Research Article

Effects of the flavonoid biochanin A on gene expression in primary human hepatocytes and human intestinal cells

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Biochanin A (BCA), a phytoestrogen present in plant food and herbal products, has been reported to have cancer-preventive effects that may be mediated, in part, through effects on carcinogen metabolism. Our objective was to examine the effect of BCA on gene expression for drug-metabolizing enzymes and transporters in human hepatocytes. Cells were exposed to 20 μM of BCA for 5 days. Gene expression was assessed by a 96-gene human drug metabolism enzyme microarray. There were seven genes that were significantly up-regulated, namely cytochrome P-450 (CYP) 2A6, CYP2B6, CYP2C9, CYP2F1, multidrug resistance gene (MDR1), thromboxane A synthase 1 (TBXAS1), and SULT1A2 (sulfotransferase). Up-regulation of MDR1, which encodes for P-glycoprotein, was confirmed using real-time RT-PCR and Western analysis in hepatocytes as well as in human colon adenocarcinoma cell line (LS-180) and the induction was dose-dependent. BCA treatment up-regulated genes mainly in the CYP2 family. This induction can influence the metabolism of xenobiotics, producing effects of pharmacological and toxicological importance.

Keywords: Biochanin A / Drug interaction / Hepatocytes / LS-180 / P-glycoprotein

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1 Introduction

Biochanin A (BCA) is a dietary isoflavone present in legumes, most notably red clover, and in many herbal dietary supplements. BCA and its metabolite genistein have been investigated for their cardiovascular and chemopreventive effects as well as in the treatment of menopausal symptoms [1 – 3]. BCA is also known to alter the enzyme activities of cytochrome P-450 (CYP) 1A1 (K_i = 4.00 μM) and CYP1B1 (K_i = 0.59 μM), CYP19 (aromatase, K_i = 12 ± 5 μM) in Chinese hamster ovary cells, and UDP glucuronosyl transferase (UGT, ten-fold induction by 5 μM of BCA) in a human prostate cancer cell line [4 – 6]. The modulation of drug-metabolizing enzymes by BCA is important in terms of human health since up-regulation of detoxifying enzymes and/or the down-regulation of activating enzymes may reduce the activity of carcinogenic compounds. Additionally, BCA may also interact with therapeutic drugs through the induction or inhibition of their metabolism.

Human hepatocytes represent a suitable model for drug metabolism studies [7], since primary hepatocytes retain most of the endogenous cellular and nuclear cofactors that are essential for normal liver function. In general, there is a good qualitative correlation between human hepatocyte results *in vitro* and clinical observations *in vivo* [8]. LS-180, human colon adenocarcinoma cell line, is commonly used in the study of induction of P-glycoprotein (P-gp) expression by xenobiotic agents [9] as well as for the *in vitro* screening of drug absorption or permeability [10]. It has been shown that the colonic absorption of BCA was the greatest for the four intestinal sites (duodenum, jejunum, ileum, and colon) evaluated using a rat intestinal perfusion

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Abbreviations: BCA, biochanin A; **CAR,** constitutive androstane receptor; **COD,** cause of death; **CYP,** cytochrome P-450; **MDR1,** multidrug resistance gene; **P-gp,** P-glycoprotein; **PXR,** pregnane X receptor; **RTQ,** real-time quantitative; **SAM,** statistical analysis of microarray; **SULT,** sulfotransferase; **TBXAS,** thromboxane A synthase; **VDR,** vitamin D receptor

model [11]. Therefore, the LS-180 cell line was used to investigate the effect of BCA on intestinal gene expression.

Our objective was to examine the effect of BCA on gene expression for drug-metabolizing enzymes and selected transporters in human hepatocytes. The genes encoding for drug transporters that are present on the gene array were metallothioneins and members of the adenosine triphosphate (ATP)-binding cassette family (ABCB1, B4, C1, C2, C3, C5, and G2). Alterations in hepatic enzyme or transport activities may represent potential mechanisms involved in the cancer-preventive effect of BCA and may be responsible for drug – food interactions.

2 Materials and methods

2.1 Materials

BCA and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). [α-^{32}P]-dCTP (2′-deoxycytidine 5′-triphosphate) (10 μCi/μL; 3000 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). InVitroGRO™ HI Medium was purchased from In Vitro Technologies (Baltimore, MD).

2.2 Cell culture

Human fresh primary hepatocytes in six-well plates were obtained from In Vitro Technologies. Cells were received on a collagen-coated well. Hepatocytes from four donors were utilized: Hispanic female, 77 years (cause of death (COD), not available; medical history of Alzheimer disease); Caucasian female, 71 years (COD, Intracerebral hemorrhagic stroke; medical history of noninsulin-dependent diabetese and hypertension); Caucasian male, 52 years (COD, aortic dissection; medical history not available); Caucasian male, 51 years (COD, stroke; medical history not available).

Cells were incubated in InVitroGRO HI Medium (commercial medium which contains fetal bovine serum, In Vitro Technologies) for 4 h at 5% CO_2/95% air in a 37°C incubator with saturating humidity upon arrival to allow for cell recovery. Then the cells were exposed to 20 μM of BCA or DMSO (vehicle control)-containing media, with fresh media replaced every 24 h for 5 days to mimic repeated consumption of BCA. Five different human hepatocyte preparations were used in this study.

The human colon adenocarcinoma cell line, LS-180, was obtained from ATCC (Manassas, VA) and cultured in minimal essential media and Earl's salts (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acid solution (Gibco BRL), and 1 mM pyruvic acid. Cells seeded in T-25 cm^2 flasks were exposed to BCA at the concentrations indicated for 1 or 5 days. Cells were then either processed for mRNA or Western analysis of P-gp expression, as described below.

2.3 Total RNA isolation and gene array

Total RNA from each sample was isolated using SV Total RNA Isolation System (Promega), as *per* the manufacturer's instructions. RNA was quantified spectrophotometrically at 260 nm (NanoDrop® Spectrophotometer, Wilmington, DE). Gene expression was assessed using the Human Drug Metabolism Enzyme Microarray (Superarray, Bethesda, MD). Each array consists of 96 genes spotted in quadruplicate, as well as control spots (PUC18 as negative control; β-actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), cyclophilin A, and ribosome). The gene arrays were used according to the manufacturer's instructions. In brief, using the reagents provided, gene-specific cDNAs were prepared and labeled from total RNA by reverse transcription with moloney murine leukemia virus (MMLV) RT (Invitrogen, Carlsbad, CA) and [α-^{32}P]-dCTP (Amersham Biosciences). Relative expression levels of each gene were analyzed using Kodak Image Station 440CF. For normalization, we chose the geometric means of four internal standards (G3PDH, β-actin, cyclophilin A, and ribosome) according to the method published by Vandesompele *et al.* [12].

2.4 Real-time quantitative (RTQ) RT-PCR (RTQ-RT-PCR)

RTQ RT-PCR was performed on β-actin (for normalization) and multidrug resistance gene (MDR1) using Stratagene's Mx4000™ Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). Total RNA (560 ng) from each sample was reverse transcribed into cDNA using a Superscript first strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. PCR reactions for MDR1 and β-actin were amplified for 40 cycles. Primers (for MDR1, forward: 5′-CTGCTTGATGGCAAAGAAATAAAG-3′, reverse: 5′-GGCTGTTGTCTCCATAGGCAAT-3′, annealing temperature: 55°C; for CYP2B6, forward: 5′-CATTCTTCCGGGGATATGGTG-3′, reverse: 5′-CCTCATAGTGGTCACAGAGAATCG-3′, annealing temperature: 60°C; for β-actin, forward: 5′-CTGGCCGGGACCTGACT, reverse: 5′-TCCTTAATGTCACGCACGATTT-3′, annealing temperature: 57°C) were designed using the computer program Primer Express® (Perkin-Elmer Applied Biosystems, Foster City, CA). The PCR product of each gene was cloned into a pCR® 2.1 TOPO® vector (Invitrogen) and transformed into One Shot chemically competent *Escherichia coli* cells (Invitrogen). Cloned PCR products were used to construct standard curves for absolute quantification of copy number.

2.5 Western analysis of P-gp

Cells were washed with PBS and harvested using a rubber policeman. Total cell lysates were prepared by adding the lysis buffer (20 mM Tris at pH 7.5, 120 mM NaCl, 100 mM

NaF, 1% Nonidet P-40, 200 μM sodium orthovanadate, 50 mM β-glycerolphosphate, 10 mM sodium pyrophosphate, 4 mM PMSF, 2 mM benzamidine, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Proteins (50 μg) were electrophoresed on 7.5% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Invitrogen, Grand Island, NY) as described by Zhang and Morris [13]. C219 (DAKO, Carpinteria, CA) was used as the primary antibody to detect P-gp. Antimouse IgG horseradish peroxidase (Amersham Biosciences) was used as the secondary antibody. As a loading control, membranes were also incubated with anti-β-actin antibody (Alltech Associates, Deerfield, IL) and antimouse IgG to detect β-actin. Membranes were washed and detected with enhanced chemiluminescence detection reagent (Amersham Biosciences). Kodak Image Station 440CF was used to analyze the Western blot results.

2.6 Statistical analysis

Significantly changed genes were identified *via* permutation analysis as implemented by the program, statistical analysis of microarray (SAM) [14]. The false discovery rate was less than 1 (0.87) among the seven genes identified as significant. For real-time RT-PCR, a Student's *t*-test with $p < 0.05$ set as the significance level was used.

3 Results and discussion

Table 1 presents the gene list for the Superarray. Forty-one out of 96 genes showed no detectable expression; some of these are not expressed in the liver, and have not been detected by other investigators using RTQ-RT-PCR (these include CYP11A, CYP24, CYP4B1, (sulfotransferase) (SULT) 1C1, SULT1C2, SULT2B1, and SULT4A1) [15–19]. For other genes, the expression level is low (including CYP1A1, CYP4A11, CRAT (CAT1,SLC7A1), GSTA4, GSTM2, GSTM3, SULT1B1, CYP7B1, GSTT1, MGST3, CHST1, CHST2, CHST3, CHST4, CST, TPST2, and COMT) [15, 18, 20], and were not detected in the Superarray.

Treatment of human hepatocytes for 5 days with BCA (20 μM) significantly altered the expression of seven genes: MDR1, CYP2A6, CYP2B6, CYP2C9, CYP2F1, thromboxane A synthase (TBXAS) 1 (CYP5A1), and SULT1A2 (Fig. 1A). All the seven genes were up-regulated. Among them, CYP2A6, CYP2B6, and CYP2F1 are part of a large cluster of CYP genes from the CYP2A, CYP2B, and CYP2F subfamilies present on chromosome 19q. Expression of MDR1, CYP2B6, and CYP2C9 are controlled by the same regulators, pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [21, 22]. Whether BCA acts as an activator of PXR/CAR is not yet known, but structurally similar compounds, steroid hormones (such as estrogens and progesterone) and dietary compounds such as coumestrol and hyperforin are ligands of PXR [23]. Hyperforin, a constituent of St. John's wort (a herbal remedy for depression), has been reported to be a potent PXR activator [23].

Using RTQ-RT-PCR and Western analysis, we confirmed the up-regulation of MDR1. After normalization to β-actin, the increase in MDR1 was 1.79 ± 0.56-fold compared to controls (mean ± SD, $n = 4$, $p = 0.060$) by RTQ-RT-PCR, compared to 3.8-fold from the gene array data (Fig. 1B). The increase in CYP2B6 mRNA was 2.46 ± 1.38-fold compared to controls (mean ± SD, $n = 4$) by RTQ-RT-PCR, although not statistically significant due to its large variability ($p = 0.064$). The changes were qualitatively but not quantitatively similar to gene array and RTQ-RT-PCR. The reason for the quantitative differences may be the following: (i) the normalization methods used were different for arrays and RTQ-RT-PCR, and (ii) some of the samples used in RTQ-RT-PCR were different from ones used in the original arrays. β-Actin (a housekeeping gene) expression did not change following BCA exposure in either the array or the RTQ-RT-PCR experiments. The protein encoded by MDR1 is the ATP-dependent drug efflux pump for xenobiotic compounds, P-gp, which has a broad substrate specificity. It is responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer drugs. Treatment with 20 μM BCA for 5 days also increased P-gp expression, as shown in Fig. 1C.

A significant induction in MDR1 by BCA, resulting in increases in both mRNA and protein expression, is consistent with our results in human intestinal LS-180 cells. In LS-180 cells, the effects of BCA were evaluated by RTQ-RT-PCR following a 5-day incubation with 20 μM BCA. The increase of MDR1 mRNA was 5.34 ± 1.06-fold compared to controls (mean ± SD, $n = 3$, $p < 0.01$); the positive control rifampicin (20 μM) increased MDR1 expression by 13.4 ± 2.00-fold (mean ± SD, $n = 3$, $p < 0.001$ (Fig. 2A). P-gp induction was dose-dependent (Fig. 2B), with 20 μM of BCA producing greater increases in P-gp expression than 1 or 2 μM BCA (Fig. 2B). However, induction by 5 days of treatment was similar to that observed following a 1-day treatment with BCA (Fig. 2B).

Although activation of P-gp might lead to the undesirable increased elimination of drug molecules, it would also result in the more rapid removal of toxic agents from the body. Besides the role of PXR and CAR in the induction of MDR1, there is evidence that the signaling processes including NF-κB [24] and the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway [25] can result in induction of MDR1. Since genistein, a metabolite of BCA, is known to regulate the activation of these pathways [26], these pathways may also represent mechanisms underlying the P-gp induction by BCA.

A number of genes encoding for CYP enzymes were up-regulated by BCA treatment, including CYP2B6 and 2C9.

Table 1. Gene list containing the list of genes present in Superarray

Phase I, P450 family		
CYP11A		
CYP1A1		
CYP1B1	Detected	
CYP24		
CYP2A6	Detected	Changed
CYP2B6	Detected	Changed
CYP2C19	Detected	
CYP2C8	Detected	
CYP2C9	Detected	Changed
CYP2D6		
CYP2E	Detected	
CYP2F1	Detected	Changed
CYP3A4	Detected	
CYP3A5	Detected	
CYP3A7	Detected	
CYP4A11		
CYP4B1		
CYP4F3	Detected	
CYP7A1		
CYP7B1		
CYP8B1	Detected	
CYP-M (CYP20A1)		
P450RAI-2		
POR		
TBXAS1	Detected	Changed
Phase II, acetyltransferases		
ACAT1	Detected	
ACAT2		
CHAT	Detected	
CRAT(CAT1, SLC7A1)		
DLAT	Detected	
HAT1	Detected	
HIBOA		
MORF(MYST4)		
NAT1	Detected	
NAT5	Detected	
Phase II, glutathine S-transferase		
GSTA2	Detected	
GSTA3	Detected	
GSTA4		
GSTM2		
GSTM3		
GSTM5	Detected	
GSTP1	Detected	
GSTT1		
GSTT2	Detected	
MGST1	Detected	
MGST2	Detected	
MGST3		
Phase II, SULT		
CHST1		
CHST2		
CHST3		
CHST4		
CHST5	Detected	
CHST6	Detected	
CHST7	Detected	
CHST8		
CST		
STE (SULT1E1)		
HNK-1ST (CHST10)		
SULT1A1	Detected	
SULT1A2	Detected	Changed
SULT1B1		
SULT1C1		
SULT1C2		
SULT2A1	Detected	
SULT2B1		
SULT4A1		
TPST1	Detected	
TPST2		
Phase II, miscellaneous		
EPHX1	Detected	
EPHX2	Detected	
LABH1(ABHD1)		
LTA4H	Detected	
UGT1A1	Detected	
UGT2A1	Detected	
UGT2B	Detected	
UGT2B10	Detected	
UGT2B4	Detected	
COMT		
HNMT	Detected	
NNMT	Detected	
TPMT	Detected	
Phase III, metallothioneins		
MT1A	Detected	
MT1E	Detected	
MT1G	Detected	
MT1H	Detected	
MT1L	Detected	
MT2A	Detected	
MT3		
MTIX	Detected	
Phase III, P-gp		
ABCB1	Detected	Changed
ABCB4	Detected	
ABCC1		
ABCC2	Detected	
ABCC3		
ABCC5		
ABCG2		

"Detected" represents genes were detected in human liver hepatocytes using Superarray technique. "Changed" represents genes were significantly changed by 5-day treatment of 20 μM BCA using SAM.

CYP2B6 is involved in the metabolism of nearly 25% of drugs in the market today [27], and is highly inducible, with significant interindividual differences in hepatic CYP2B6 expression reported [28]. Induction of CYP2B gene expression is predominantly regulated at the transcriptional level for CYP2B genes [27]. CYP2C9 ranks second, after CYP3A4, for the highest protein expression of all the drug-metabolizing CYP isoforms presenting in human liver [29]. The vitamin D receptor (VDR) pathway can regulate the expression of CYP2B6 and CYP2C9 [30]. Since genistein,

which is a metabolite of BCA in the human liver [31], can up-regulate the transcription of the VDR gene and also increases VDR protein [32, 33], the induction of CYP2B6 and CYP2C9 after a 5-day treatment with BCA may be related to the induction of VDR.

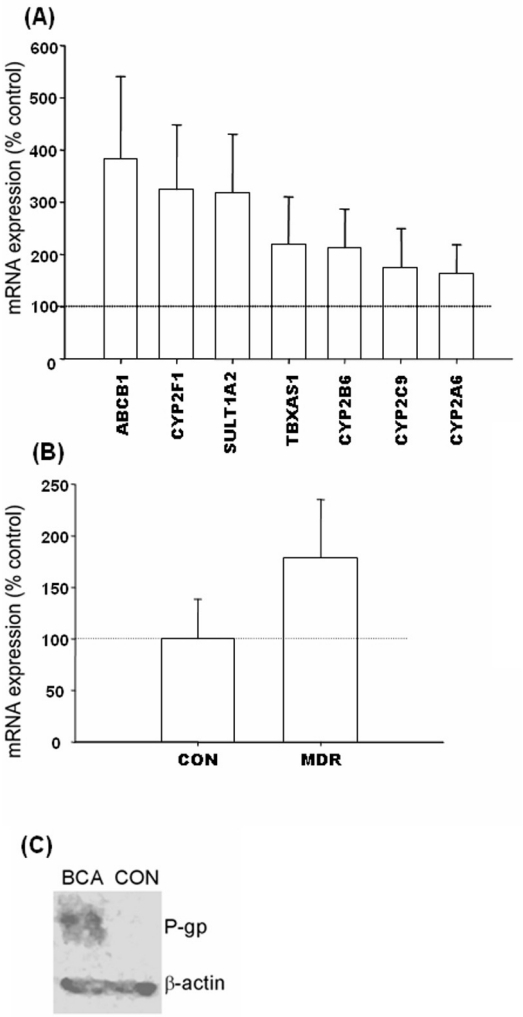

Figure 1. (A) Significantly changed genes following treatment with 20 μM of BCA in primary human hepatocytes ($n = 5$). Results are expressed as percent of control. Error bars represent SDs. Comparisons of treated *vs.* control were evaluated using SAM, with the geometric means of four internal standards (G3PDH, β-actin, cyclophilin A, and ribosome) used for data normalization. (B) Effect of 20 μM BCA on MDR1 mRNA expression in primary human hepatocytes ($n = 4$). The effects of BCA were evaluated by RTQ-RT-PCR following a 5-day incubation with 20 μM BCA. Results were normalized by β-actin and expressed as percent of control. Error bars represent SDs. $p = 0.059$, Student's *t*-test. (C) Western blot analysis of P-gp expression in primary human hepatocytes in vehicle-treated (CON) and following 5 days of treatment with 20 μM BCA. Cellular P-gp level was examined by Western blot analysis, as described under Section 2. P-gp was detected with the primary antibody C219. β-Actin was used to confirm equal protein loading. Two separate experiments were conducted and similar results were obtained.

BCA treatment also increased the expression of CYP2A6, CYP2F1, and thromboxane synthase (TBXAS1 (CYP5A1)). Thromboxane synthase catalyzes the conversion of the prostaglandin endoperoxide into thromboxane A2, a potent vasoconstrictor and inducer of platelet aggregation [34]. A decrease in CYP2 gene expression, especially that of CYP2A6, has been reported to be a crucial

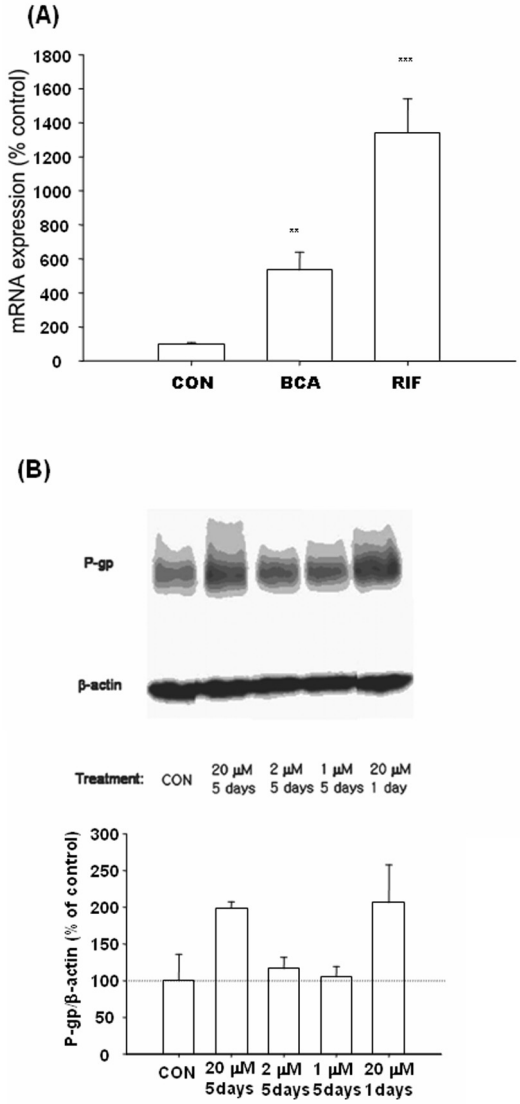

Figure 2. (A) Effect of BCA and rifampicin (RIF) on MDR1 mRNA expression in LS-180 cells ($n = 3$). The effects of BCA were evaluated by RTQ-RT-PCR following a 5-day incubation with 20 μM BCA and 20 μM rifampicin. Results were normalized by β-actin and expressed as percent of control. Error bars represent SDs.$^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t*-test. (B) Western blot analysis of P-gp expression in LS-180 cells in vehicle-treated (CON) and following 5 days of treatment with BCA 1, 2, and 20 μM, and 1 day treatment with 20 μM BCA. Relative protein expression (vehicle-treated control is set as 100) normalized to β-actin represents two independent experiments.

step toward hepatocarcinogenesis in humans [17]. There are no studies that have examined the regulation of these enzymes by flavonoids.

One phase II enzyme, SULT1A2 was also up-regulated (3.18-fold) by BCA treatment. SULTs are important in the regulation of levels and activities of neurotransmitters and hormones as well as in the elimination of xenobiotics [35]. SULT1A2 is the most efficient enzyme in sulfating some aromatic hydroxylamines [36]. The isoflavones genistein and equol have been shown to be potent mixed inhibitors of hepatic estrogen SULT, with inhibitory constant values of 500 and 400 nM, respectively [37]; induction has not been reported.

The *in vivo* intestinal concentration of BCA upon consumption of herbal products containing this flavonoid is not known, but it is likely that the concentrations we used in this study could be achievable *in vivo* after the ingestion of herbal preparations. As Zhang and Morris [38] described in their report, the calculated maximum clinical intestinal concentrations of BCA is about 366 μM, assuming a tablet of red clover extracts was ingested along with 250 mL of water. Plasma concentrations of 10 – 25 μM were obtained after oral administration of flavonoid genistein in humans [39]: BCA is the *O*-methylated form of genistein. Therefore, the 20 μM concentration of BCA represents a reasonable estimate of pharmacological BCA concentrations in the liver.

4 Concluding remarks

In summary, this is the first study to demonstrate interactions between BCA, a dietary component present in food and herbal products, and drug-metabolizing enzyme expression in the primary hepatocytes after chronic exposure (5 days) to 20 μM BCA. The microarray data indicated that the flavonoid BCA is able to induce five phase I enzymes, one phase II enzyme, and the transporter MDR1 in the liver. Enzyme induction can influence the metabolism of xenobiotics, producing effects of pharmacological and toxicological importance. This induction is also important in carcinogenesis because many carcinogens are metabolized by CYP enzymes to either biologically inactive metabolites or to chemically reactive electrophilic metabolites that covalently bind to DNA producing carcinogenicity.

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5 References

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