and from 10 to 500 nM (PCs A2, B2, B5, C1, and CB1). To obtain the same amount of matrix in each sample, the transport medium from cells, without a compound applied, was added to the calibration standards during dilution.

Control compounds were quantified by external calibration using matrix-matched standards. The transport medium from cells, without a compound applied, was added as matrix in calibration standards.

Statistical Analysis. Data are expressed as mean values ± SD. Statistical analysis was performed with a two-sided Student's *t* test with $p < 0.05$ considered as statistically significant. All experiments were performed $n \geq 9$ in at least three replicates, unless specified otherwise. The cytotoxicity assay (CCK-8) and the experiments with ^{14}C-glucose were carried out $n \geq 3$.

RESULTS

Establishment and Validation of the Caco-2 Model System. Prior to the transport studies with model compounds, the Caco-2 cell monolayer model was established and validated. Monolayer integrity and reliability were ensured by transport experiments with several control compounds (FD4, ^{14}C-sucrose, propranolol, rhodamine 123 (Rho123), and ^{14}C-glucose) and by measuring the TEER values. With our experimental conditions, the TEER values remained stable above 3000 Ω·cm^2 during the whole experiment. Further details concerning the validation of the system can be found in the Supporting Information, including Figures S3−S5.

Cytotoxicity Study. The cytotoxicity of the used model compounds in differentiated Caco-2 cells was examined by the CCK-8 assay to ensure that the compounds do not modify cell viability in the used concentrations. As no reduction in the cell viability was observed, all examined concentrations could be used for permeability studies (for details, see the Supporting Information and Figure S6).

Stability of Model Compounds and Setup of the Caco-2 System. The stability of the model compounds had to be investigated prior to the permeability experiments and was tested in the cell culture medium and transport buffer. Our first stability studies revealed that rapid degradation of C8Cglc occurs by using HBSS/HEPES at pH 7.4, 37 °C, and 5% CO_2, although this medium has often been described for transport experiments in the literature.[14,16,27] The instability of the compounds was challenging, and different transport media and buffers were tested regarding stability of the compounds and

the Caco-2 cell monolayers. Our experiments showed that a transport medium (MEM with PIPES, pH 6.5, or MEM, pH 7.4, without $NaHCO_3$, 0% CO_2, 37 °C) was the best choice to obtain stable pH values and stable compounds and ensured cell viability (as measured by TEER values) (for further details see the Supporting Information, Figures S1 and S2).

Permeability of Model Compounds. *Permeation of Catechin-C-glucosides (CCglcs).* CCglcs were applied in concentrations of 50 and 100 μM to apical (ap), basolateral (bl) or in both compartments. In the first studies with model compounds, samples were taken at different points up to 24 h (2, 4, 8, and 24 h). As a representative example, the concentration of C8Cglc in the acceptor compartment (in percentages) at different points in time and with different types of application is shown in Figure 2. The compound

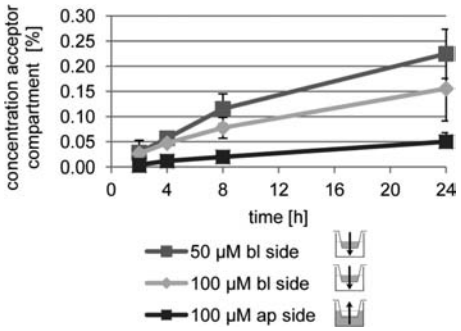

Figure 2. Permeability of C8Cglc during 24 h. Relative concentration in the acceptor compartment with apical or basolateral application of 50 and 100 μM ($n \geq 3$, mean values ± SD).

concentration increased linearly and slowly over time in the acceptor compartment. Different types of application (apical or basolateral or different concentrations) had no influence on this increase.

Because of the linear increase in concentration, only the 24 h samplings were analyzed and evaluated for the following experiments. This strategy of sampling was chosen to simulate an ongoing consumption of flavanols and their *C*-glucosides in foods such as cocoa, fruits, berries, wine, and tea.

After 24 h, an average of about 0.1% of each applied (ap or bl application as well as apical application with a pH gradient in the compartments) CCglc (C6Cglc and C8Cglc) had permeated to the acceptor compartment (see Figure 3). An application in both compartments (ap and bl) did not show any significant differences in each of the compartments after 24 h (data not shown). As far as we know, these are the first data on the permeability of CCglcs, and they demonstrate only low permeability.

The apparent permeability coefficient (P_{app}) was calculated for the apical as well as the basolateral application of C6Cglc and C8Cglc, and the values are summarized in Table 1. The values ranged from $(0.004 \pm 0.002) \times 10^{-6}$ to $(0.031 \pm 0.022) \times 10^{-6}$ cm/s for the CCglcs, including all forms of application.

Table 1. Apparent Permeability Coefficient (P_{app}) Values for Model Compounds[a]

transport direction and additional conditions	concn applied (μM)	P_{app} ($\times 10^{-6}$ cm/s)
C6Cglc		
ap-to-bl	100	0.017 ± 0.007
ap (pH 6.5)-to-bl (pH 7.4)	100	0.015 ± 0.008
ap-to-bl + verapamil	100	0.014 ± 0.008
ap (pH 6.5)-to-bl (pH 7.4) + verapamil	100	0.018 ± 0.012
ap-to-bl	50	0.017 ± 0.005
bl-to-ap	100	0.010 ± 0.005
bl (pH 7.4)-to-ap (pH 6.5)	100	0.004 ± 0.002
bl-to-ap	50	0.006 ± 0.003
C8Cglc		
ap-to-bl	100	0.025 ± 0.017
ap (pH 6.5)-to-bl (pH 7.4)	100	0.009 ± 0.006
ap-to-bl + verapamil	100	0.014 ± 0.004
ap-to-bl	50	0.031 ± 0.022
bl-to-ap	100	0.010 ± 0.006
bl (pH 7.4)-to-ap (pH 6.5)	100	0.006 ± 0.002
bl-to-ap	50	0.008 ± 0.005
PC B2 ap-to-bl	300	0.001 ± 0.000
PC B5 ap-to-bl	300	0.003 ± 0.001
CB1 ap-to-bl	300	0.002 ± 0.001
A-type dimers[b] ap-to-bl	300	0.010 ± 0.000
PC C1 ap-to-bl	300	no permeability

[a]If not specified, transport medium MEM (minimum essential medium (Eagle)), pH 6.5, was used ($n \geq 9$; apical application of procyanidin C1 (PC C1) and the catechin-*C*-glucosides in basolateral-(pH 7.4)-to-apical (pH 6.5) application: $n = 3$). Results are given as mean values ± SD. ap, apical; bl, basolateral; C6Cglc, (−)-catechin-6-*C*-glucoside; C8Cglc, (−)-catechin-8-*C*-glucoside; PC, procyanidin; CB1, procyanidin A-type trimer cinnamtannin B1. [b]Due to a rearrangement of PC A2 under transport conditions, the sum of all A-type configurational isomers formed was quantified.

Influence of P-Glycoprotein (Pgp) on the Permeability of CCglcs. By adding verapamil to the cells prior to the permeability experiments, inhibition of the efflux transporter Pgp, which is expressed only on the apical side of Caco-2 cells, is achieved. If Pgp acts as an efflux pump, inhibition would lead to increased permeation from the apical (luminal) to the

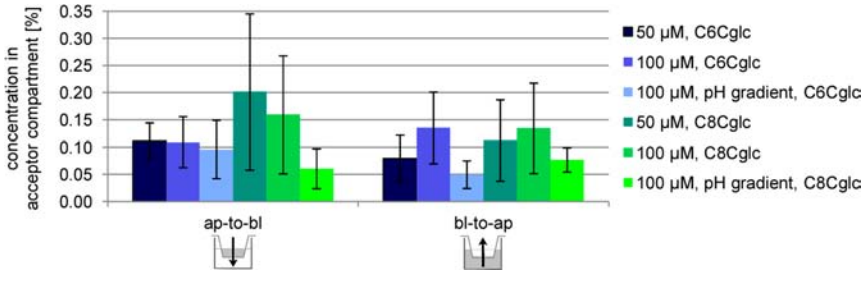

Figure 3. Relative concentrations of C6Cglc and C8Cglc in the acceptor compartment after application of 50 or 100 μM to the apical or basolateral compartments and sampling after 24 h ($n \geq 9$ in at least three different replicates, mean values ± SD).

basolateral (serosal) side, due to the absence of efflux transport. C8Cglc and C6Cglc transfer is not affected by verapamil, because ap-to-bl permeability of C8Cglc and C6Cglc does not increase with Pgp inhibition by verapamil (data not shown), which indicates that CCglcs are not substrates for Pgp.

Permeation of Procyanidins. The PCs were applied at a concentration of 300 μM apical (ap), basolateral (bl), or in both compartments. In contrast to the permeation rate of the CCglcs, PCs B2 and CB1 permeated about 0.02% from the ap to the bl sides. PC B5 showed ap-to-bl permeation of 0.04% (Figure 4). PC A2 exhibited a larger permeation rate from ap-

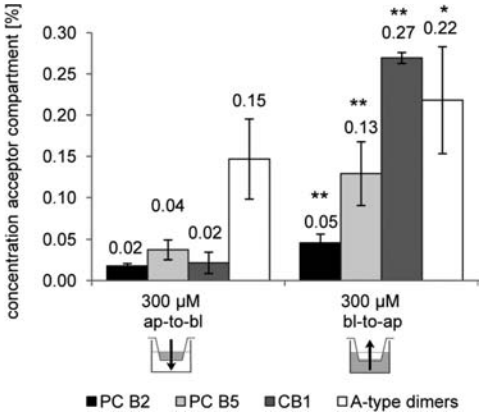

Figure 4. Relative concentrations of PCs B2, B5, nad CB1 and A-type dimers in the acceptor compartments after apical (ap) or basolateral (bl) application of 300 μM (n = 9) and sampling after 24 h. CB1 bl-to-ap: 100 μM application and n = 3 (mean values ± SD; ∗ ($p \leq 0.05$) and ∗∗ ($p \leq 0.01$) compared to ap application of corresponding compound).

to-bl side of about 0.15% (Figure 4), whereas, in contrast, ap-to-bl and bl-to-ap permeation of the trimer PC C1 was not detectable in the respective acceptor compartment. Under experimental conditions (and also without cells) it revealed that PC A2 showed a rearrangement (which was confirmed by MS^2 experiments), and therefore this PC was quantified as the sum of all configurational isomers of PC A2 formed (for further details see specific section and Figures S7 and S8 in the Supporting Information). The application of the model compound on both sides revealed no significant differences in the concentration in one of the compartments (data not shown), and these results gave no hints for an active transport. However, the bl-to-ap transport direction revealed significantly higher permeation rates than the opposite direction for the PCs (Figure 4). Higher transport in one direction may indicate an active transport.[27] To study this in more detail additional experiments with inhibition of an efflux transporter were therefore performed (see Influence of P-Glycoprotein (Pgp) on the Permeability of PCs).

The P_{app} values were calculated for the PCs after apical application. They ranged between no permeability (PC C1) and (0.010 ± 0.000) × 10^{-6} cm/s (A-type dimers) and are all listed in Table 1.

Influence of Pgp on the Permeability of PCs. The ap-to-bl permeability of PC B5 and the A-type dimers (sum of isomers observed after application of PC A2) was not enhanced as a consequence of the inhibition of Pgp. Surprisingly, the situation was different when the ap-to-bl transport of PCs B2 and CB1 was examined. Inhibition of Pgp resulted in a 3-fold increase in permeability for these PCs (Figure 5). This increase is significant, which means that Pgp seems to be involved in an active efflux transport of PCs B2 and CB1.

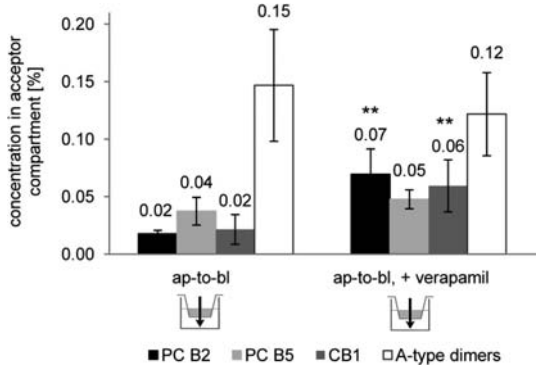

Figure 5. Relative concentrations of PCs B2, B5, and CB1 and A-type dimers in the basolateral compartments after apical application of 300 μM, with and without Pgp inhibitor verapamil, and sampling after 24 h ($n \geq 6$) (mean values ± SD; ∗ ($p \leq 0.05$) and ∗∗ ($p \leq 0.01$) compared to corresponding compound application without verapamil preincubation).

Formation of PC Metabolites. After permeability experiments with PCs, the collected samples were screened for possible metabolites, which already have been observed in other studies.[29] The PCs themselves can be methylated or degraded to the monomeric units. These monomeric units can, furthermore, be methylated, sulfated, or glucuronidated.[29] In our experiments the PCs mainly permeated unmodified, so that no metabolites were found for PCs B5 and CB1 and the A-type dimers. However, the metabolite screening revealed metabolites for PC B2. When PC B2 was applied on the basolateral side, three metabolites (EC, 3′-*O*-, and 4′-*O*-methyl-EC) could be identified in small amounts and quantified in the apical compartment after 24 h (see chromatograms, Figure S9, in the Supporting Information). For the transport studies in the opposite direction (ap-to-bl), no metabolites were detected, probably because of the larger volume of the basolateral compartment (1.5 mL) in comparison to the apical one (0.7 mL) and the limit of detection. In Figure 6, the relative concentrations of the metabolites compared to the unmodified PC B2 are represented in percentages of the applied compound.

Permeability of Glucose in the Presence of Flavan-3-ol-C-glucosides or Procyanidins. Another reason for positive

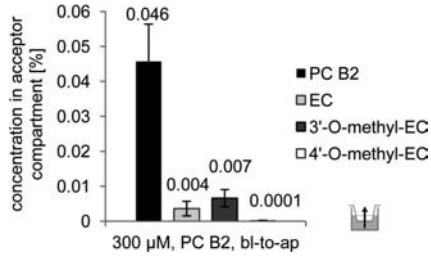

Figure 6. Relative concentrations of PC B2 and its metabolites in the apical compartment, 24 h after application of PC B2 in the basolateral compartments (n = 9 in three different replicates; mean values ± SD). Each metabolite was quantified with a corresponding external standard and matrix calibration.

health effects could be related to a described reduction of glucose absorption induced by flavonoids, which can occur, for example, by inhibition of digestive enzymes such as α-amylase, α-glucosidase, maltase, and lactase or by inhibition of glucose transporters SGLT1 or GLUT2, due to the binding of polyphenols to proteins.[30] This aspect was further investigated in our study. The first preliminary results showed that ap-to-bl glucose transport was significantly reduced (P_{app} value of (0.97 $\pm$ 0.50) $\times$ 10^{-6} cm/s) when cells were preincubated with PC A2 in comparison with the control ((6.23 $\pm$ 0.89) $\times$ 10^{-6} cm/s; without any preincubation of flavonoids). Due to the limited availability of the isolated PCs the experiments could only be performed in triplicate in one replicate.

DISCUSSION

The aim of this study was to analyze the bioavailability of flavan-3-ol-*C*-glucosides (catechin-6- and -8-*C*-glucoside) and dimeric and trimeric procyanidins (PCs B2, B5, C1, A2, and CB1) in the Caco-2 model system. It was investigated whether there are differences in the absorption of these related compounds and if a *C*-linked sugar moiety causes changes in the permeation. The Caco-2 cell model was used as it is a well-established in vitro model for the investigation of the intestinal permeability of different compounds or drugs. Artursson et al.[31] reviewed the suitability of Caco-2 monolayers as a tool to simulate drug transport close to the in vivo situation. The selected concentrations for the application of the model compounds (50−300 μM) were chosen as a reasonable compromise between already published amounts used for transport studies and the assumed human consumption. Depending on diet, age, and gender a total intake of catechins and dimeric procyanidin of up to 50−60 mg/day has been described.[32] Furthermore, the limited availability of the reference compounds as well as the detection limits of the analytical methods had to be considered.

Permeation of PCs and CCglcs. In the present study the tested PCs (except PC C1) showed ap-to-bl permeation rates between 0.02 and 0.15% within 24 h through the Caco-2 cell model. PC C1 did not show any permeability after 24 h neither after apical nor after basolateral application in our experiments. Nevertheless, some studies describe a slight absorption of PC C1.[15,18] The reason for that discrepancy compared to literature data is probably a very low permeability, which lies below the limit of detection.

The analyzed CCglcs showed permeation rates of about 0.1% to the acceptor compartment within 24 h after an ap or bl application. The current study revealed a 3-fold higher permeation of CCglcs than of PCs B2, B5, and CB1 from the apical to the basolateral side, even though the permeability of the CCglcs is low. However, low permeability does not automatically imply that the compounds have no positive impact on health, because other means of action are possible. Flavanols and PCs may, for example, protect the intestinal mucosa from oxidative stress or act against carcinogens.[33] This possible effect could be supported in the present study by a recovery of the model compounds of about 100% in the donor compartments (data not shown).

With regard to the P_{app} values for the tested model compounds, the CCglcs seem to be transported in the same way as the PCs due to their similarly low P_{app} values (Table 1). A very slow permeation with a linear increase in concentration in the opposite compartment within 24 h, which could be shown for the CCglcs (Figure 2), in addition to the general low transport ratios suggests that the CCglcs are transported by passive diffusion. The passive paracellular route of transport has already been discussed for the PCs and other flavonoids in the literature.[15,34]

The P_{app} values for the PCs are quite low and support the hypothesis of transport by passive diffusion via the paracellular route. The observation of low permeation confirms the results from previously published studies on PCs.[14,15,17,35] Zumdick et al.[15] reported P_{app} values for an ap-to-bl transport in Caco-2 cells of PC B2 of about 0.5 $\times$ 10^{-6} cm/s and of 0.3 $\times$ 10^{-6} cm/s for PC C1. Ou et al.[14] described P_{app} values of about 2 $\times$ 10^{-6} and 1 $\times$ 10^{-6} cm/s, respectively, after apical application of PCs A2 and B2. The P_{app} values for the PCs in the present study are lower than described by other groups (ranging from 0.3 $\times$ 10^{-6} to 5 $\times$ 10^{-6} cm/s) that used Caco-2 cells.[14−16] A possible reason for that could be the about 10-fold higher TEER values measured in our study.

The comparison of the absorption rates of monomeric *C*-glucosides (CCglcs), the PC dimers (PCs B2 and B5), and PC trimer (PC C1) confirms the observation of former in vitro and in vivo studies that the absorption rate depends on the degree of polymerization.[14,16,17] A higher absorption rate is assumed for compounds with a lower molecular weight than for those with higher molecular weight.[16] This correlation is also observed in our study due to the absorption of the A-type dimer (PC A2) and the A-type trimer (CB1). Furthermore, the results of the present study confirm the findings of Appeldoorn et al.[17] and Ou et al.[14] that A-type PCs are absorbed at higher rates than B-type PCs. The results concerning the low absorption rates for PCs obtained in the present Caco-2 in vitro studies are in good accordance with the results of in vivo studies in humans and animals. Two in vivo studies with humans detected low absorptions of 10.6 nmol/L PC B1 (Sano et al.[36]) and 41 nmol/L PC B2 (Holt et al.[37]) after intake of a proanthocyanidin-rich grape seed extract (2 g) or a flavanol-rich cocoa (0.375 g/kg body weight), respectively. In a feeding study with pigs, which have a gastrointestinal tract similar to that of humans, Rzeppa et al.[18] quantified a relative urinary excretion of 0.008% PC B2 and 0.004% PC C1.

An enhanced permeability of CCglcs due to the sugar moiety is theoretically conceivable. Kreuz et al.[38] detected aspalathin (a *C*-linked dihydrochalcone glucoside) in small but higher amounts than its aglycone in the urine of pigs, and Hollman et al.[19] showed a higher absorption of dietary quercetin glycosides (52% for glucosides and 17% for rutinosides) than for the quercetin aglycone (24%) in healthy ileostomy volunteers. These values were achieved on the basis of oral intake and ileostomy excretion, considering the degradation in the ileostomy bag. In contrast, a poor absorption of *C*-linked flavones has been shown by Zhang et al.[39] investigating the absorption of the flavone-*C*-glucosides luteolin-8- and -6-*C*-glucoside and apigenin-8- and -6-*C*-glucoside in rats. Although in our study flavan-3-ol derivatives were used rather than flavones, these data are in good accordance with our findings.

Furthermore, we proved that the CCglcs are not a substrate for the efflux transporter Pgp. If an efflux transporter is actively participating in the transport of the CCglcs, transport ratios in the bl-to-ap direction would be higher than in the ap-to-bl direction,[27] and after an application to both compartments, higher concentrations in the apical compartment should be detectable. This could not be shown in our study.

In contrast, our studies revealed that Pgp seems to be involved in an active efflux transport of PCs B2 and CB1.

Zumdick et al.[15] have presented similar results for PC B2 from an extract of hawthorn leaves and flowers. However, to our knowledge it has not been described yet that the A-type trimer CB1 is a substrate for the Pgp transporter. Both results in combination emphasize a possible involvement of Pgp in an active efflux transport of some PCs. Due to an increased bl-to-ap transport of all tested PCs, presumably other ABC transporters may be involved.

Influence on the Glucose Transport. The ap-to-bl glucose transport was significantly reduced when cells were preincubated with PC A2 in comparison with the control. A decreased glucose uptake might be positive with regard to various beneficial health effects, for example, type 2 diabetes,[30] but a reduced uptake could also have negative effects due to the necessity of glucose as an energy source.[40] A study by Khan et al. in humans with type 2 diabetes revealed reduced plasma glucose levels after intake of cinnamon.[41] Prominent components of cinnamon are the PCs, especially the A-type PCs.[42] The result obtained in our study, that PC A2 decreased the glucose transport, might explain the reduced serum glucose levels in the study of Khan et al.;[41] however, further studies are needed to support this hypothesis.

In summary, we could show a slight permeation of several procyanidins and catechin-*C*-glucosides of about 0.02−0.27% (ap-to-bl and bl-to-ap) after 24 h in the Caco-2 model and only the trimeric procyanidin C1 was not transported. Pgp seems to be involved in the efflux transport of PCs B2 and CB1 as the ap-to-bl permeation increased when Pgp was inhibited. These are the first results showing the permeability of flavan-3-ol-*C*-glucosides across the Caco-2 cell monolayer and CB1 as a Pgp substrate.

■ ASSOCIATED CONTENT

Supporting Information

Methods and results for the establishment and validation of the Caco-2 model system, cytotoxicity assay, and quantitation of PC A2; stability of CCglcs in MEM at pH 6.5 and 7.4 (Figure S1), TEER values of Caco-2 cells in MEM (pH 6.5 and 7.4) prior to and during permeability experiments (Figure S2), transport experiment of FD4 (Figure S3), transport of Rho123 (Figures S4 and S5), cytotoxicity of model compounds (Figure S6), HPLC-MS/MS chromatogram of PC A2 (Figure S7), total ion chromatogram and ESI-MS-MS product spectra of PC A2 and newly formed peaks (Figure S8), and HPLC-MS/MS chromatogram of PC B2 and its metabolites (Figure S9). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(H.-U.H.) Phone: +49 251 8333391. Fax: +49 251 8333396. E-mail: humpf@wwu.de.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

3′-*O*-methyl-EC, 3′-*O*-methylepicatechin; 4′-*O*-methyl-EC, 4′-*O*-methylepicatechin; ACN, acetonitrile; ap, apical; bl, basolateral; C6Cglc, (−)-catechin-6-*C*-glucoside; C8Cglc, (−)-catechin-8-*C*-glucoside; CB1, procyanidin A-type trimer cinnamtannin B1; CCglcs, (−)-catechin-*C*-glucosides; CCK-8, Cell Counting Kit-8; CE, collision energy; CXP, cell exit potential; DP, declustering potential; EC, epicatechin; EP, entrance potential; FA, formic acid; FCglcs, flavan-3-ol-*C*-glucosides; FD4, fluorescein isothiocyanate-dextran 4000; HBSS, Hank's balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; MEM, minimum essential medium (Eagle); MeOH, methanol; MW, molecular weight; P_{app}, apparent permeability coefficient; PC, procyanidin; Pgp, P-glycoprotein; PIPES, piperazine-*N*,*N*′-bis(2-ethansulfonic acid); Rho123, rhodamine 123; SD, standard deviation; TEER, transepithelial electrical resistance; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt

■ REFERENCES

(1) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79* (5), 727−747.

(2) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds − nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.

(3) Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl.), 243S−255S.

(4) Rios, L. Y.; Bennett, R. N.; Lazarus, S. A.; Remesy, C.; Scalbert, A.; Williamson, G. Cocoa procyanidins are stable during gastric transit in humans. *Am. J. Clin. Nutr.* **2002**, *76* (5), 1106−1110.

(5) Rzeppa, S.; Von Bargen, C.; Bittner, K.; Humpf, H. U. Analysis of flavan-3-ols and procyanidins in food samples by reversed phase high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (RP-HPLC-ESI-MS/MS). *J. Agric. Food Chem.* **2011**, *59* (19), 10594−10603.

(6) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **2004**, *134* (3), 613−617.

(7) Krafczyk, N.; Glomb, M. A. Characterization of phenolic compounds in rooibos tea. *J. Agric. Food Chem.* **2008**, *56* (9), 3368−3376.

(8) Stark, T.; Hofmann, T. Application of a molecular sensory science approach to alkalized cocoa (*Theobroma cacao*): structure determination and sensory activity of nonenzymatically C-glycosylated flavan-3-ols. *J. Agric. Food Chem.* **2006**, *54* (25), 9510−9521.

(9) Kashiwada, Y.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XLV. Rhubarb. (5): Isolation and characterization of flavan-3-ol and procyanidin glucosides. *Chem. Pharm. Bull.* **1986**, *34* (10), 3208−3222.

(10) Aron, P. M.; Kennedy, J. A. Flavan-3-ols: nature, occurrence and biological activity. *Mol. Nutr. Food Res.* **2008**, *52* (1), 79−104.

(11) Talhi, O.; Silva, A. M. S. Advances in C-glycosylflavonoid research. *Curr. Org. Chem.* **2013**, *16* (7), 859−896.

(12) Andujar, I.; Recio, M. C.; Giner, R. M.; Rios, J. L. Cocoa polyphenols and their potential benefits for human health. *Oxid. Med. Cell. Longevity* **2012**, DOI: 10.1155/2012/906252.

(13) Tanwar, B.; Modgil, R. Flavonoids: dietary occurrence and health benefits. *Spatula DD* **2012**, *2* (1), 59−68.

(14) Ou, K.; Percival, S. S.; Zou, T.; Khoo, C.; Gu, L. Transport of cranberry A-type procyanidin dimers, trimers, and tetramers across monolayers of human intestinal epithelial Caco-2 cells. *J. Agric. Food Chem.* **2012**, *60* (6), 1390−1396.

(15) Zumdick, S.; Deters, A.; Hensel, A. In vitro intestinal transport of oligomeric procyanidins (DP 2 to 4) across monolayers of Caco-2 cells. *Fitoterapia* **2012**, *83* (7), 1210−1217.
(16) Kosinska, A.; Andlauer, W. Cocoa polyphenols are absorbed in Caco-2 cell model of intestinal epithelium. *Food Chem.* **2012**, *135* (3), 999−1005.
(17) Appeldoorn, M. M.; Vincken, J. P.; Gruppen, H.; Hollman, P. C. Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. *J. Nutr.* **2009**, *139* (8), 1469−1473.
(18) Rzeppa, S.; Bittner, K.; Doll, S.; Danicke, S.; Humpf, H. U. Urinary excretion and metabolism of procyanidins in pigs. *Mol. Nutr. Food Res.* **2012**, *56* (4), 653−665.
(19) Hollman, P. C.; de Vries, J. H.; van Leeuwen, S. D.; Mengelers, M. J.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62* (6), 1276−1282.
(20) Day, A. J.; Canada, F. J.; Diaz, J. C.; Kroon, P. A.; McLauchlan, R.; Faulds, C. B.; Plumb, G. W.; Morgan, M. R.; Williamson, G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **2000**, *468* (2−3), 166−170.
(21) Hollman, P. C.; Katan, M. B. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed. Pharmacother.* **1997**, *51* (8), 305−310.
(22) Manach, C.; Morand, C.; Texier, O.; Favier, M. L.; Agullo, G.; Demigne, C.; Regerat, F.; Remesy, C. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J. Nutr.* **1995**, *125* (7), 1911−1922.
(23) Sanugul, K.; Akao, T.; Li, Y.; Kakiuchi, N.; Nakamura, N.; Hattori, M. Isolation of a human intestinal bacterium that transforms mangiferin to norathyriol and inducibility of the enzyme that cleaves a C-glucosyl bond. *Biol. Pharm. Bull.* **2005**, *28* (9), 1672−1678.
(24) Hasslauer, I.; Oehme, A.; Locher, S.; Valotis, A.; Van't Slot, G.; Humpf, H. U.; Schreier, P. Flavan-3-ol C-glycosides − preparation and model experiments mimicking their human intestinal transit. *Mol. Nutr. Food Res.* **2010**, *54*, 1−10.
(25) Stalmach, A.; Mullen, W.; Pecorari, M.; Serafini, M.; Crozier, A. Bioavailability of C-linked dihydrochalcone and flavanone glucosides in humans following ingestion of unfermented and fermented rooibos teas. *J. Agric. Food Chem.* **2009**, *57* (15), 7104−7111.
(26) Van't Slot, G.; Mattern, W.; Rzeppa, S.; Grewe, D.; Humpf, H. U. Complex flavonoids in cocoa: synthesis and degradation by intestinal microbiota. *J. Agric. Food Chem.* **2010**, *58* (15), 8879−8886.
(27) Hubatsch, I.; Ragnarsson, E. G.; Artursson, P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat. Protoc.* **2007**, *2* (9), 2111−2119.
(28) Walgren, R. A.; Walle, U. K.; Walle, T. Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochem. Pharmacol. (Amsterdam, Neth.)* **1998**, *55* (10), 1721−1727.
(29) Monagas, M.; Urpi-Sarda, M.; Sanchez-Patan, F.; Llorach, R.; Garrido, I.; Gomez-Cordoves, C.; Andres-Lacueva, C.; Bartolome, B. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food Funct.* **2010**, *1* (3), 233−253.
(30) Williamson, G. Possible effects of dietary polyphenols on sugar absorption and digestion. *Mol. Nutr. Food Res.* **2013**, *57* (1), 48−57.
(31) Artursson, P.; Palm, K.; Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Delivery Rev.* **2012**, *64*, 280−289.
(32) de Pascual-Teresa, S.; Moreno, D. A.; Garcia-Viguera, C. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. *Int. J. Mol. Sci.* **2010**, *11* (4), 1679−1703.
(33) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl.), 230S−242S.
(34) Tian, X. J.; Yang, X. W.; Yang, X.; Wang, K. Studies of intestinal permeability of 36 flavonoids using Caco-2 cell monolayer model. *Int. J. Pharm. (Amsterdam, Neth.)* **2009**, *367* (1−2), 58−64.
(35) Déprez, S.; Mila, I.; Huneau, J. F.; Tome, D.; Scalbert, A. Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. *Antioxid. Redox. Signaling.* **2001**, *3* (6), 957−967.
(36) Sano, A.; Yamakoshi, J.; Tokutake, S.; Tobe, K.; Kubota, Y.; Kikuchi, M. Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Biosci., Biotechnol., Biochem.* **2003**, *67* (5), 1140−1143.
(37) Holt, R. R.; Lazarus, S. A.; Sullards, M. C.; Zhu, Q. Y.; Schramm, D. D.; Hammerstone, J. F.; Fraga, C. G.; Schmitz, H. H.; Keen, C. L. Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am. J. Clin. Nutr.* **2002**, *76* (4), 798−804.
(38) Kreuz, S.; Joubert, E.; Waldmann, K. H.; Ternes, W. Aspalathin, a flavonoid in *Aspalathus linearis* (rooibos), is absorbed by pig intestine as a C-glycoside. *Nutr. Res. (N.Y.)* **2008**, *28* (10), 690−701.
(39) Zhang, Y.; Tie, X.; Bao, B.; Wu, X.; Zhang, Y. Metabolism of flavone C-glucosides and p-coumaric acid from antioxidant of bamboo leaves (AOB) in rats. *Br. J. Nutr.* **2007**, *97* (3), 484−494.
(40) Pellerin, L. Food for thought: the importance of glucose and other energy substrates for sustaining brain function under varying levels of activity. *Diabetes Metab.* **2010**, *36*, S59−S63.
(41) Khan, A.; Safdar, M.; Ali Khan, M. M.; Khattak, K. N.; Anderson, R. A. Cinnamon improves glucose and lipids of people with type 2 diabetes. *Diabetes Care* **2003**, *26* (12), 3215−3218.
(42) Mateos-Martin, M. L.; Fuguet, E.; Quero, C.; Perez-Jimenez, J.; Torres, J. L. New identification of proanthocyanidins in cinnamon (*Cinnamomum zeylanicum* L.) using MALDI-TOF/TOF mass spectrometry. *Anal. Bioanal. Chem.* **2012**, *402* (3), 1327−1336.