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Pharmacokinetics and Bioavailability of the Bioflavonoid Biochanin A: Effects of Quercetin and EGCG on Biochanin A Disposition in Rats

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Abstract: Little is known regarding pharmacokinetic (PK) or pharmacodynamic interactions of flavonoids with each other: this is of significance since multiple flavonoids are present in the diet and in dietary supplements. Our objective was to determine the effect of quercetin and (-)-epigallocatechin-3-gallate (EGCG), major flavonoids present in the diet, on the PK and bioavailability of biochanin A, a flavonoid with chemopreventive properties. BCA was administered to rats intravenously (5 mg/kg) or orally (16.67 or 50 mg/kg) with or without concomitant EGCG and quercetin. In vitro studies with the human intestinal Caco-2 and human hepatic HepG2 cell lines were performed to evaluate the effects of quercetin and EGCG on the cellular metabolism of BCA, and studies with human breast cancer MCF-7 cells that overexpress P-glycoprotein or BCRP (MCF-7/ADR and MCF-7/MX100 cells, respectively) or MDCK cells that express MRP2 (MDCK-MRP2) were performed to evaluate the effects of cellular efflux. An HPLC assay was used to determine plasma, urine, and cellular concentrations of BCA and the conjugated metabolites of BCA (following enzymatic hydrolysis). The coadministration of quercetin and EGCG significantly increased the BCA area under the plasma concentration vs time curve (AUC) in rats, after both iv and oral administration of BCA. The AUC of total BCA (unchanged + conjugated) was also increased. The increases in BCA AUC reflected predominantly increased bioavailability; this was true even after iv administration due to an apparent increase in the enterohepatic cycling of BCA. Our findings demonstrate for the first time that the administration of multiple flavonoids results in increased flavonoid bioavailability, as well as a decrease in clearance, potentially due to increased enterohepatic cycling.

Keywords: Biochanin A; bioavailability; combinations of flavonoids; transporter; metabolism; quercetin; EGCG

Introduction

Flavonoids are components in a wide range of fruits, vegetables, and plant-derived beverages and have low toxicity. Biochanin A (BCA) (5,7-dihydroxy-4′-methoxy-isoflavone, Figure 1) is the major isoflavone constituent in red clover and certain herbal products marketed for the alleviation of menopausal symptoms.[1] It also has been reported to have antioxidant,[2] anti-inflammatory,[3] antiviral,[4] and anticarcinogenic effects,[5] and protective effects on endothelial integrity and function.[6] We have reported that BCA treatment produces beneficial chemopreventive alter-

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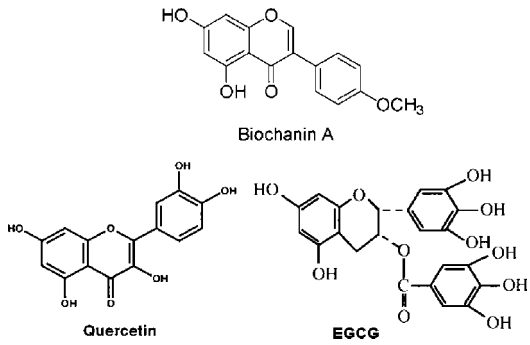

Figure 1. Chemical structures of BCA, quercetin, and EGCG.

ations in gene expression at concentrations that can be obtained following dietary intake.[7]

However, a major concern has been the poor oral bioavailability of BCA, similar to other flavonoids. Our previous studies indicated that BCA exhibits a high clearance and a high volume of distribution in rats and has a poor bioavailability (1–2%), due, at least in part, to extensive metabolism to glucuronide and sulfate conjugates both in the intestine and liver.[8] The main conjugation reaction of BCA in rat liver and intestinal microsome preparations is glucuronidation (K_m of 2.7–7.0 μM; V_{max}/K_m of 0.12 in liver and 0.40 in duodenum).[9] BCA can also undergoes *O*-demethylation to genistein or hydroxylation by cytochrome P450 (CYP) enzymes.[10,11] In human or rat liver microsomes, the hydroxylated products are 3′-, 6-, or 8-hydroxy BCA or GEN,[10,12] but the in vivo significance of these oxidative metabolites is unknown. Even though BCA is a highly permeable compound, intestinal absorption of BCA is limited by the high rate of intestinal metabolism to conjugated metabolites and the potential efflux of BCA back into the intestine by ATP binding cassette (ABC) proteins present in intestinal cell apical membranes.[9] Biliary excretion of BCA mediated by ABC transport proteins, followed by enterohepatic recirculation, also plays an important role in the disposition of BCA.[8]

There may be possible ways to improve the oral bioavailability of BCA. Methylation of BCA at the 7-position may inhibit glucuronidation/sulfation, as the presence of free hydroxyl groups may result in increased glucuronidation and sulfation.[13] Fully methylated flavones have higher metabolic stability than their unmethylated forms.[13] However, methylation of BCA may reduce the affinity to the receptors (i.e., estrogen receptor) resulting in decreased activity, although Walle et al.[14] reported that 5,7-dimethylflavone and 5,7,4′-trimethylflavone were more potent than their unmethylated analogs with regard to inhibition of cellular proliferation. In addition, methylation does not inhibit cytochrome P450 oxidation.[13] Another method that has been used to increase bioavailability is through the incorporation of flavonoids in liposomal or nanoparticle drug delivery systems. The formation of pegylated liposomal quercetin has been reported to improve the solubility and bioavailability of quercetin in BALB/c mice bearing CT26 colon adenocarcinoma and H22 hepatoma.[15] Permeation of ellagic acid (polyphenol antioxidant) was increased by the preparation of nanoparticles using a cosolvent in an *in situ* intestinal permeability study.[16]

In the present study, our hypothesis was that multiple flavonoids alter the pharmacokinetics (PK) and bioavailability

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of individual flavonoids and may represent a strategy to increase the bioavailability of individual flavonoids; additionally, such combinations may result in additive or synergistic pharmacodynamic effects due to differing mechanisms of action. In the present study, we examined the effect of quercetin and (–)-epigallocatechin-3-gallate (EGCG) (Figure 1) on the absorption and disposition of BCA in rats. We chose quercetin and EGCG because they are among the most widely consumed flavonoids. Our studies found an increased AUC after both iv and oral administration. Mechanisms underlying the observed interaction were investigated using human intestinal Caco-2 cells, human hepatic HepG2 cells, Madin–Darby canine kidney (MDCK) cells transfected with human multidrug resistance-associated protein 2 (MRP2, ABCC2), and human breast cancer MCF-7 cells overexpressing P-glycoprotein (P-gp, ABCB1) or breast cancer resistance protein (BCRP, ABCG2).

Experimental Methods

Materials. BCA, quercetin, EGCG, flavone (internal standard), mitoxantrone, and β-glucuronidase/sulfatase from *Helix pomatia* were purchased from Sigma (St. Louis, MO). RPMI 1640, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were from Gibco BRL (Buffalo, NY). Caco-2 and HepG2 cells were obtained from American type Cell Culture Collection (ATCC, Manassas, VA). MCF-7/sensitive and MCF-7/MX100 were kind gifts from Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD). MCF-7/Adr cells were obtained from the National Cancer Institute. MDCK-WT (parent) and MDCK cells transfected with the human MRP2 gene (MDCK-MRP2) were gifts from Dr. Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands).

Pharmacokinetic Studies in Rats. Male Sprague–Dawley rats weighing 200–300 g were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN), and used for all the kinetic studies. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee guidelines and followed approved protocols. Animals were acclimated with regular rat chow and drinking water ad libitum for 1 week before the study. A jugular vein cannula was implanted two days prior to the study and used for iv dosing and blood sampling. For the intravenous dose, BCA was dissolved in DMSO and then diluted 20-fold with 25% hydroxypropyl-β-cyclodextrin (HPBCD) and dosed at a 5 mg/kg concentration alone or concomitantly with 5 mg/kg quercetin + 5 mg/kg EGCG. Oral doses of BCA were given by oral gavage in DMSO and olive oil at the ratio of 1:20 with four treatment regimens: (1) 50 mg/kg (176 μmol/kg) of BCA, (2) 50 mg/kg (176 μmol/kg) of BCA + 5 mg/kg (14.8 μmol/kg) of quercetin + 5 mg/kg (10.9 μmol/kg) of EGCG, (3) 50 mg/kg (176 μmol/kg) of BCA + 50 mg/kg (148 μmol/kg) of quercetin + 50 mg/kg (109 μmol/kg) of EGCG, (4) 16.67 mg/kg (58.6 μmol/kg) of BCA, and (5) 16.67 mg/kg (58.6 μmol/kg) of BCA + 16.67 mg/kg (49.3 μmol/kg) of quercetin + 16.67 mg/kg (36.4 μmol/kg) of EGCG.

Approximately 0.2 mL of blood was harvested from the jugular vein cannula at various time points and replaced with the same volume of saline. For the iv studies, the first 0.2 mL of blood was discarded to avoid contamination. Urine was collected over 24 h. Samples were stored in a −80 °C freezer until the HPLC analysis of BCA.

Hydrolysis of Conjugates. "Total" BCA (free aglycon plus aglycon following the hydrolysis of the glucuronide and sulfate conjugates) in plasma and urine were measured following incubation with glucuronidase/sulfatase type H-5 (from *Helix pomatia*, Sigma Chemical, St. Louis, MO) as previously described.[8] Fifty microliters of 0.2% sodium chloride containing sulfatase/glucuronidase (1000 units/mL) and 100 μL of 0.2 M sodium acetate (pH 5.0 at 37 °C) were added to 50 μL of cell lysate and incubated at 37 °C for 90 min. The reaction was stopped by adding a 1 mL of ether and centrifuging. The supernatant was removed, dried under nitrogen, and then resuspended in 50/50 methanol/acetonitrile. Free BCA concentrations were measured in the same fashion as described for the total BCA except that 50 μL of 0.2% sodium chloride without enzymes was added to samples. BCA sulfate and/or glucuronide conjugate levels were determined by subtracting the free aglycon level from the level determined after glucuronidase/sulfatase digestion.

High-Performance Liquid Chromatography Analysis. The analysis was carried out on a Waters HPLC system (Milford, MA) equipped with an Alltech Alltima column (C18, 250 mm × 4.6 mm i.d., 5 μm, Grace, Deerfield, IL). The mobile phase consisted of 45% acetonitrile in 1% of acetic acid. The UV detector was set at a single wavelength of 260 nm. The lower limit of quantification was 5 ng/mL. The assay was evaluated for any potential interferences by quercetin, EGCG, or their metabolites with the BCA peak. Quercetin is more polar than BCA in this assay and elutes earlier (5.8 min retention time compared with 12 min retention time for BCA), and the metabolites of quercetin (isorhamnetin and the conjugated metabolites) are all more polar than BCA. EGCG was not detected under our assay conditions.

PK Data Analysis. The pharmacokinetic parameters of BCA were analyzed by noncompartmental analysis using WinNonlin Professional version 2.1 (Pharsight Corp., Mountain View, CA). The area under the concentration–time curve (AUC) of BCA was calculated by the trapezoidal method; the maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration were obtained by visual inspection of the experimental data; the terminal half-life ($t_{1/2}$) was calculated as $0.693/k$, with k representing the slope of the terminal regression line. The bioavailability (F) of BCA was calculated by the following equation:

$$F = \frac{\text{oralAUC}_{0-\infty}}{\text{ivAUC}_{0-\infty}} \times \frac{\text{ivDose}}{\text{oralDose}} \times 100$$

One-way ANOVA followed by Newman–Keuls test and Student's unequal variance t test with $p < 0.05$ set as the significance level were used for statistical analysis.

Cell Culture. Caco-2 cells were cultured in 75 cm^2 flasks with MEM culture medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids (Gibco BRL, Buffalo, NY), and 1% antibiotic–antimycotic solution (Gibco BRL, Buffalo, NY). Human liver cancer cells (HepG2) were cultured in 75 cm^2 flasks with minimal essential media and Earl's salts (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acid solution (Gibco BRL), and 1 mM pyruvic acid. MDCK-WT and MDCK-MRP2 cells were grown in 75 cm^2 flasks with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. MCF-7 cells (both parent and MDR subtype) were cultured in 75 cm^2 flasks with RPMI 1640 culture media supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 10% CO_2/90% air. Culture media also contained 100 units/mL of penicillin and 100 μg/mL of streptomycin. For mitoxantrone-selected MCF-7/MX100 cells, the culture media also contained 100 nM mitoxantrone. All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2/95% air. For the drug accumulation studies, cells were seeded onto 35 mm^2 dishes at a density of approximately 5×10^5 cells per dish.

Metabolism Studies in Caco-2 Cells and HepG2 Cells. BCA alone (3.33 μM) or a mixture of BCA, quercetin, and EGCG (3.33 μM each) was added to the cells to assess the metabolism of BCA. Both Caco-2 cells and HepG2 cells demonstrate good activity for glucuronidation and sulfation,[17–19] the two major metabolic pathways for BCA. The concentration was chosen since it represents an intestinal concentration that could be achieved in vivo.[20] All experiments were performed in triplicate. After 1-h incubations, media were aspirated, and the cells were washed with 10 mL of cold 1 × PBS three times. Cells were harvested by scraping, lysed by Branson SONIFIER ultrasonic cell disruptor (Branson Ultrasonics Corp., Danbury, CT) in 300 μL of double distilled water. BCA conjugates were determined as described in the Hydrolysis of Conjugates section.

Accumulation Studies in MCF-7, Caco-2, and MDCK Cells. Cells grown in 35 mm^2 culture dishes were washed twice with the assay buffer (137 mM NaCl, 54 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.4) and then incubated with 24 nM ^{3}H-BCA (Moravek Biochemicals, Brea, CA) containing 16.67 μM of BCA or BCA with quercetin and EGCG (16.67 μM each) at room temperature for 5 min. The rationale for choosing this time point was to capture initial cellular concentrations of BCA since BCA is rapidly absorbed and efficiently conjugated, and the conjugates are quickly excreted.[9] The uptake reaction of ^{3}H-BCA was stopped by rinsing the cells four times with ice-cold buffer (137 mM NaCl, and 14 mM Tris base, pH 7.4). Cells were then solubilized using a solution of 0.3 N NaOH and 1% SDS, and aliquots were used to determine the radioactivity by liquid scintillation counting (1900 CA, Tri-Carb Liquid Scintillation Analyzer, Packard) and protein content using the BCA protein assay. Results were normalized to the protein content of the cells in each dish, and ^{3}H-BCA uptake values were expressed as percent accumulation in the control group.

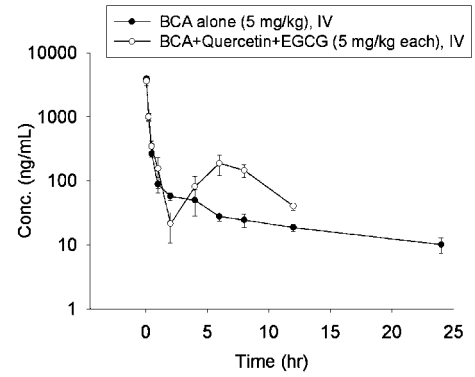

Figure 2. Mean (±SD) plasma concentration–time curves of BCA administered intravenously in the presence (open circle) and absence (closed circle) of 5 mg/kg of quercetin and 5 mg/kg of EGCG (n = 3–4).

Results

Biochanin A Concentrations in Plasma and Urine. The pharmacokinetic profiles of BCA after administration of BCA alone and in combination are shown in Figure 2 (intravenous administration) and Figure 3 (oral administration). C_{max} and AUC values are summarized in Table 1. The iv studies indicated that BCA exhibits a high volume of distribution in rats, likely due to the hydrophobicity of BCA. The plasma concentration versus time plots for intravenous BCA indicated that administration of BCA with quercetin and EGCG resulted in a large re-entry peak (Figure 2), not evident in rats receiving BCA alone. The $AUC_{0–12}$ was significantly increased by the coadministration of quercetin and EGCG (1.54-fold, Table 1A), and subsequently, CL was significantly decreased by the coadministration of 5 mg/kg of quercetin and 5 mg/kg of EGCG intravenously (0.65-fold and 0.50-fold, respectively, Table 1A).

Following the oral administration of both 50 mg/kg of BCA alone and 50 mg/kg of BCA with 5 mg/kg of quercetin and 5 mg/kg of EGCG, a re-entry peak was observed. The C_{max} values of BCA were not significantly changed (Table 1B). The plasma concentration of BCA at 24 h was measurable in only one rat that received the mixture of flavonoids (data not shown), but the $AUC_{0–8}$ values were not significantly different (Table 1B).

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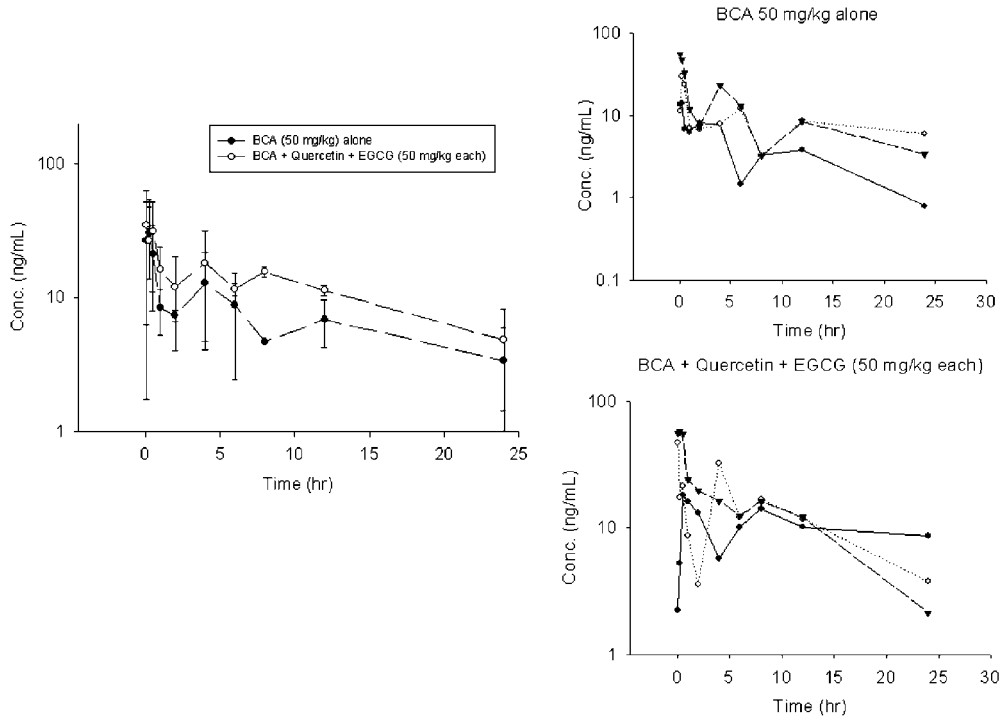

Figure 3. Mean (±SD) plasma concentration–time curves (left) and individual profiles (right) of BCA administered orally in the presence of and absence of 50 mg/kg of quercetin and 50 mg/kg of EGCG ($n = 3$).

When rats received 50 mg/kg of BCA orally, with 50 mg/kg of quercetin and 50 mg/kg of EGCG, the AUC_{0-24} of BCA was significantly increased compared to the AUC_{0-24} obtained after administration of 50 mg/kg of BCA alone (2.03-fold, $p < 0.01$, Table 1C, Figure 3). The oral bioavailability (F) of BCA was significantly increased by coadministration of 50 mg/kg of quercetin and 50 mg/kg of EGCG (3.44-fold, $p < 0.05$), although it still remained low. The 24 h urinary excretion of BCA was significantly higher in rats fed 50 mg/kg of BCA with 50 mg/kg of quercetin and 50 mg/kg of EGCG compared to animals who received 50 mg/kg of BCA alone (2.97-fold, $p < 0.05$, Figure 4A).

When rats received a lower dose of BCA (16.67 mg/kg) with 16.67 mg/kg of quercetin and 16.67 mg/kg of EGCG orally, concentrations of BCA in plasma samples of rats were only measurable in the combination group (data not shown); BCA could not be detected in the plasma samples for the animals receiving BCA alone. Consistent with the plasma data, the 24 h urinary excretion of BCA was significantly higher in rats fed the mixture of flavonoids compared to rats fed 16.67 mg/kg BCA alone (2.69-fold, $p < 0.05$, Figure 4B).

Biochanin A Glucuronide and Sulfate Conjugates. To examine if the coadministration of quercetin and EGCG could affect the extent of BCA conjugation, we measured total BCA glucuronide and sulfate conjugate concentrations in plasma and urine, following enzymatic hydrolysis. Almost all BCA was conjugated after oral administration (% conjugates = 98.9%), whereas a larger percentage of BCA remained unconjugated after iv administration (% conjugates = 30.8%) (Table 2). This suggests that the intestine represents the major site of BCA conjugation. The AUC of both total BCA (unchanged plus its conjugated metabolites) and BCA conjugates significantly increased following the intravenous administration of BCA with quercetin and EGCG (Table 2). When rats received the 50 mg/kg dose of BCA with 5 mg/kg of quercetin and 5 mg/kg of EGCG or with 50 mg/kg of quercetin and 50 mg/kg of EGCG orally, the mean AUC values for both total BCA and BCA conjugates were increased, but these changes are not statistically significant due to the large variability observed (Table 2B,C). The 24 h urinary excretion of total BCA, BCA conjugates, and % conjugates increased with the coadministration of quercetin and EGCG in all cases (Table 3).

Effects of Quercetin and EGCG on the Metabolism of BCA in Caco-2 and HepG2 Cells. Studies were performed using human intestinal Caco-2 cells and human hepatic HepG2 cells to investigate if the coincubation of BCA with quercetin and EGCG could affect the metabolism of BCA in human intestinal and hepatic cells. Significantly higher concentrations of BCA aglycon were observed in both Caco-2 and HepG2 cells after a 1 h incubation with BCA, quercetin, and EGCG (3.33 μM each) than in cells incubated with BCA alone (3.33 μM) (1.51-fold in Caco-2 cells, $p < 0.05$, Figure 5A; 3.06-fold in HepG2 cells, $p < 0.05$, Figure 5B). Significantly less conjugated metabolites were formed in Caco-2 cells incubated with the mixture than in cells incubated with BCA alone (0.225-fold, $p < 0.001$, Figure 5A). Conjugation of BCA was also reduced in HepG2 cells (0.490-

Table 1. PK Parameters of BCA after Intravenous (A) and Oral (B and C) Administration (Mean ± SD)

(A) (5 mg/kg of BCA IV) vs (5 mg/kg of BCA with 5 mg/kg of EGCG and 5 mg/kg of Quercetin IV)			
	5 mg/kg of BCA IV	5 mg/kg of MIX IV	fold change[a]
CL (l/h/kg)	2.76 ± 0.232	1.78 ± 0.401	**0.646***
V_{ss} (l/kg)	15.5 ± 3.99	11.7 ± 5.19	0.750
AUC_{0-12} (ng·h/mL)	1210 ± 159	1870 ± 399	**1.54***
(B) (50 mg/kg of Oral BCA) vs (50 mg/kg of BCA with 5 mg/kg of EGCG and 5 mg/kg of Quercetin)			
	50 mg/kg of BCA PO	5 mg/kg of MIX PO	fold change
$t_{1/2}$ (h)	8.78 ± 6.80	16.3 ± 13.0	1.86
C_{max} (ng/mL)	33.3 ± 21.0	49.5 ± 29.1	1.49
AUC_{0-8} (ng·h/mL)	87.9 ± 37.6	186 ± 127	2.12
F	0.00802 ± 0.00258	0.0151 ± 0.0100	1.88
(C) (50 mg/kg of Oral BCA) vs (50 mg/kg of BCA with 50 mg/kg of EGCG and 50 mg/kg of Quercetin)			
	50 mg/kg of BCA PO	50 mg/kg of MIX PO	fold change[a]
$t_{1/2}$ (h)	8.78 ± 6.80	14.4 ± 14.2	1.64
C_{max} (ng/mL)	33.3 ± 21.0	40.2 ± 19.9	1.21
AUC_{0-24} (ng·h/mL)	137 ± 45.4	278 ± 28.4	**2.03****
F	0.00802 ± 0.00258	0.0233 ± 0.00980	**3.44***

[a] Statistically significant difference: *$P < 0.05$; **$P < 0.01$.

fold, Figure 5B), but it was not statistically significant. A mixture of quercetin and EGCG also resulted in less of the metabolite genistein being produced from BCA in HepG2 cells, compared to BCA treatment alone (0.850-

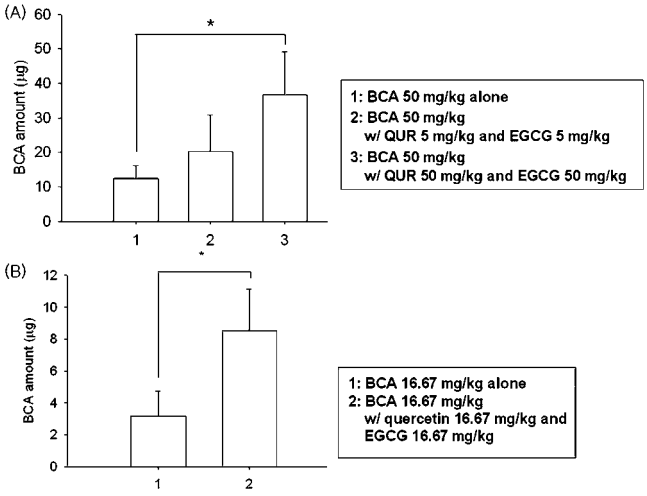

Figure 4. Mean (±SD) amount of BCA in urine after 24 h when 50 mg/kg of BCA was given orally in the presence of and absence of 5 mg/kg of quercetin and 5 mg/kg of EGCG or 50 mg/kg of quercetin and 50 mg/kg of EGCG ($n = 3$) (A). Mean (±SD) amount of BCA in urine when 16.67 mg/kg BCA was given orally in the presence of and absence of 16.67 mg/kg quercetin and 16.67 mg/kg EGCG ($n = 3$) (B).

Table 2. The AUC_{0-t} of Total BCA (Unchanged Plus Its Conjugated Metabolites) and Conjugated Metabolites (Mean ± SD)

(A) (5 mg/kg of BCA IV) vs (5 mg/kg of BCA with 5 mg/kg of EGCG and 5 mg/kg of Quercetin IV)			
	BCA alone IV	with 5 mg/kg of MIX	fold change[a]
AUC_{012} of total BCA (ng·h/mL)	2340 ± 633	7260 ± 2850	**3.10***
AUC_{0-12} of conjugates (ng·h/mL)	769 ± 506	3830 ± 207	**4.98****
(B) (50 mg/kg of Oral BCA) vs (50 mg/kg of Oral BCA with 5 mg/kg of EGCG and 5 mg/kg of Quercetin)			
	BCA alone PO	with 5 mg/kg of MIX	fold change
AUC_{0-8} of total BCA (ng·h/mL)	10400 ± 7230	25000 ± 10600	2.40
AUC_{0-8} of conjugates (ng·h/mL)	10200 ± 7160	25000 ± 10500	2.45
(C) (50 mg/kg of Oral BCA) vs (50 mg/kg of Oral BCA with 50 mg/kg of EGCG and 50 mg/kg of Quercetin)			
	BCA alone PO	with 50 mg/kg of MIX	fold change
AUC_{0-24} (ng·h/mL)	13500 ± 11700	33100 ± 11800	2.44
AUC_{0-24} of conjugates (ng·h/mL)	13400 ± 11600	33000 ± 11900	2.46

[a] Statistically significant difference: *$P < 0.05$; **$P < 0.01$.

fold, Figure 5B), but the changes were not statistically significant.

Effects of Quercetin and EGCG on the ^{3}H-BCA Accumulation in MCF-7/Adr, MCF-7/MX100, Caco-2, MDCK/WT, and MDCK/MRP2 Cells. To investigate the effects of quercetin and EGCG on ABC transporter-mediated cellular efflux, we first confirmed the presence of P-gp and BCRP in MCF-7 cells and of MRP2 in the MDCK cells used in our experiments by Western blot analysis (data not shown). P-gp and BCRP were not detectable in the negative control MCF-7/sensitive cells, consistent with previous studies.[20,21]

The absolute values (radioactivity of ^{3}H-BCA/protein amount) were largest in MCF-7/sensitive, followed by in MCF-7/Adr, and smallest in MCF-7/MX100, suggesting that BCA is effluxed by BCRP and P-gp. Quercetin and EGCG inhibited P-gp, but not BCRP, at the concentrations used in this investigation. Similarly, the absolute values (radioactivity of ^{3}H-BCA/protein amount) were larger in MDCK/WT than in MDCK/MRP2 cells, suggesting that BCA is an MRP2 substrate. In all the studies, accumulation was examined at 5 min to minimize the influence of metabolism on the intracellular radioactivity measurements; however, the conjugates may be substrates for BCRP and MRP2 and may contribute to the changes in the accumulation of radioactivity in these studies.

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Table 3. Urinary Excretion of Total BCA (Unchanged Plus Its Conjugated Metabolites) and Conjugated Metabolites (Mean ± SD)

(A) (50 mg/kg of Oral BCA) vs (50 mg/kg of Oral BCA with 5 mg/kg of EGCG and 5 mg/kg of Quercetin)

	BCA alone PO	with 5 mg/kg of MIX	fold change
total BCA (μg)	214 ± 33.4	497 ± 304	2.32
conjugates (μg)	202 ± 35.1	477 ± 296	2.37

(B) (50 mg/kg of Oral BCA) vs (50 mg/kg of Oral BCA with 50 mg/kg of EGCG and 50 mg/kg of Quercetin)

	BCA alone PO	with 50 mg/kg of MIX	fold change
total BCA (μg)	214 ± 33.4	627 ± 312	2.93
conjugates (μg)	202 ± 35.1	593 ± 300	2.94

(C) (16.67 mg/kg of Oral BCA) vs (16.67 mg/kg of Oral BCA with 16.67 mg/kg of EGCG and 16.67 mg/kg of Quercetin)

	BCA alone PO	with 16.67 mg/kg of MIX	fold change[a]
total BCA (μg)	102 ± 36.1	579 ± 154	**5.65****
conjugates (μg)	99.2 ± 37.6	570 ± 156	**5.75****

[a] Statistically significant difference: $^{**}P < 0.01$.

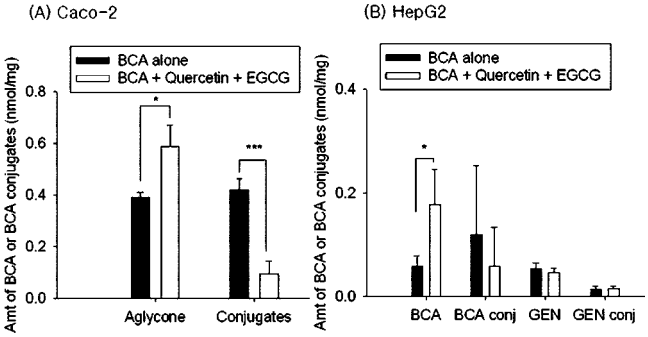

Figure 5. Metabolism of BCA in Caco-2 (A) and HepG2 (B) cells. Metabolism of BCA was determined after 1-h incubations with BCA (3.33 μM) or BCA + quercetin + EGCG (3.33 μM each). Results are presented as amounts of BCA, BCA conjugates and total BCA normalized to the amount of cell lysates protein ± SD, $n = 3$.

The cellular uptake of ^{3}H-BCA in MCF-7/Adr cells was significantly increased by 16.67 μM quercetin and 16.67 μM EGCG (177 ± 36.3% of the control, $p < 0.05$, Figure 6A). In contrast, the accumulation in MCF-7/sensitive and MCF-7/MX100 cells was not statistically different. These results suggest that quercetin and EGCG can inhibit P-gp mediated cellular efflux in MCF-7/Adr cells.

Discussion

One of the main reasons why BCA has poor bioavailability is because it undergoes extensive first pass extraction due to metabolism in the intestine and liver. The aim of this study was to examine the effect of quercetin and EGCG on the absorption and disposition of BCA in rats, and the mechanism(s) underlying this interaction. The important finding in the present study was that the coadministration of quercetin and EGCG with BCA significantly increased the BCA AUC in rats following both iv and oral administration, likely due to an increase in BCA bioavailability after its oral administration and/or upon enterohepatic cycling. The 24 h urinary excretion of BCA was also significantly higher in rats fed the mixture compared to rats fed 16.67 mg/kg of BCA alone. This also may be due to an increase in BCA bioavailability resulting in increased systemic exposure. The increased AUC resulted in a decreased estimate of clearance after its intravenous administration; half-life could not be determined due to the presence of re-entry peaks.

The magnitude of re-entry peaks, indicating the extent of enterohepatic recycling, was increased by the coadministration of quercetin and EGCG when rats received 5 mg/kg (17.6 μmol/kg) of BCA with 5 mg/kg (14.8 μmol/kg) of quercetin and 5 mg/kg (10.9 μmol/kg) of EGCG intravenously and when rats received 50 mg/kg (176 μmol/kg) of BCA with 50 mg/kg (148 μmol/kg) of quercetin and 50 mg/kg (109 μmol/kg) of EGCG orally. The re-entry peaks resulted in significantly increased AUC values for the BCA aglycon. The percentage increase in AUC for the BCA aglycon was greater following its oral administration than after its intravenous administration.

The mechanism underlying the increased bioavailability or decreased elimination may involve inhibition of the metabolism of BCA. BCA undergoes extensive metabolism to glucuronide and sulfate conjugates in both the intestine and the liver.[8] Since both quercetin and EGCG are also reported to undergo extensive conjugation by UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT),[22,23] decreased conjugation in intestinal cells may be attributed to competitive inhibition of glucuronidation/sulfation reactions. Moreover, quercetin is known as a weak inhibitor of estradiol 3-glucuronidation at the concentrations tested (5–100 μM),[24] and a potent inhibitor of estrogen sulfation (IC_{50} of 60 nM).[25] In the present study, the total amounts of BCA conjugates were decreased when the mixture was incubated in both intestinal Caco-2 and hepatic HepG2 cell lines. This finding contrasts with the increase in the ratio of BCA conjugates/total BCA observed in the in vivo studies. Interpretation of this increased ratio is not possible without further investigation. Among other possibilities, an increased ratio of conjugates to total BCA may reflect a decreased elimination of the conjugates due to inhibition of ABC transporter-mediated elimination or inhibition of other transporters; a change in the

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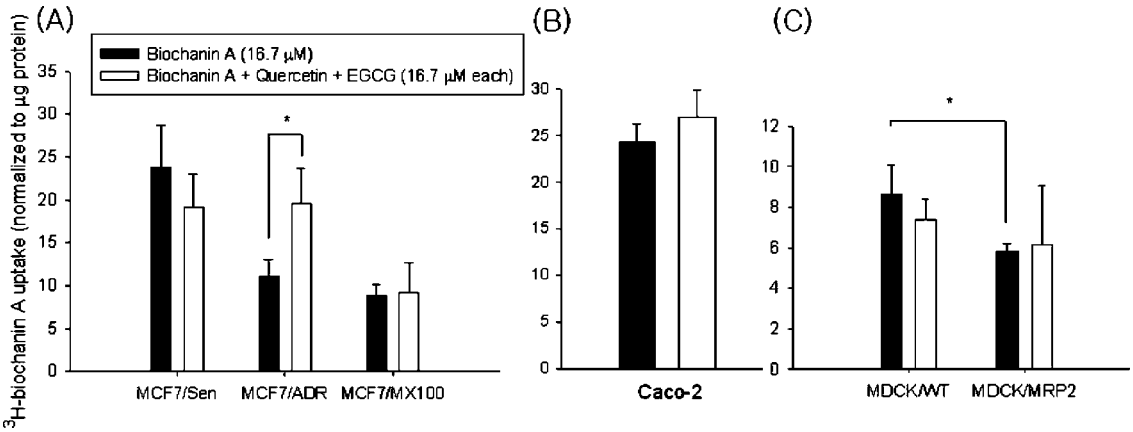

Figure 6. Effects of quercetin and EGCG on the uptake of ^{3}H-BCA in MCF-7/sensitive, MCF-7/Adr, and MCF-7/MX100 cells (A), in Caco-2 cells (B), and in MDCK/WT and MDCK/MRP2 cells (C). The 5 min uptake of ^{3}H-BCA (24 nM) in cells grown in 35 mm^2 culture dishes in the presence of 16.67 μM BCA only (considered as 100%) or 16.67 μM quercetin and 16.67 μM EGCG was determined as described in the Materials and Methods. Data are expressed as mean ± SD, $n = 3$.

type of conjugate formation, for example, more glucuronidation and less sulfation with differences in volume of distribution and elimination for the different types of conjugates; inhibition of the metabolism of BCA by other parallel metabolic pathways, such as CYP-mediated metabolism. This latter hypothesis is supported by the decreased concentrations of BCA's demethylated metabolite, genistein, that was observed in HepG2 studies, and the reports that quercetin can inhibit CYP1A1, CYP1A2 and CYP1B1.[26,27] Quercetin can inhibit human recombinant enzyme activities of CYP1A1 and 1B1 with $IC_{50} = 6.0$ μM and $K_i = 0.25$ μM (CYP1A1), and $IC_{50} = 0.55$ μM and $K_i = 0.12$ μM (CYP1B1).[27] Quercetin can also significantly inhibit of ethoxyresorufin-*O*-deethylase (EROD) activity with an IC_{50} of 4.14 μM.[27]

The mechanism of increased absorption may also involve inhibition of efflux transporter(s). BCA and/or BCA conjugates are potential substrates for the efflux transporters P-glycoprotein, BCRP, and MRP2[20,21,28] and quercetin and EGCG are known to interact with these transporters.[29–31] Therefore, we hypothesized that quercetin and EGCG inhibition of these intestinal transporters may contribute to the increased BCA absorption. Our results suggest that inhibition of P-glycoprotein may be involved in the increase of BCA absorption by quercetin and EGCG, at least at our test concentration. BCA may represent a P-glycoprotein–substrate, since sulfate and glucuronide conjugates generally are not transported by P-glycoprotein.

In conclusion, our findings demonstrate for the first time that the administration of multiple flavonoids results in the increased bioavailability of BCA. Although the bioavailability of BCA increased by about 3-fold, it still remains very low. The increase in bioavailability resulted in an increased extent of enterohepatic cycling for BCA. Therefore, combinations of flavonoids present in herbal preparations or in the diet may alter the bioavailability and enterohepatic cycling of individual flavonoids resulting in higher plasma concentrations and potentially enhancing its pharmacological effects. Further studies are needed to determine the specific pharmacokinetic interactions between BCA, quercetin and EGCG.

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