JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1001–1005

Received February 16, 2004
Accepted May 18, 2004
DOI 10.1211/0022357044003
ISSN 0022-3573

Inhibition of P-glycoprotein function by tea catechins in KB-C2 cells

Shuji Kitagawa, Tomohiro Nabekura and Shizu Kamiyama

Abstract

We studied the effects of tea catechins, (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin gallate (EGCG) on the P-glycoprotein (P-gp) function in multidrug-resistant P-gp over-expressing KB-C2 cells. EC did not have any effects on cellular accumulation of P-gp substrates, rhodamine-123 and daunorubicin, but the other catechins increased the accumulation in the order of EGC < ECG < EGCG. The effects of EGCG were larger than those of verapamil and quercetin. Since these catechins inhibited the efflux of P-gp substrates, the elevation of substrate accumulation seemed to be induced by the inhibition of the efflux transporter. The results showed that the inhibitory effects of the catechins did not depend on their total hydrophobicity, but significantly depended on their chemical structure. The presence of the galloyl moiety on the C-ring markedly increased the *n*-octanol/PBS partition coefficients of the catechins and their activity on P-gp. On the other hand, the presence of the trihydric pyrogallol group as the B-ring decreased the partition coefficients but increased the activity on P-gp, compared with the action of the corresponding catechins with a dihydric catechol B-ring.

Introduction

Over-expression of P-glycoprotein (P-gp), a plasma membrane transporter that extrudes chemotherapeutic agents out of cells, has been associated with the multidrug resistance of cancer cells. This ATP-dependent transporter extrudes a wide variety of structurally unrelated compounds, such as vinca alkaloids, etoposides, taxenes and anthracyclines (Bosch & Croop 1996; Sharom 1997). P-gp-mediated multidrug resistance is also reversed by various compounds such as verapamil, dihydropyridine analogs, quinidine and ciclosporin due to their inhibition of transporter activity (Bosch & Croop 1996; Sharom 1997). Recently, it has been revealed that various polyphenols such as quercetin also modulate P-gp activity (Castro & Altenberg 1997; Shapiro & Ling 1997; Conseil et al 1998; Zhang & Morris 2003). Their inhibitory effects have been reported in many cell lines, whereas in some other cell lines their stimulatory effects have been reported (Critchfield et al 1994). Among polyphenols, green tea flavonoids such as (−)-epigallocatechin gallate (EGCG) have also been recently reported to modulate P-gp activity. However, the effects seem to differ for each catechin. That is, the inhibitory effects of EGCG on P-gp activity have been reported in CH^RC5 cells and Caco-2 cells (Jodoin et al 2002). On the other hand, the elevation of P-gp function by (−)-epicatechin (EC) has been reported in NIH-3T3-G185 cells (Wang et al 2002).

Six catechins are present in green tea, the most abundant being (−)-epigallocatechin gallate (EGCG), followed by (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-catechin gallate (CG) and (+)-catechin (C) (Chu & Juneja 1997). Since the relationship of the effects of polyphenols on P-gp with either their chemical structure or physicochemical properties is also unknown, we tried to clarify it for four major catechins, EC, EGC, ECG and EGCG, the chemical structures of which are shown in Figure 1. We tried to clarify the relationship of the number of hydroxyl groups in the B-ring and the presence of the galloyl moiety on the C-ring with the activity of the catechins, as well as that between their total hydrophobicity and their activity. For this purpose we used multidrug-resistant human epidermal carcinoma cell line KB-C2 cells which over-express P-gp (Yoshimura et al 1989). We used

Niigata University of Pharmacy and Applied Life Sciences, Kamishin'ei-cho 5-13-2, Niigata 950-2081, Japan

Shuji Kitagawa, Tomohiro Nabekura, Shizu Kamiyama

Correspondence: S. Kitagawa, Niigata University of Pharmacy and Applied Life Sciences, Kamishin'ei-cho 5-13,2, Niigata 950-2081, Japan. E-mail:kitagawa@niigata-pharm.ac.jp

Funding: This study was supported by a grant from the Promotion and Mutual Aid Corporation for Private schools in Japan.

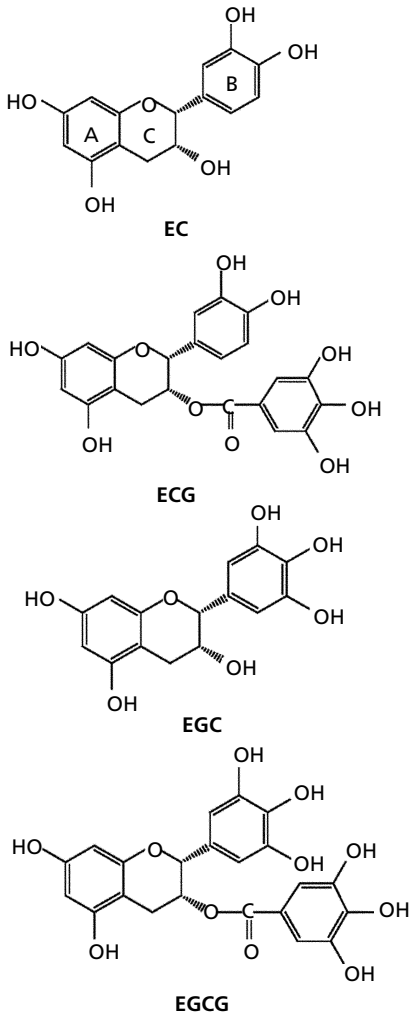

Figure 1 Chemical structure of tea catechins. EC, (−)-epicatechin; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin gallate; EGCG, (−)-epigallocatechin gallate.

fluorescent rhodamine-123 and daunorubicin as the substrates for P-gp; these have often been used for the study of various P-gp transport modulators, including flavonoids (Castro & Altenberg 1997; Jodoin et al 2002; Zhang & Morris 2003). Daunorubicin has also been revealed to be a substrate of multidrug resistance protein 1 (MRP1) (Renes et al 1999). However, since MRP1 is scarcely found in KB-C2 cells (Okumura et al 2000), its involvement in the substrate efflux is negligible.

Materials and Methods

Materials

EC, EGC, ECG and EGCG were purchased from Nakarai Tesque, Inc. (Kyoto, Japan). Rhodamine-123 was from Molecular Probe (Junction City, OR). Dulbecco's modified Eagle medium (D-MEM) and fetal bovine serum were from Invitrogen Co. (Carlsbad, CA). Daunorubicin hydrochloride and all other reagents were from Wako Pure Chemical Industries, Ltd (Osaka, Japan). KB-C2 cells were kindly provided by Prof. Shin-ichi Akiyama (Kagoshima University, Japan).

Cell culture

KB-C2 cells were cultured in D-MEM culture medium supplemented with 10% fetal bovine serum and $2\,\mu g\,mL^{-1}$ colchicine. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2–95% air.

Measurement of cellular accumulation of substrates

Cells were plated at 2.5×10^5 cells/35-mm dish and cultured for 24 h in a CO_2 incubator. Cells were then washed with D-MEM without serum and the medium was exchanged to D-MEM without serum. After addition of tea catechins, either 20 μM rhodamine-123 or 50 μM daunorubicin was added, and cells were incubated for another 2 h in a CO_2 incubator. Cells were then washed twice with an excess volume of ice-cold phosphate-buffered saline (PBS) and lysed with either 0.1% Triton X-100 (for rhodamine-123) or 1% sodium dodecyl sulfate (SDS) (for daunorubicin). Fluorescence intensity was measured with an F-4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan), and the accumulation amount of the probes was calculated. The excitation and emission wavelengths used for rhodamine-123 and daunorubicin were 485 and 532 nm and 502 and 588 nm, respectively.

Efflux of rhodamine-123

The efflux of rhodamine-123 was also measured using cells incubated with 20 μM rhodamine-123, without tea catechins, for 2 h as described above. Cells were then washed twice with D-MEM, without serum, to remove the fluorescence probe in the medium and incubated again with the medium in the presence or absence of tea catechins. After incubation for various times, cells were then washed twice with an excess volume of ice-cold PBS, lysed with 0.1% Triton X-100, and fluorescence intensity was measured as described above. The amount of rhodamine-123 retained in the cells was obtained.

Measurement of partition coefficients of tea catechins and quercetin between *n*-octanol and PBS

Tea catechin or quercetin solution (0.1–1.0 mM) in PBS (3 mL) was mixed with 3 mL *n*-octanol in test tubes with glass stoppers. The PBS and *n*-octanol used were pre-saturated with either *n*-octanol or PBS and de-oxygenized with a nitrogen stream. The test tubes were set at 37°C for 18 h in a shaking water bath. After stopping the shaking, the incubation continued for another 1 h. The concentration of tea catechins or quercetin in both the PBS phase and the *n*-octanol phase was determined

by HPLC (L-6000; Hitachi, Tokyo, Japan) with an L-4000 UV detector (Hitachi) at 280 nm (EC), 271 nm (EGC), 279 nm (ECG), 276 nm (EGCG) and 360 nm (quercetin). Separation was achieved on a reversed-phase column (Mightysil RP-18 GP, 4.6 mm i.d., 250 mm) using a mobile phase consisting of methanol–water–phosphoric acid (100:100:1) at a flow rate of 0.7 mL min^{-1}. Ferulic acid was used as an internal standard.

FACS flow cytometry

Fluorescence measurements of individual cells were performed using a Becton-Dickinson FACScalibur fluorescence-activated cell sorter (San Jose, CA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530 ± 15 nm band-pass filters). Analysis was gated to include single viable cells on the basis of forward and side light scatter and was based on acquisition of data from 10 000 cells. Log fluorescence was collected and displayed as single-parameter histograms representing the distribution of cells with different levels of fluorescence substrates.

Statistical analysis

One way analysis of variance and Bonferroni's post-hoc test was used to analyse the difference between the sets of data. A P-value less than 0.05 was considered statistically significant.

Results

Effect of tea catechins on accumulation of P-gp substrates

We first examined the effects of tea catechins on the accumulation of rhodamine-123 in KB-C2 cells. As shown in Figure 2, at 100 μM, ECG and EGCG, which are gallic acid esters and have a galloyl moiety on the C-ring (Figure 1), increased cellular accumulation of the fluorescent substrate 2.1 fold and 3.7 fold, respectively. The effect of EGCG was more significant than that of verapamil and quercetin. Increased accumulation of rhodamine-123 in individual cells in the presence of these catechins was also confirmed by flow cytometry for EGCG (Figure 3).

We also examined the effects of the same catechins on intracellular accumulation of daunorubicin. For the dose-dependent effects, increasing effects were observed at lower concentrations in the order of EGC < ECG < EGCG (Figure 4). These catechins significantly increased the accumulation at and above the concentrations of 200 μM (EGC), 100 μM (ECG) and 25 μM (EGCG) ($P < 0.01$). EC had no effect within 300 μM. Gallic acid had no effect.

Effect of tea catechins on efflux of P-gp substrates

The enhanced accumulation of rhodamine-123 and daunorubicin in the presence of tea catechins, such as EGCG, mentioned above seemed to be due to the inhibition of the P-gp-mediated efflux of these substrates by the tea catechins. Therefore, we examined the effects of EGCG, which increased the accumulation most markedly, on the efflux of rhodamine-123. After loading rhodamine-123 and removing the fluorescent substrate in the medium, the amount of substrate remaining in the cells was monitored in the presence or absence of EGCG. As shown in Figure 5, at 100 μM, the amount of rhodamine-123 remaining in KB-C2 cells was higher in the presence of EGCG than in its absence, suggesting that EGCG decreased the efflux of rhodamine-123 by blocking P-gp.

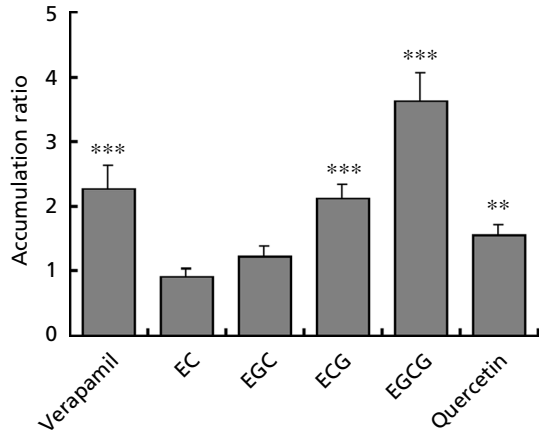

Figure 2 Effects of 100 μM tea catechins, verapamil and quercetin on the relative accumulation amount of rhodamine-123 in multidrug-resistant P-glycoprotein over-expressing KB-C2 cells. EC, (−)-epicatechin; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin gallate; EGCG, (−)-epigallocatechin gallate. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the value in the absence of tea catechins. Data are means ± s.d. of six experiments. The control value of accumulation, which was $(3.03 \pm 0.56) \times 10^8$ molecules/cell for eleven experiments, was defined as 1.00.

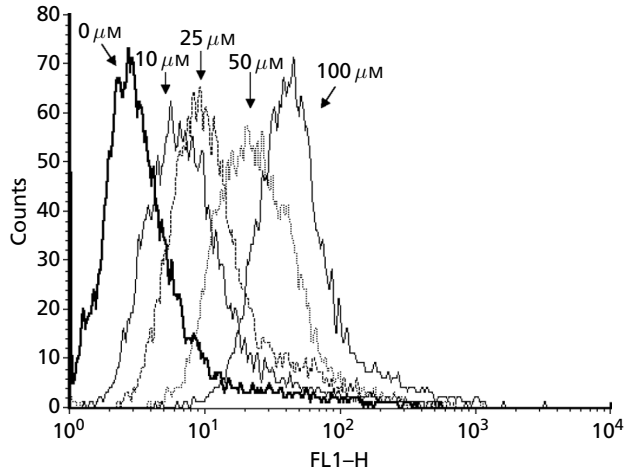

Figure 3 Analysis by flow cytometry of effects of (−)-epigallocatechin gallate (EGCG) (0–100 μM) on intracellular retention of rhodamine-123 in the accumulation phase of multidrug-resistant P-glycoprotein over-expressing KB-C2 cells.

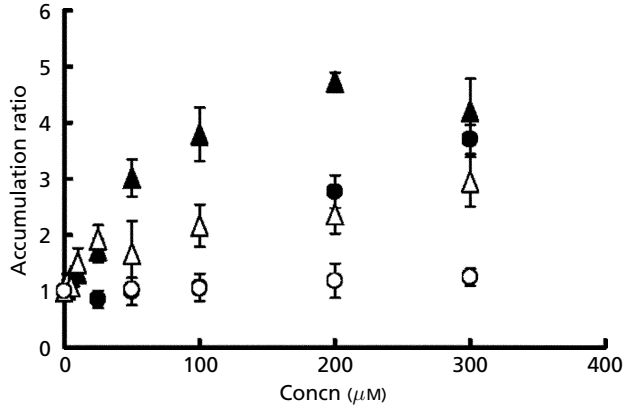

Figure 4 Effect of tea catechins (○, (−)-epicatechin (EC); ●, (−)-epigallocatechin (EGC); △, (−)-epicatechin gallate (ECG); ▲, (−)-epigallocatechin gallate (EGCG)) on accumulation of daunorubicin in multidrug-resistant P-glycoprotein over-expressing KB-C2 cells. Data are means ± s.d. of six experiments. The control value of accumulation, which was $(1.27 \pm 0.23) \times 10^9$ molecules/cell for nine experiments, was defined as 1.00.

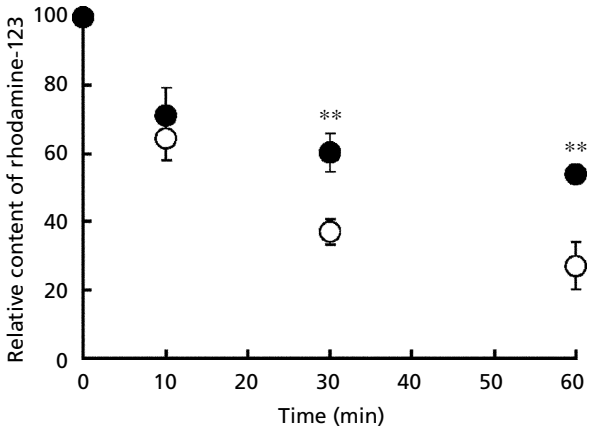

Figure 5 The efflux of rhodamine-123 in the presence (●) or absence (○) of 100 μM (−)-epigallocatechin gallate (EGCG). Data are means ± s.d. of six experiments. $^{**}P < 0.01$ compared with the control value.

Relationship between partition coefficients of tea catechins and their effects on P-gp

Since most of the compounds which have been revealed to interact with P-gp are relatively hydrophobic (Sharom 1997), there is a possibility that hydrophobicity is important for the inhibitory effects of catechins on P-glycoprotein transport activity. Therefore, we measured the partition coefficients P_{oct} of the four catechins between *n*-octanol and PBS at 37°C. As shown in Table 1 for the logarithm values, the P_{oct} values of gallic acid esters of the catechins EGCG and ECG were larger than those of the others, and increased in the order of EGC < EC < EGCG < ECG. This result indicated that the presence of a galloyl residue on the C-ring increased the partition coefficients, which was consistent with the increase in the inhibitory effects on P-gp. On the other hand, the presence of the *ortho*-trihydroxy group in the B-ring (gallyl group), as a substitute for the dihydric catechol group, decreased the partition coefficients, although the inhibitory effects of the catechins with the gallyl group were larger than those of the corresponding catechins with the catechol group. Therefore, a correlation between partition coefficient and inhibitory effect on P-glycoprotein was not observed. Likewise, the *n*-octanol/PBS partition coefficient of quercetin was much larger than that of EGCG, but its inhibitory effect on P-gp activity was smaller.

Table 1 Partition coefficients of tea catechins and quercetin between *n*-octanol and PBS at 37°C

Catechins	log P_{oct}
EC	−0.079 ± 0.026
EGC	−0.44 ± 0.04
ECG	1.28 ± 0.08
EGCG	0.61 ± 0.03
Quercetin	2.69 ± 0.08

Data are means ± s.d. of nine experiments at three different concentrations.

Discussion

Recently, various flavonoids and isoflavones have been revealed to interact with P-gp. That is, flavonoids such as quercetin and apigenin have been reported as modulators of P-gp in cells over-expressing the transporter (Shapiro & Ling 1997). The isoflavone genistein inhibited the efflux of rhodamine-123 and the binding of the P-gp substrate azidopine to the transporter in BC19/3 cells (Castro & Altenberg 1997). Flavonoids such as morin and silymarin have also been reported to inhibit azidopine binding in MDA435/LCC6 cells (Zhang & Morris 2003). As for the mechanisms of the modification, flavonoids like kaempheride have also been revealed as modulators with bifunctional interactions at vicinal ATP-binding sites and steroid-interacting hydrophobic regions within the cytoplasmic domain of P-gp (Conseil et al 1998).

Among flavonoids, green tea catechins such as EGCG have also been recently reported to inhibit rhodamine-123 efflux and iodoarylazidoprazosin binding to P-gp in CH^RC5 cells (Jodoin et al 2002). On the other hand, EC has been reported to enhance P-gp activity in NIH-3T3-G185 cells (Wang et al 2002). According to the present findings in KB-C2 cells, EC did not have any effect on P-gp activity. The present findings also revealed that the effects on P-gp activity in KB-C2 cells markedly depended on the chemical structure of the tea catechins, and did not depend on their total hyrophobicity. It has been reported that a galloyl moiety on the C-ring produces the hydrophobic region of ECG and EGCG and increases their affinity for the lipid bilayer (Hashimoto et al 1999). That hydrophobic moiety seems to be very important for the interaction

of the catechins with P-gp. Additionally, although hydrophobicity decreases in the presence of a larger number of hydroxyl groups in the B-ring, the presence of the trihydric pyrogallol group was more favourable for the interaction with P-gp than the dihydric catechol group as the B ring. EGCG is relatively hydrophilic, but its amphiphilic chemical structure may be favourable for the interaction with either the substrate binding sites or the activity-modulating sites of P-glycoprotein.

Tea catechins have also been revealed to inhibit the activity of other membrane transporters (Murakami et al 1992; Shimizu et al 2000; Naftalin et al 2003). However, the order of the effects differs for each membrane transporter. For example, EGC was the most potent inhibitor of the human erythrocyte glucose transporter GLUT1 (Naftalin et al 2003), whereas EGCG was the most potent inhibitor of H^+,K^+-ATPase (Murakami et al 1992), as revealed here for P-gp. Therefore, the relationship between the chemical structure of the tea catechins and their inhibitory activity seems to depend on the characteristics of the binding sites of each transporter.

Tea catechins, especially EGCG, seem to be a promising natural dietary compound for overcoming the multidrug resistance that derives from the active efflux of anti-tumour drugs by P-gp. Further work is necessary to clarify the effects, especially in-vivo, of EGCG on multidrug resistance associated with P-gp in chemotherapy using anti-tumour drugs.

Conclusions

This study revealed that tea catechins increased the cellular accumulation of substrates of P-glycoprotein by inhibiting their efflux by the transporter. From the results, it was shown that the activity of the tea catechins depended on the substituents of the flavonoid ring. The presence of a galloyl residue on the C-ring markedly increased the activity of the catechins. As the B-ring, the trihydric pyrogallol group was more favourable to the activity than the dihydric catechol group. However, no relationship was found between the partition coefficients of the catechins and their effects.

References

Bosch, I., Croop, J. (1996) P-glycoprotein multidrug resistance and cancer. *Biochim. Biophys. Acta* **1288**: F37–F54

Castro, A. F., Altenberg, G. A. (1997) Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. *Biochem. Pharmacol.* **53**: 89–93

Chu, D.-C., Juneja, L. R. (1997) General chemical composition of green tea and its infusion. In: Yamamoto, T., Juneja, L. R., Chu, D.-C., Kim, M. (eds) *Chemistry and applications of green tea*. CRC Press, Boca Raton, pp 13–22

Conseil, G., Baubichon-Cortay, H., Dayan, G., Jault, J.-M., Barron, D., Di Pietro, A. (1998) Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. *Proc. Natl Acad. Sci. USA* **95**: 9831–9836

Critchfield, J. W., Welsh, C. J., Phang, J. M., Yeh, G. C. (1994) Modulation of adriamycin accumulation and efflux by flavonoids in HCT-15 colon cells. *Biochem. Pharmacol.* **48**: 1437–1445

Hashimoto, T., Kumazawa, S., Nanjo, F., Hara, Y., Nakayama, T. (1999) Interaction of tea catechins with lipid bilayers investigated with liposome systems. *Biosci. Biotechnol. Biochem.* **63**: 2252–2255

Jodoin, J., Demeule, M., Beliveau, R. (2002) Inhibition of the multidrug resistance P-glycoprotein activity by green tea polyphenols. *Biochim. Biophys. Acta* **1542**: 149–159

Murakami, S., Muramatsu, M., Otomo, S. (1992) Gastric H^+, K^+-ATPase inhibition by catechins. *J. Pharm. Pharmacol.* **44**: 926–928

Naftalin, R. J., Afzal, I., Cunningham, P., Halai, M., Ross, C., Salleh, N., Milligan, S. R. (2003) Interactions of androgens, green tea catechins and the antiandrogen flutamide with the external glucose-binding site of the human erythrocyte glucose transporter GLUT1. *Br. J. Pharmacol.* **140**: 487–499

Okumura, H., Chen, Z.-S., Sakou, M., Sumizawa, T., Furukawa, T., Komatsu, M., Ikeda, R., Suzuki, H., Hirota, K., Aikou, T., Akiyama, S.-I. (2000) Reversal of P-glycoprotein and multidrug-resistance protein-mediated drug resistance in KB cells by 5-O-benzoylated taxinine K. *Mol. Pharmacol.* **58**: 1563–1569

Renes, J., de Vries, E. G. E., Nienhuis, E. F., Jansen, P. L. M., Muller, M. (1999) ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. *Br. J. Pharmacol.* **126**: 681–688

Shapiro, A. B., Ling V. (1997) Effect of quercetin on Hoechst 33342 transport by purified and reconstituted P-glycoprotein. *Biochem. Pharmacol.* **53**: 587–596

Sharom, F. J. (1997) The P-glycoprotein efflux pump: How does it transport drugs? *J. Membrane Biol.* **160**: 161–175

Shimizu, M., Kobayashi, Y., Suzuki, M., Satsu, H., Miyamoto, Y. (2000) Regulation of intestinal glucose transport by tea catechins. *BioFactors* **13**: 61–65

Wang, E.-J., Barecki-Roach, M., Johnson, W. W. (2002) Elevation of P-glycoprotein function by a catechin in green tea. *Biochem. Biophys. Res. Commun.* **297**: 412–418

Yoshimura, A., Kuwazuru, Y., Sumizawa, T., Ikeda, S.-I., Ichikawa, M., Usagawa, T., Akiyama, S.-I. (1989) Biosynthesis processing and half-life of P-glycoprotein in a human multidrug-resistant KB cell. *Biochim. Biophys. Acta* **992**: 307–314

Zhang, S., Morris, M. E. (2003) Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *J. Pharmacol. Exp. Ther.* **304**: 1258–1267