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Involvement of multidrug resistance-associated proteins in regulating cellular levels of (−)-epigallocatechin-3-gallate and its methyl metabolites☆,☆☆

Jungil Hong,[a] Joshua D. Lambert,[a] Sung-Hack Lee,[b] Patrick J. Sinko,[b] and Chung S. Yang[a,*]

[a] *Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA*
[b] *Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA*

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Abstract

(−)-Epigallocatechin-3-gallate (EGCG), a major polyphenol of green tea, has many interesting biological activities. The uptake of EGCG and involvement of specific efflux pumps were studied in MDCKII cells transfected with hPgp, hMRP1, and hMRP2 genes. Total cell associated [^{3}H]EGCG increased 7-fold in the presence of the MRP inhibitors, indomethacin and probenecid, in MDCKII/MRP1 cells, compared to a 2-fold increase in wild-type cells. Intracellular levels of EGCG, 4″-*O*-methyl EGCG, and 4′,4″-di-*O*-methyl EGCG were increased by 13-, 11-, and 3-fold, respectively, by indomethacin in MDCKII/MRP1 cells. Accumulation of EGCG and its methyl metabolites was also increased ~10-fold in the presence of MK-571 in MDCKII/MRP2 cells. Co-treatment with isoflavones, curcumin and tetrahydrocurcumin, increased [^{3}H]EGCG accumulation significantly in MDCKII/MRP1 and HT-29 cells. The results indicate that EGCG and its methyl metabolites are substrates for MRP1 and MRP2, but not for Pgp. MRP type efflux pumps may limit the bioavailability of EGCG.

Keywords: EGCG; Efflux; Pgp; MRP; MDCKII cell

Epigallocatechin-3-gallate (EGCG) is the most abundant catechin found in green tea (*Camellia sinensis*). Consumption of green tea has been suggested to have a number of beneficial effects, including the prevention of cancer [1]. Studies of cell lines have demonstrated many potential mechanisms of action for tea polyphenols, including antioxidative activity, inhibition of AP-1 transactivation, inhibition of EGFR and other growth factor signals, inhibition of topoisomerase II, inhibition of telomerase, and inhibition of the proteasome activity [1–9]. The concentrations necessary to observe these activities, however, often far exceed those available after consumption of tea.

Studies in rats, humans, and mice have shown that the bioavailability of EGCG is low [10,11] (Lambert, unpublished). Cai et al. [12] have demonstrated in cannulated-rats that the limited bioavailability of EGCG is due largely to poor absorption in the small intestine rather than extensive extraction by the liver. EGCG is a substrate for UDP-glucuronosyltransferase, sulfotransferase, and catechol-*O*-methyltransferase, and there are significant species differences in the amount of EGCG conjugate found in the plasma [13,14] (Lambert, unpublished). EGCG is largely conjugated in the plasma of mice and rats, whereas EGCG exists mostly in free form in human plasma [10,11]. Apparently, phase II biotransformation and the efflux of EGCG and its metabolites affect the bioavailability of EGCG, and more research in this area is needed.

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☆☆ *Abbreviations:* EGCG, (−)-epigallocatechin-3-gallate; MeEGCG, 4″-*O*-methyl-EGCG; DiMeEGCG, 4′,4″-di-*O*-methyl-EGCG; MRP, multidrug resistance-associated proteins; Pgp, *P*-glycoprotein; HBSS, Hanks' balanced salt solution.
* Corresponding author. Fax: 1-732-445-0687.
E-mail address: csyang@rci.rutgers.edu (C.S. Yang).

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The multidrug resistance-associated proteins (MRP) are ATP-dependent efflux transporters that are expressed in many tissues and are overexpressed in many types of human tumors. MRP1 (ABCC1) is located on the basolateral side of most cells and is present in nearly all tissues. The physiological function of this protein is to transport compounds from the interior of the cells into the interstitial space [15]. MRP1(−/−) mice are more sensitive to etoposide phosphate-induced damage to the oropharyngeal mucosal layer and testicular tubules than are wild-type animals [16]. In contrast, MRP2 (ABCC2) is located on the apical surface of the intestine, kidney, and liver, where it transports compounds from the bloodstream into the lumen, urine, and bile, respectively [15]. Rats deficient in MRP2 have impaired biliary excretion of organic anions and accumulate heme degradation products [17]. There is limited information available on the role of MRPs in the bioavailability of dietary components such as flavonoids. Quercetin-4′-β-glucoside and (−)-epicatechin have been previously reported as substrates for MRP2 [18,19]. The role of MRP or other multidrug-resistance pumps in affecting cellular level of EGCG and EGCG bioavailability, however, has not been reported previously. Using Madin–Darby canine kidney (MDCKII) cells overexpressing MRP1, MRP2, and Pgp, we have determined the effects of MRP1 and 2 and Pgp on the accumulation of EGCG and its methylated metabolites. Herein, we report the results of this study and discuss implications for the bioavailability of EGCG and its metabolites.

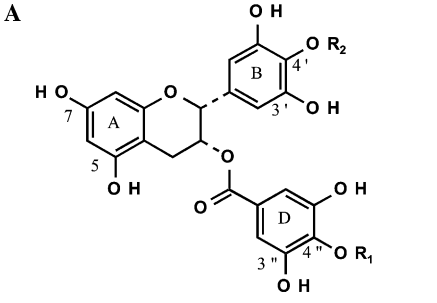
Materials and methods

Chemicals. [^{3}H]EGCG (13 Ci/mmol) was synthesized as described previously [20] and was generously provided by Dr. Yukihiko Hara of Mitsui Norin (Fujieda City, Japan). Unlabeled EGCG and other green tea catechins were a generous gift from Unilever Bestfoods (Englewood Cliffs, NJ). 4″-*O*-Methyl EGCG (MeEGCG) and 4′,4″-Di-*O*-methyl EGCG (DiMeEGCG) were chemically synthesized in our laboratory [21]. The structures of these compounds are shown in Fig. 1A. Indomethacin was purchased from Cayman Chemical Company (Ann Arbor, MI). GF120918 and MK-571 were provided by GlaxoSmithKline (Research Triangle Park, NC) and Merck Laboratories (Whitehouse, NJ), respectively. Isoflavones, including genistein, daidzein, and biochanin A, were purchased from LKT Laboratories (St. Paul, MN). Curcuminoids and formononetin were generously provided by Drs. Mou-Tuan Huang and Chi-Tang Ho, respectively (Rutgers University, New Brunswick, NJ). Probenecid, vinblastine, cyclosporin A, and all other chemicals were from Sigma Chemical (St. Louis, MO).

Cell culture. Madin–Darby canine kidney type II (MDCKII) cells were generously provided by Drs. R. Evers and P. Borst (The Netherlands Cancer Institute, Amsterdam). Wild-type MDCKII cells (MDCKII/wt) and MDCKII cells stably expressing human transporters including hMRP1 (MDCKII/MRP1), hMRP2 (MDCKII/MRP2), and hPgp (MDCKII/Pgp) at 5–30 passages were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% nonessential amino acid, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in 95% humidity and 5% CO_2. HT-29 cells (American Type Culture Collection, Rockville, MD) at 30–40 passages were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in 95% humidity and 5% CO_2.

Cellular uptake of [^{3}H]EGCG. To evaluate the effect of efflux pump inhibitors on EGCG uptake by cells, HT-29 and MDCKII cells (∼90 and ∼100% confluency, respectively, in a 24-well plate) were preincubated with serum free Hanks' balanced salt solution (HBSS) for 2 h. The cells were then treated with [^{3}H]EGCG (10 μM, 0.25 μCi/ml) in 0.4 ml HBSS including 100 μM ascorbic acid with different efflux inhibitors or vehicle (DMSO). After 1 h incubation, cells were washed three times with ice-cold PBS. The attached cells were lysed by 200 μl of 0.2 N NaOH and the well was washed with 300 μl of distilled water two times, which was then combined as total cell lysates. The radioactivity from total cell associated EGCG was analyzed by a scintillation counter (Model LS3801, Beckman Coulter, Fullerton, CA).

HPLC analysis of EGCG and metabolites. To analyze cellular accumulation of EGCG and its methyl metabolites, MDCKII cells were cultured in a six-well plate. After a 2 h preincubation in HBSS, the



	R_1	R_2
I. EGCG	H	H
II. 4"-*O*-MeEGCG	CH_3	H
III. 4'4"-di-*O*-MeEGCG	CH_3	CH_3

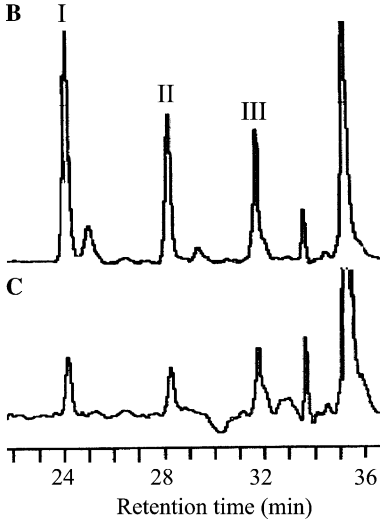


Fig. 1. Structure (A) and HPLC chromatogram (B,C) of EGCG and its methyl metabolites. Chromatogram shows cytosolic EGCG (I), MeEGCG (II), and DiMeEGCG (III) after incubation of these compounds (each 5 μM) for 1 h with MDCKII/MRP1 in the presence (B) or absence (C) of 10 μM indomethacin. Scales shown in (B) and (C) are 1× and 2×, respectively.

EGCG, MeEGCG or DiMeEGCG (10 μM) was incubated with efflux pump inhibitors or vehicle (DMSO) for 1 h in HBSS containing 100 μM ascorbic acid. In some experiments, EGCG and its metabolites (each 5 μM) were added simultaneously. The attached cells were then washed with ice-cold PBS three times and the harvested cells were homogenized using an ultrasonic processor, five times for 5 s intervals at level 3 (Model XL2015, Misonic, Farmingale, NY) in ice-cold EGCG stabilizing buffer (1% ascorbic acid and 0.01% EDTA in distilled water). The cell lysates were centrifuged at 10,000*g* for 20 min at 4 °C. After mixing the supernatant with an equal volume of methanol, the mixture was centrifuged again for 20 min at 10,000*g* to precipitate proteins. The supernatant (50 μl) was analyzed by HPLC according to the method described previously [11]. The protein concentration of the cell lysates was determined by the method of Bradford (Bio-Rad, Hercules, CA).

Data analysis. Statistical significance was evaluated using Student's *t* test. The significance level was defined as a minimum *p* value of 0.01.

Results

Cellular accumulation of [^{3}H]EGCG affected by MRP inhibitors

The accumulation of [^{3}H]EGCG in MDCKII/wt and Pgp, MRP1, and MRP2 overexpressing cells was investigated in the presence of different efflux pump inhibitors. The stable overexpression of Pgp or MRP1 in each transfectant was confirmed by Western blot analysis. The overexpression of mRNA and protein levels of MRP2 in MDCKII/MRP2 cells was also determined by RT-PCR and Slot blot analysis [22]. The amount of total cell associated radioactivity from [^{3}H]EGCG was significantly increased (over 2-fold) by the MRP inhibitors, probenecid and MK-571, in all MDCKII cells (Fig. 2). Indomethacin, an MRP1 inhibitor, also caused a significant increase of [^{3}H]EGCG accumulation in all cell lines, except the MRP2 transfectant. The increase caused by indomethacin and probenecid was much more prominent in MDCKII/MRP1 cells (~7-fold vs. ~2-fold increase in wild-type cells) (Fig. 2). Known inhibitors of MRP2, MK-571, probenecid, cyclosporin A, or vinblastine, all caused more extensive accumulation of [^{3}H]EGCG in MDCKII/MRP2 than in MDCKII/wt (Fig. 2). GF120918, a specific inhibitor of Pgp and breast cancer resistance protein (BCRP), however, did not affect [^{3}H]EGCG accumulation in either MDCKII/wt or transfected cell lines. Other Pgp inhibitors, including cyclosporin A and vinblastine, also did not increase [^{3}H]EGCG uptake in MDCKII/wt and its Pgp transfectant (Fig. 2).

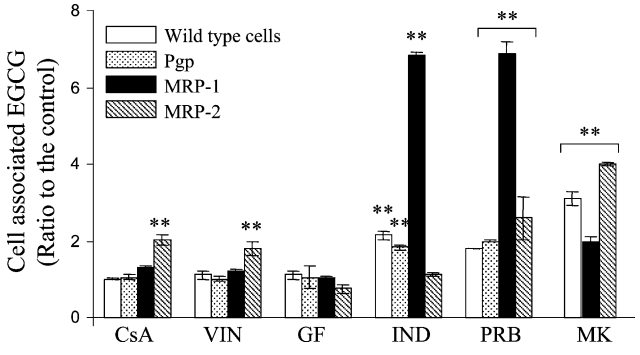


Fig. 2. Effects of several efflux pump inhibitors on cell associated [^{3}H]EGCG in wild-type, Pgp-, MRP1-, and MRP2-overexpressed MDCKII cells. The cells were incubated with [^{3}H]EGCG (10 μM, 0.25 μCi/ml) in HBSS containing 100 μM ascorbic acid in the presence of different efflux pump inhibitors (CsA, 10 μM cyclosporin A; VIN, 50 μM vinblastine; GF, 1 μM GF120918; IND, 10 μM indomethacin; PRB, 1 mM probenecid; and MK, 50 μM MK-571). After incubation for 1 h at 37 °C, cells were lysed with 0.2 N NaOH. The cell associated radioactivity was analyzed by a scintillation counter and expressed as ratio to the control (in the absence of the inhibitors). The results are means ± SD ($n = 4$) (**$p < 0.001$).

Cellular accumulation of EGCG and its methyl metabolites by MRP inhibitors

The cellular accumulation of EGCG, possible biotransformation of EGCG, and efflux of EGCG metabolites in MDCKII cells were investigated. The retention times of EGCG, MeEGCG, and DiMeEGCG were 24, 28.5, and 32 min, respectively, in the current HPLC system (Figs. 1B and C). No significant oxidation of EGCG occurred in the presence of 100 μM ascorbic acid during the 1 h incubation (data not shown). No major metabolites (methylated or other conjugated forms) were detected after 1 h incubation with any of the MDCKII cells (data not shown). In the presence of 10 μM indomethacin, cytosolic accumulation of EGCG, MeEGCG, or DiMeEGCG in MDCKII/MRP1 cells was increased by 10-, 11-, or 3-fold, respectively (Fig. 3A). When EGCG, MeEGCG, and DiMeEGCG were added together to MDCKII/MRP1 cells, the pattern of accumulation caused by this MRP1 inhibitor was similar to that when these compounds were added individually, suggesting that there is little competition between EGCG and its metabolites for the efflux pump (Fig. 3B). Similarly, cytosolic accumulation of EGCG, MeEGCG, and DiMeEGCG in MDCKII/MRP2 was increased 10-, 15-, and 12-fold, respectively, by a MRP inhibitor, MK-571 (75 μM) (Fig. 3C). The accumulation of EGCG, MeEGCG, and DiMeEGCG, however, was not affected by GF120918, a Pgp inhibitor, in Pgp overexpressing MDCKII cells (data not shown).

Effects of other dietary phenolic compounds on EGCG accumulation

The effect of other dietary phenolic compounds on EGCG efflux was investigated. At 10 μM, (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin-3-gallate did not affect [^{3}H]EGCG accumulation in either MDCKII/MRP1 or MRP2 overexpressing cells. The isoflavones, daidzein, genistein, biochanin A, and formononetin, significantly increased EGCG accumu-

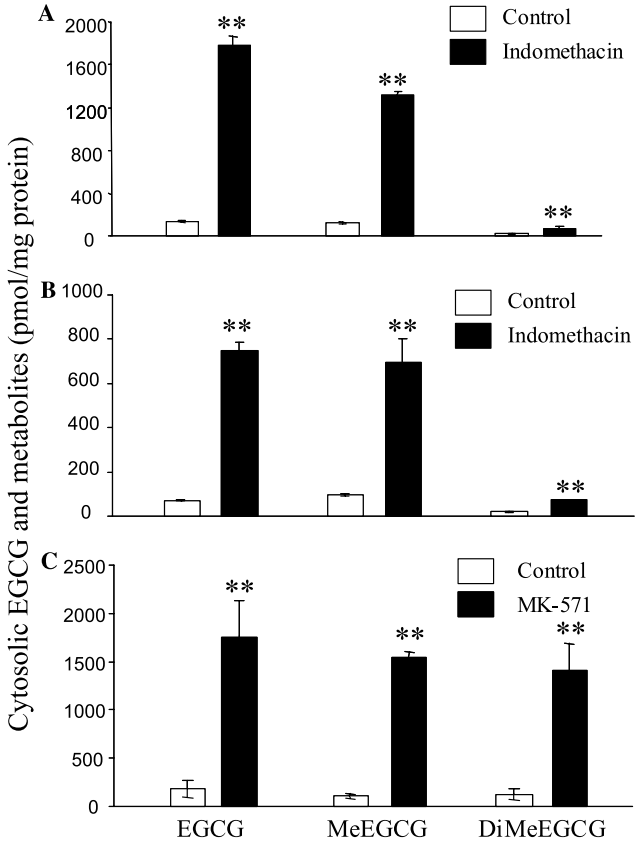


Fig. 3. Accumulation of EGCG and its methyl metabolites in the cytosolic fraction of MDCKII/MRP1 (A,B) or MRP2 (C) overexpressing cells in the presence of MRP inhibitors. EGCG, 4″MeEGCG, and 4′4″-DiMeEGCG (10 μM), individually (A,C) or in a mixture (each 5 μM) (B), were incubated in the presence of indomethacin (10 μM), MK-571 (75 μM), or the vehicle (DMSO) in HBSS containing 100 μM ascorbic acid. After incubation for 1 h at 37 °C, cell lysates were prepared as described in Materials and methods; intracellular EGCG and metabolites were analyzed by HPLC. The results are means ± SD ($n = 3$) (**$p < 0.001$).

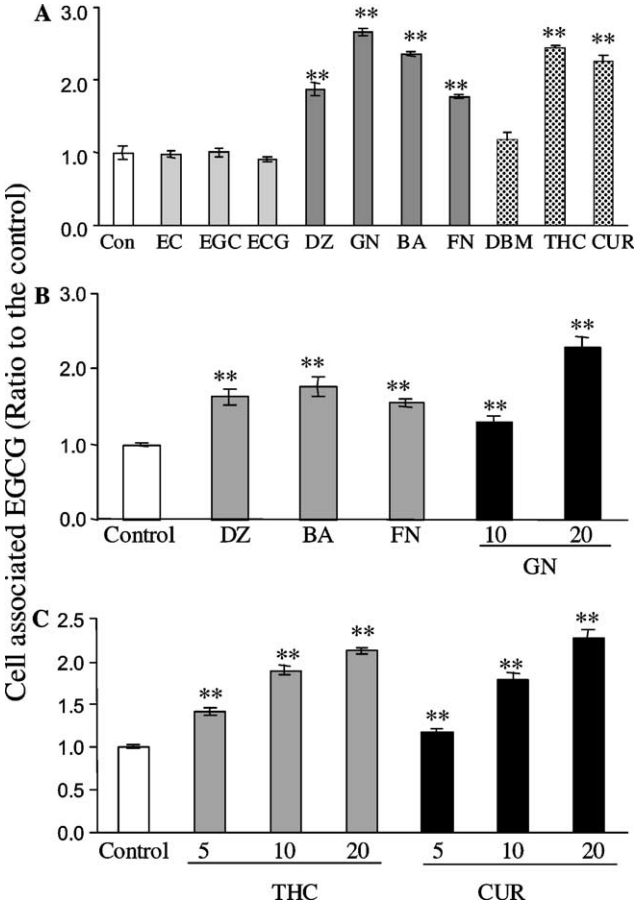


Fig. 4. Effect of dietary polyphenols on the accumulation of [^{3}H]EGCG in MDCKII/MRP1 overexpressed cells (A) and HT-29 cells (B,C). (A) MDCKII cells were incubated with [^{3}H] EGCG (0.25 μCi, 10 μM) for 1 h in HBSS containing 100 μM ascorbic acid in the presence of tea catechins, EC (epicatechin), EGC (epigallocatechin), and ECG (epicatechin 3-gallate), in the presence of isoflavones, DZ (daidzein), GN (genistein), BA (Biochanin A), and FN (Formononetin), or in the presence of curcuminoids, DBM (dibenzoyl methane), THC, (tetrahydrocurcumin), and CUR (curcumin) (each 10 μM). (B,C) [^{3}H]EGCG [0.25 μCi, 10 μM in (B), or 5 μM in (C)] was incubated for 2 h in HBSS containing 100 μM ascorbic acid with HT-29 cells in the presence of 10 μM of DZ, BA, FN, or 10 or 20 μM of GN, and in the presence of three concentrations (in μM) of THC and CUR. The results are means ± SD ($n = 6$–8) (**$p < 0.001$).

lation in MDCKII/MRP1 overexpressing cells. Among the curcuminoids, tetrahydrocurcumin (THC) and curcumin also increased EGCG uptake by greater than 2-fold. Dibenzoyl methane, which is the simplest curcuminoid structure with a β-diketone group, did not increase EGCG accumulation (Fig. 4A). The effect of the compounds on EGCG accumulation was much less prominent in MDCKII/MRP2 cells; curcumin increased the accumulation by 40%, and THC and isoflavones by 20–30% (data not shown). The increased EGCG accumulation by dietary constituents was also observed in HT-29 human colon adenocarcinoma cells. All isoflavones significantly increased EGCG uptake by 50–100% (Fig. 4B). Curcumin and THC also increased EGCG accumulation in a concentration-dependent manner. THC and curcumin (20 μM) showed similar effects on EGCG accumulation, causing an increase over 2-fold (Fig. 4C).

Discussion

The family of ATP-binding cassette transporters, including Pgp and the MRPs, plays an important role in the efflux of many drugs and in drug resistance. Previously we reported that cell associated radioactivity from [^{3}H]EGCG, and intracellular 4″-*O*-methyl-EGCG and EGCG 4″-glucuronide increased after incubation of EGCG by MRP inhibitors in HT-29 cells [23]. The present study shows that the MRP inhibitor-induced accumulation of [^{3}H]EGCG was more pronounced in MDCKII/MRP1 and 2 transfected cells than in wild-type cells. This increased accumulation was even more

prominent when cytosolic EGCG was examined. Since a significant portion of EGCG is bound nonspecifically to the membrane and cell surface [23], the increase of cytosolic EGCG by MRP inhibitors is therefore greater than that of total cell associated EGCG. The results suggest that EGCG is a substrate of MRPs. Interestingly, EGCG metabolites, MeEGCG and DiMeEGCG, also accumulated in the presence of MRP inhibitors. These pumps are generally thought to efflux hydrophilic metabolites such as glutathione- and glucuronide-conjugates. To our knowledge, this is the first report establishing that EGCG and the methyl metabolites of EGCG are MRP substrates. Judging from their accumulation profile (Fig. 3), EGCG and its methyl metabolites may have a similar affinity for MRP2, but EGCG and MeEGCG are apparently better substrates for MRP1 than DiMeEGCG.

Based on the apical location of MRP2 in the intestine, kidney, and liver, we hypothesize that a fraction of EGCG absorbed from the intestine is secreted back into the intestinal lumen by means of MRP2 before or after being methylated by cytosolic COMT. EGCG that is not secreted from the enterocyte into the intestinal lumen would be absorbed into the portal circulation, enter the liver, and could subsequently be effluxed by MRP2 located on the canalicular membrane of hepatocytes. MRP1 is located on the basolateral membrane of enterocytes, hepatocytes, and other tissues. Substrates of this pump are effluxed from the interior of the cells into the bloodstream or intestinal space. The role of MRP1 would be expected to increase the bioavailability of EGCG in vivo. The influence of MRP1 and 2 on the bioavailability of EGCG in vivo, however, is likely to depend on the relative expression and functional activity of each pump in a particular tissue. It was reported that the transcript level of MRP2 was over 10-fold higher than that of MRP1 in the human jejunum [24]; therefore efflux of EGCG by MRP2 may be predominant in the intestine, resulting in decrease of bioavailability.

MRP1 is known to be important in acquired resistance to various chemotherapy drugs in many cancer cells. As substrates of MRP1, EGCG and its methyl metabolites may act as competitive inhibitors and increase the efficacy of therapeutic agents whose efflux is mediated by MRP1. Although our preliminary results indicated that the accumulation of etoposide was not increased by EGCG and its methyl metabolites (at 10 μM) in both MDCKII and HT-29 cells, the possible inhibition of efflux of other chemotherapy drugs needs to be further investigated. The accumulation of EGCG and its methyl metabolites, however, was not affected by GF120918 in all cell lines, indicating that EGCG and its methyl metabolites are not substrates of Pgp or BCRP. It has been reported that green tea catechins, including EGCG, inhibited Pgp [25]. This inhibition may be due to binding to the pump rather than a competition with Pgp substrates.

Genistein is known to inhibit MRP-mediated drug transport in a competitive manner [26]. The presently observed increase of EGCG accumulation in cells by isoflavones is consistent with this concept and suggests that the competitive inhibition involves MRP1 rather than MRP2. It has been reported that curcumin inhibits efflux by Pgp and suppresses the expression of Pgp protein [27]. The present results are not related to this mechanism and suggest that curcumin and THC can also modulate MRP type efflux pumps. The increase of cellular accumulation of EGCG by other commonly consumed dietary phenolic compounds may produce a synergy in their biological activities.

In conclusion, the present results provide evidence that EGCG and its methyl metabolites are transported by MRP1 and MRP2. The efflux of these compounds by MRP2 may play a vital role in limiting the bioavailability of EGCG in vivo. Further, the results suggest that a combination of dietary compounds such as tea and isoflavones or curcumin may enhance the potential of beneficial effects of EGCG in tea by increasing its bioavailability. These findings represent a new line of research and should be explored further.

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