Inhibition of the multidrug resistance P-glycoprotein activity by green tea polyphenols

Julie Jodoin [a,b], Michel Demeule [a,b], Richard Béliveau [a,b,*]

[a] *Laboratoire de Médecine Moléculaire, Centre de Cancérologie Charles Bruneau, Université du Québec à Montréal – Hôpital Sainte-Justine, C.P. 8888, Succursale Centre-Ville, Montréal QC, Canada H3C 3P8*
[b] *Groupe de Recherche en Transport Membranaire, Université de Montréal, Montréal, QC Canada*

Abstract

Many beneficial proprieties have been associated with polyphenols from green tea, such as chemopreventive, anticarcinogenic, antiatherogenic and antioxidant actions. In this study, we investigated the effects of green tea polyphenols (GTPs) and their principal catechins on the function of P-glycoprotein (P-gp), which is involved in the multidrug resistance phenotype of cancer cells. GTPs (30 μg/ml) inhibit the photolabeling of P-gp by 75% and increase the accumulation of rhodamine-123 (R-123) 3-fold in the multidrug-resistant cell line CH^{R}C5, indicating that GTPs interact with P-gp and inhibit its transport activity. Moreover, the modulation of P-gp transport by GTPs was a reversible process. Among the catechins present in GTPs, EGCG, ECG and CG are responsible for inhibiting P-gp. In addition, EGCG potentiates the cytotoxicity of vinblastine (VBL) in CH^{R}C5 cells. The inhibitory effect of EGCG on P-gp was also observed in human Caco-2 cells, which form an intestinal epithelial-like monolayer. Our results indicate that, in addition to their anti-cancer properties, GTPs and more particularly EGCG inhibit the binding and efflux of drugs by P-gp. Thus, GTPs or EGCG might be potential agents for modulating the bioavailability of P-gp substrates at the intestine and the multidrug resistance phenotype associated with expression of this transporter in cancer cells. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

P-glycoprotein (P-gp) was first studied in the context of cancer research where its overexpression in tumor cells has been associated with the multidrug resistance (MDR) phenotype [1–3]. In cancer cells, P-gp acts as an efflux pump that extrudes chemotherapeutic agents out of the cells, decreasing their intracellular concentration. This ATP-dependent transporter also exports a wide variety of structurally unrelated compounds such as vinka alkaloids, antibiotics, anthracyclines, etoposides, cytokines, opioids and steroids [3–8]. P-gp is also expressed in normal tissues. High levels of P-gp expression have been observed in the endothelial cells of brain capillaries, in kidney and in adrenal glands

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while moderate expression has been detected in lung, liver and intestines [9–13]. P-gp is involved in organism detoxification by excreting toxic compounds into the bile, urine and gastrointestinal tract [14–16]. Moreover, it seems to play an important role in brain protection at the blood-brain barrier [14,17]. Localized at the luminal side of endothelial cells in brain capillaries, P-gp prevents the passage of many drugs into the brain [18,19].

Recently, many groups have studied the beneficial effects of natural products in cancer prevention or treatment. Studies have suggested that polyphenols from green tea could have chemopreventive, anti-atherogenic, anticarcinogenic and antioxidant proprieties [20]. Epidemiological studies suggest a chemopreventive effect of green tea against breast, prostate, esophagus, stomach, pancreas and colon cancers [20–22]. Moreover, green tea consumption in animal models inhibits cancer angiogenesis [23] and metastasis [24], and reduced tumor formation in skin, lung, liver, pancreas and the gastrointestinal tract [20,21]. However, nothing is known about the effects of polyphenols from green tea on the activity of P-gp. Green tea contains many polyphenolic compounds. Flavanols, also called catechins, are the major polyphenols found in green tea. 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 (GC) and (+)-catechin (C) [25].

In the present study, we investigated the effects of green tea polyphenols (GTPs) and the six principal catechins of GTPs on P-gp function. Photoaffinity labeling experiments using [125I]iodoarylazidoprazosin (IAAP) show that GTPs interact with P-gp. More specifically, the catechins EGCG, ECG and CG interact with P-gp. Furthermore, GTPs and the principal catechin of green tea, EGCG, inhibit the transport of two P-gp substrates, R-123 and [3H]VBL, into both Chinese hamster ovary resistant cells (CH^{R}C5) and human colon adenocarcinoma cells (Caco-2). These results suggest that GTPs and EGCG, which interact with P-gp and inhibit its transport activity, could be used to modulate the function of P-gp.

2. Materials and methods

2.1. Materials

Sensitive (AuxB1) and resistant (CH^{R}C5) cell lines were provided by Dr. V. Ling and were grown in α-MEM from Gibco BRL (Burlington, ON, Canada) with 10% calf serum from HyClone Laboratories (Logan, UT, USA). Caco-2 human colon adenocarcinoma cells were purchased from ATCC (Rockville, MD, USA). Caco-2 cells were grown in DMEM high glucose (Gibco BRL) containing 10 mg/l transferrin, 110 mg/l pyruvate and 10% calf serum from HyClone Laboratories. Electrophoresis reagents were purchased from BioRad (Mississauga, ON, Canada). MAb C219, directed against P-gp, was from ID Labs (London, ON, Canada). Anti-mouse IgG horseradish peroxidase-linked whole antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfée, QC, Canada). [125I]Iodoarylazidoprazosin (IAAP) (2200 Ci/mmol) and [14C]sucrose (671 mCi/mmol) were purchased from NEN (Boston, MA, USA) whereas [3H]vinblastine (VBL) (11.3 Ci/mmol) was from Amersham Pharmacia Biotech (Oakville, ON). Cyclosporin A and PSC 833 were provided by Novartis (Basel, Switzerland). Green tea polyphenols (GTPs) were purchased from LKT Laboratories (St-Paul, MN, USA) and catechins were purchased from Funakoshi Co (Tokyo, Japan), Sigma-Aldrich Canada (Oakville, ON, Canada) and from ICN Biomedicals (Aurora, OH, USA). All other reagents were from Sigma–Aldrich Canada (Oakville, ON).

2.2. Photoaffinity labeling with [125I]IAAP

Membranes from CH^{R}C5 cells were prepared as described previously [26]. CH^{R}C5 membranes (50 μg) were incubated with 20 nM IAAP (2.3 μCi) in 10 mM Tris–HCl (pH 7.5) for 1 h at 25°C in the dark with 0.1% DMSO (control), verapamil (50 μM), cyclosporin A (5 μM), PSC 833 (5 μM), resveratrol (100 μM), GTPs (30 μg/ml) or catechins (50 μM). The membranes were cross-linked under an UV Stratalinker 2400 lamp (Stratagene, La Jolla, CA, USA) at 254 nm for 5 min at 4°C. Laemmli

electrophoresis buffer (62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) was added to the membrane samples and the proteins were resolved by SDS–PAGE on 6.25% acrylamide/bisacrylamide (29.2:0.8) gels. The gels were dried and the photolabeled P-gp was detected following exposure of the gels to Fuji films for 1–3 h at −80°C.

2.3. Rhodamine-123 accumulation

CH^{R}C5 cells were seeded at 130 000 cells per well in 24-well plates and cultured for 3 days at 37°C and 5% CO_2. At confluence, cells were washed twice with Hank's balanced salt solution (HBSS) (1.3 mM $CaCl_2$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.83 mM $MgSO_4$, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4 and 25 mM D-glucose, pH 6.5) at 37°C. Cells were pre-incubated 30 min at 37°C with HBSS containing DMSO (0.1% v/v) or tested compounds (30 and 100 μg/ml GTPs, 100 μM catechins, 5 μM CsA, 5 μM PSC 833, 50 μM verapamil or 100 μM resveratrol). R-123 (20 μM) was then added for an incubation of 2 h at 37°C. R-123 accumulation was stopped by washing the cells five times with cold phosphate-buffered saline (PBS) (150 mM NaCl, 2.7 mM KCl, 1.3 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and the cells were lysed with 0.1% Triton X-100 at room temperature. Fluorescence of R-123 in cell lysates was measured using a spectrofluorometer (SpectraMax Gemini, Molecular Devices) at a wavelength of 485 nm for excitation and 538 nm for emission.

2.4. Efflux of Rhodamine-123

At confluence, CH^{R}C5 cells in 24-well plates were washed twice with HBSS and incubated 2 h at 37°C with 20 μM R-123 and 10 μM CsA in order to load the cells with R-123. Cells were then washed five time with HBSS, following which HBSS containing 100 μM EGCG, 10 μM CsA, 10 μM PSC 833 or 0.1% DMSO was added. After incubation at 37°C for 0 to 60 min, cells were washed five times with cold PBS and lysed with 0.1% Triton X-100 at room temperature. The fluorescence of R-123 in cell lysates was measured as described above.

2.5. Analysis of catechins in green tea polyphenols

The proportions of the individual catechins CG, ECG and EGCG in green tea polyphenol extracts were analyzed by high-performance liquid chromatography (HPLC) on a C18 column with an isocratic mobile phase composed of $CH_3CN/C_2H_2OCOCH_3$/ 0.05% H_3PO_4 (12:0.6:90). For each catechin, a standard curve was made and its percentage (w/w) of green tea polyphenols was calculated.

2.6. P-gp detection

P-gp was immunodetected by Western blot analysis. Proteins from CH^{R}C5 or AuxB1 cells were resuspended in Laemmli electrophoresis buffer without prior heating, loaded and separated on 7.5% acrylamide/bisacrylamide (29.2:0.8) SDS–PAGE gels. Gels were electroblotted onto PVDF membranes using standard procedures. Blots were blocked overnight in Tris-buffered saline (TBS: 147 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween 20 and 5% non-fat dry milk followed by a 1 h 30 min incubation with the primary antibody against P-gp (mAb C219) as described previously [12]. Blots were washed three times with TBS containing 0.3% Tween 20 followed by a 1 h incubation with horseradish peroxidase-conjugated antibodies directed against mouse IgGs. Blots were again washed three times with TBS and 0.3% Tween 20, and detection was performed with ECL reagents (Amersham) according to the manufacturer's instructions.

2.7. Cell proliferation assay

CH^{R}C5 or AuxB1 cells were seeded at 5000 and 3000 cells per well, respectively, in 96-well plates. Varying concentrations of EGCG (0–50 μM) with or without VBL (0–100 μM) were added and cells were grown for 3 days at 37°C and 5% CO_2. To measure cell proliferation, a crystal violet assay was used. Briefly, the cells were washed three times with PBS and incubated 5 min at room temperature with 0.2% crystal violet in 10% ethanol. The crystal violet stain was removed and the plate was washed three times with PBS. Cells were lysed with 2% SDS and absorbance was read at 560 nm with a spectrophotometer (ThermoMax, Molecular Devices).

2.8. Vinblastine transport and accumulation in Caco-2 cells

Apical-to-basal transport of VBL across a monolayer of Caco-2 cells was measured in the presence of P-gp or MRP inhibitors. The intracellular accumulation of VBL in Caco-2 cells was also measured and the passage of sucrose across monolayers was used to verify the integrity of the monolayer barrier during all experiments. Caco-2 cells were seeded at 1500 cells per filter (Transwell, 24 mm diameter, 0.4 μm pore size, Costar, Cambridge, MA, USA) in six-well plates and cultured at 37°C and 5% CO_2. At the 20th day of culture, Caco-2 cells were used for the experiment. The transepithelial resistance, measured with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FA, USA), was generally 800 Ω/ cm^2. All of the experiments were performed at 37°C. Caco-2 cell filters were washed with HBSS and transferred into fresh six-well plates containing 3 ml of HBSS. In the upper chamber of each filter, 1 ml of HBSS with 20 nM [^{3}H]VBL (0.23 μCi) or 74.5 nM [^{14}C]sucrose (0.05 μCi) with or without EGCG (100 μM), PSC 833 (10 μM) or indomethacin (10 μM). After 30 and 60 min, filters were moved into fresh wells to limit basal-to-apical flux. After 120 min, filters were placed into fresh six-well plates, washed three times with cold PBS and the polyester membranes containing Caco-2 cells were removed and placed into vials containing 10 ml of scintillation cocktail solution in order to quantify the [^{3}H]VBL accumulation in cells. Five hundred μl from 3 ml of each lower chamber were also put into scintillation vials and the radioactivity was measured. The apical-to-basolateral fluxes of [^{3}H]VBL and [^{14}C]sucrose were calculated as described by Takanaga et al. [27] and Fenart et al. [28].

2.9. Densitometric and statistical analysis

All of the experiments were performed at least three times. The intensities of the bands obtained from the photolabeling experiment were calculated with a Personal densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA). Statistical analysis was performed using Student's paired *t*-test. $P < 0.05$ was considered significant.
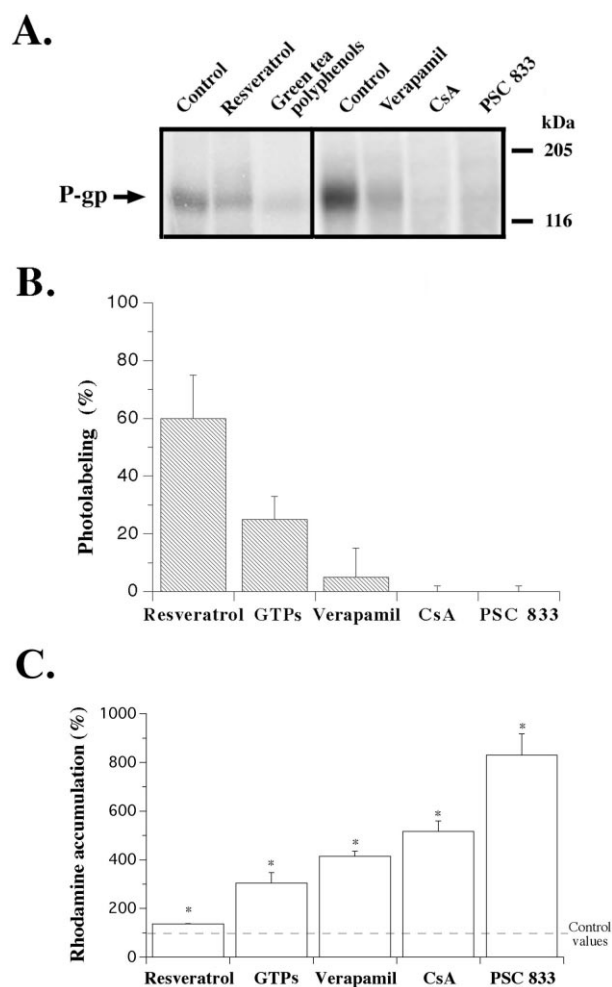

Fig. 1. Inhibition of P-gp photolabeling and transport. (A) For P-gp photolabeling, CH^{R}C5 cell membranes (50 μg protein) were incubated in the presence of 0.1% DMSO (control), 100 μM resveratrol, 30 μg/ml green tea polyphenols (GTPs), 50 μM verapamil, 5 μM cyclosporin A (CsA) or 5 μM PSC 833 along with IAAP (20 nM) followed by cross-linking with UV light. Proteins were resolved by SDS–PAGE using 6.25% polyacrylamide gels as described in Section 2. For the detection of photolabeled P-gp with [^{125}I]IAAP, gels were exposed to Fuji films at −80°C. (B) The bands corresponding to photolabeled P-gp were analyzed by laser densitometry. Results are presented as the percentage of photolabeled P-gp ± S.D. in the presence of individual agents as compared to the control (100%). One representative experiment is shown (*n* = 3). (C) To study the transport activity of P-gp, the accumulation of R-123 in CH^{R}C5 cells was measured over 2 h as described in Section 2. R-123 accumulation in CH^{R}C5 cells in the presence of 0.1% DMSO, resveratrol (100 μM), green tea polyphenols (30 μg/ml), verapamil (50 μM), CsA (5 μM) or PSC 833 (5 μM) was measured, and the results are expressed as a percentage of R-123 accumulation in the control cells ± S.D. (*n* = 3).

3. Results

3.1. Effect of green tea polyphenols on binding and transport activities of P-gp

We first investigated the inhibition of P-gp photo-labeling in resistant cells overexpressing P-gp (CH^{R}C5) by GTPs and resveratrol, a polyphenol from red wine (Fig. 1A,B). Levels of photolabeled
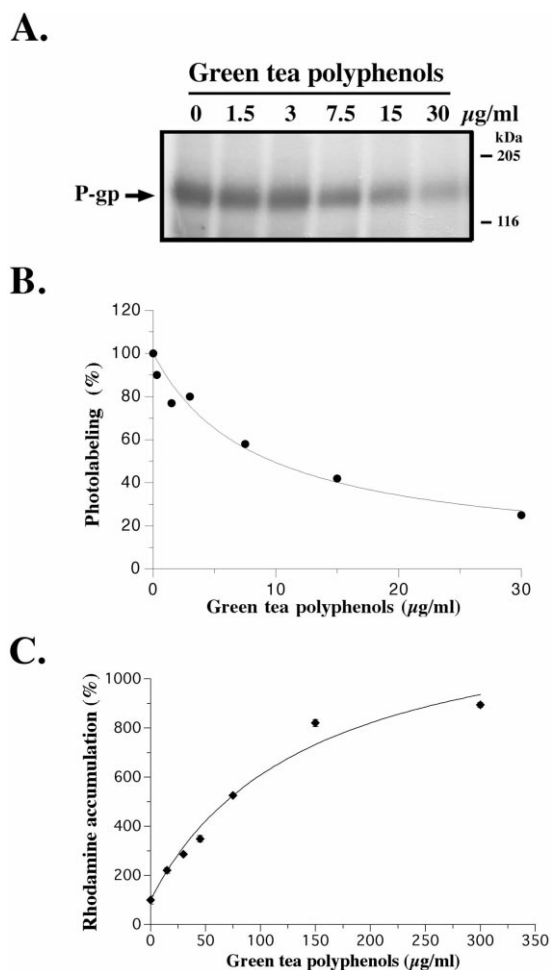
Fig. 2. Inhibition of P-gp photolabeling and transport by green tea polyphenols. (A) CH^{R}C5 membrane proteins (50 μg) were incubated with different concentrations of GTPs along with IAAP (20 nM) and then cross-linked with UV light. Proteins were resolved by SDS–PAGE using 6.25% polyacrylamide gels. (B) The levels of photolabeled P-gp were estimated by laser densitometry and expressed as a percentage of control level ($n = 3$). (C) The R-123 accumulation in CH^{R}C5 cells was measured in the presence of varying concentrations of GTPs. Results are expressed as a percentage of R-123 accumulation in the control cells ± S.D. ($n = 3$).
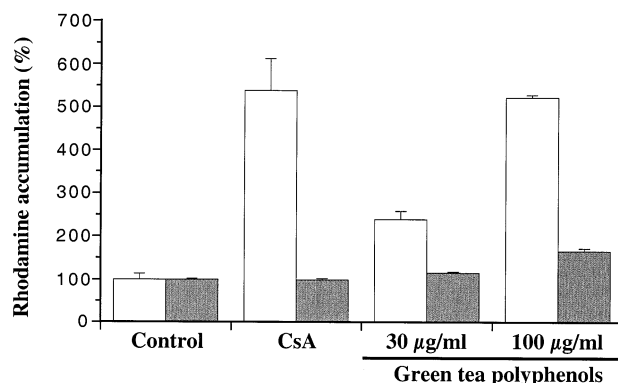
Fig. 3. Reversibility of P-gp transport inhibition by GTPs. To verify whether the inhibition of P-gp transport in CH^{R}C5 by GTPs was reversible, R-123 accumulation was measured under standard conditions (open bars) as described in Section 2, or after washing the cells to remove CsA or GTPs (closed bars). CH^{R}C5 cells were pre-incubated with 0.1% DMSO (control), 5 μM CsA or 30 and 100 μg/ml GTPs for 30 min, washed five times with HBSS (where applicable) and R-123 (20 μM) was added for 2 h at 37°C. Results are expressed as a percentage of R-123 accumulation in the control cells ± S.D. ($n = 3$).

P-gp were decreased by 40% and 75% in the presence of resveratrol (100 μM) and GTPs (30 μg/ml), respectively. Known P-gp inhibitors verapamil (50 μM), CsA (5 μM) and PSC 833 (5 μM) decreased the photolabeling of P-gp by more than 90%. Increasing the concentration of GTPs augmented the inhibition of P-gp photolabeling (Fig. 2A,B). The concentration of GTPs which inhibited 50% of P-gp photolabeling was 10 μg/ml.

The uptake of R-123, a P-gp substrate, by CH^{R}C5 cells was used to study P-gp transport. R-123 accumulation was increased in cells incubated with resveratrol, GTPs, verapamil, CsA and PSC 833 by 1.4-, 3.1-, 4.2-, 5.2- and 8.3-fold, respectively (Fig. 1C). To further examine the inhibition of P-gp by GTPs, R-123 accumulation was evaluated in the presence of increasing concentrations of GTPs (Fig. 2C). GTPs increased the accumulation of R-123 in CH^{R}C5 cells in a dose-dependent manner. At the lowest concentration of GTPs used, 15 μg/ml, R-123 accumulation was increased 2.2-fold compared to 8.3-fold at 300 μg/ml. At this higher concentration of GTPs, R-123 accumulation was similar to that measured in the presence of 5 μM PSC 833, a well known and potent P-gp inhibitor.

Following these experiments, we sought to determine whether the inhibition of P-gp transport caused
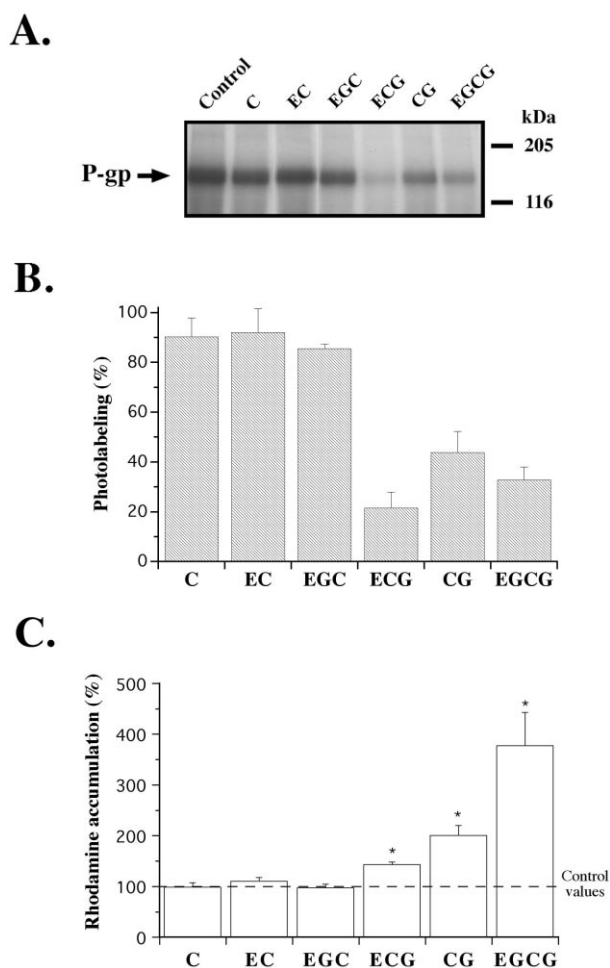
A.

B.

C.

Fig. 4. Effect of individual catechins from GTPs on P-gp photolabeling and transport. (A) CH^{R}C5 membrane proteins (50 μg) were incubated with 50 μM of each catechin (C, EC, EGC, ECG, CG and EGCG) and IAAP (20 nM) followed by cross-linking under UV light. Proteins were resolved by SDS–PAGE using 6.25% polyacrylamide gels. (B) The levels of photolabeled P-gp were estimated by laser densitometry and expressed as a percentage of control level (n = 3). (C) R-123 accumulation in CH^{R}C5 cells was measured in the presence of 100 μM of each catechin (C, EC, EGC, ECG, CG and EGCG). Results are expressed as a percentage of R-123 accumulation in the control cells (n = 4).

by GTPs was reversible (Fig. 3). In this approach, CH^{R}C5 cells were pre-incubated with 30 or 100 μg/ml GTPs or 5 μM CsA for 30 min. After this pre-incubation, cells were washed five times before the addition of 20 μM R-123. Compared to non-washed cells, the accumulation of R-123 in washed cells returned to the control values, indicating that inhibitors were removed during the washing procedures.

Thus, the inhibition of P-gp transport by GTPs, as well as by CsA, was reversible.

3.2. Effects of individual catechins from green tea on the activity of P-gp

We investigated the inhibition of P-gp photolabeling and transport by the major catechins found in green tea (Fig. 4). At a concentration of 50 μM, the catechins C, EC and EGC decreased P-gp photolabeling with IAAP by 10% whereas CG, EGCG and ECG inhibited 56%, 67% and 78% of P-gp labeling, respectively (Fig. 4A,B). Among the catechins tested, only ECG, CG and EGCG had an effect on P-gp transport (Fig. 4C). EGCG, CG and ECG increased the accumulation of R-123 in CH^{R}C5 cells by 3.7-, 2.0- and 1.5-fold, respectively. The proportions of these three catechins in the GTPs used in the present study were analyzed by HPLC. EGCG, ECG and CG comprised 33%, 4.1% and less than 1% (w/w) of GTPs, respectively, suggesting that the effect of these polyphenols on P-gp was mainly caused by EGCG.

To further study the inhibition of P-gp transport by EGCG, the efflux of R-123 was measured as a direct representation of P-gp transport (Fig. 5). Efflux experiment was performed in order to verify that EGCG affects P-gp and not the membrane permeability, since EGCG could increase R-123 accumula-
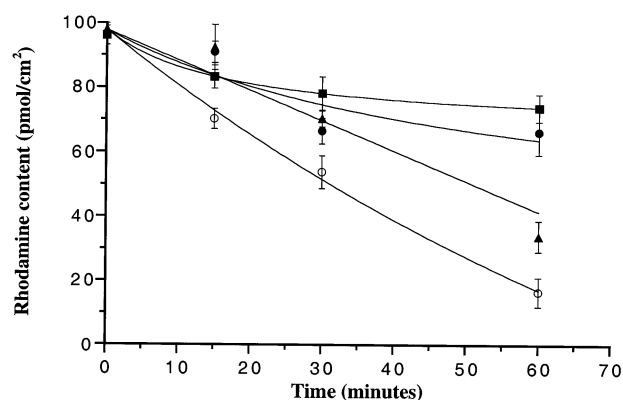

Fig. 5. Efflux of R-123 from CH^{R}C5 cells. Cells were pre-incubated with R-123 (20 μM) and CsA (10 μM) for 2 h followed by three washes with HBSS. Cells were then incubated in HBSS with 0.1% DMSO as a control (○), 100 μM EGCG (▲), 10 μM CsA (●) or 10 μM PSC 833 (■). The R-123 remaining in the CH^{R}C5 cells was measured at intervals over 60 min (n = 4).

tion in CH^RC5 cells by increasing membrane permeability. CH^RC5 cells were loaded with the fluorescent dye, washed and the R-123 remaining in the cells was then monitored in the presence or absence of P-gp modulators. A diminution over time of the R-123 in the cells indicates that R-123 was expelled from the cells. In the presence of EGCG, like CsA and PSC 833, the R-123 remaining in CH^RC5 cells was higher than in the control cells, suggesting that EGCG decreased the efflux of R-123 by blocking P-gp.

3.3. Proliferation of CH^RC5 and AuxB1 cells in the presence of EGCG

P-gp expression in the parental sensitive cell line (AuxB1) and the resistant cell line overexpressing P-gp (CH^RC5) was monitored by Western blots using the mAb C219 (Fig. 6A). Immunodetection results showed that CH^RC5 cells express a higher level of P-gp than do AuxB1 cells. These two cell lines were used to determine the effect of EGCG on cell proliferation. Cells were grown for 3 days in the presence of different concentrations of EGCG (0–50 μM) without reaching confluence and the crystal violet assay was used to measure the effect of this catechin on cell growth. The proliferation of CH^RC5 cells was less affected than AuxB1 cells by the presence of EGCG (Fig. 6B). Similar results were obtained with CH^RC5 and AuxB1 cells when using 0 to 100 μg/ml of GTPs (data not shown). Furthermore, the combination of EGCG with VBL was tested on CH^RC5 cell proliferation to determine whether EGCG potentiates the toxicity of the chemotherapeutic agent VBL (Table 1). The concentration of

Table 1
Proliferation of AuxB1 and CH^RC5 cells in the presence of vinblastine

	EGCG (μM)	IC_{50} for vinblastine (nM)
AuxB1	0	9 ± 4
CH^RC5	0	1000 ± 200
	10	600 ± 100
	50	9 ± 5

AuxB1 and CH^RC5 cells were grown at subconfluence in the presence of VBL and the concentration (IC_{50}) of VBL for which cell growth was decreased by 50% was determined. In addition to VBL, CH^RC5 cells were incubated with 10 and 50 μM EGCG. Crystal violet assay was used to measure cell proliferation ($n = 3$).
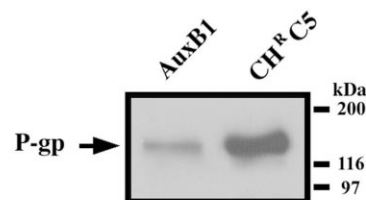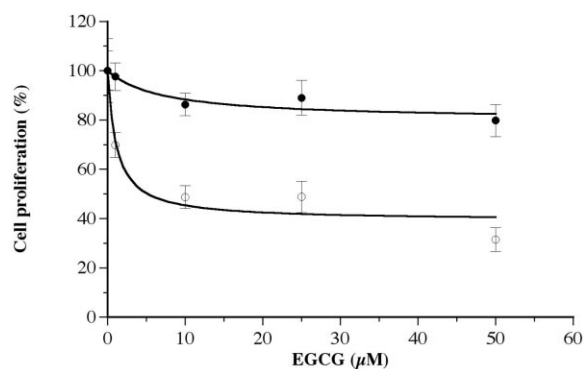

Fig. 6. Proliferation of CH^RC5 and AuxB1 cells in the presence of EGCG. (A) Immunodetection of P-gp in AuxB1 (20 μg of protein) and CH^RC5 (2 μg) cells by Western blot. (B) AuxB1 (○) and CH^RC5 (●) cells were grown at subconfluence in the presence of 0–50 μM EGCG. The crystal violet assay was used to measure the proliferation of cells as described in Section 2 ($n = 3$).

VBL which decreases cell growth by 50% (IC_{50}) was 1000 nM for CH^RC5 cells and 9 nM for the sensitive AuxB1 cells. When CH^RC5 cells were grown in the presence of VBL and 10 or 50 μM of EGCG, the IC_{50} values decreased to 600 and 9 nM, respectively.

3.4. Vinblastine transport and accumulation in Caco-2 cells

Human intestinal adenocarcinoma cells (Caco-2), which form an epithelial monolayer, are often used as a model for intestinal transport studies mediated by P-gp. [^{3}H]VBL flux across the intestinal barrier is represented by the passage of [^{3}H]VBL, a P-gp substrate, from the apical compartment towards the basolateral side. After 2 h incubation the amount of [^{3}H]VBL was measured in the lower chamber of the well, which corresponds to the basolateral side.
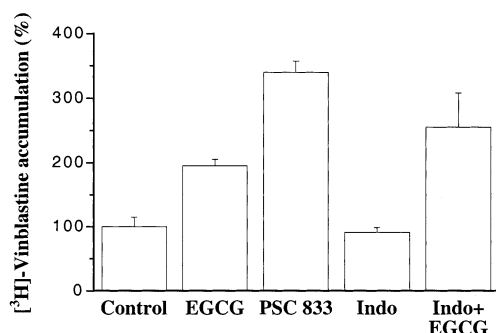
A.
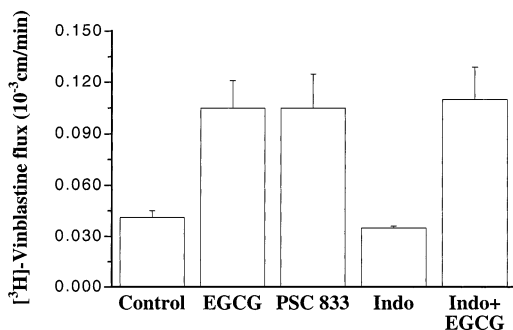
B.

Fig. 7. Inhibition of vinblastine transport in Caco-2 cells by EGCG. (A) Caco-2 cells were used to measure apical-to-basal transport of [³H]VBL in the presence of 0.1% DMSO (control), 100 μM EGCG, 10 μM PSC 833, 10 μM indomethacin (Indo) or a combination of 100 μM EGCG with 10 μM indomethacin (Indo+EGCG). Apical-to-basolateral flux of [³H]VBL was calculated as previously described in Section 2. (B) [³H]VBL accumulation in Caco-2 cells was measured by recovering the filters containing the cells exposed to 100 μM EGCG, 10 μM PSC 833, 10 μM indomethacin or 100 μM EGCG with 10 μM indomethacin and comparing radiolabel content to that found in the control samples (100%) ($n = 4$).

EGCG (100 μM) and the P-gp inhibitor PSC 833 (10 μM) each increased by 2.6-fold the apical-to-basal flux of [³H]VBL across the Caco-2 cell monolayer (Fig. 7A) and increased the accumulation of [³H]VBL within the cells by 2.2- and 3.4-fold, respectively (Fig. 7B). Indomethacin (10 μM), a MRP inhibitor, did not increase the [³H]VBL flux and the intracellular accumulation. The passage of [³H]VBL across Caco-2 cells exposed to EGCG was not further increased when indomethacin was present along with EGCG. The permeability coefficient for the apical-to-basolateral passage of [¹⁴C]sucrose across the Caco-2 cell monolayer was also measured. Under all conditions of this experiment, the permeability coefficient was the same $(0.45 \pm 0.02 \times 10^{-3}$ cm/min), indicating that the integrity of the monolayer barrier was unaffected by P-gp and MRP modulators. Thus, the passage of [³H]VBL through the intestinal barrier and its uptake into cells were both increased by the presence of EGCG without affecting the integrity of this barrier.

4. Discussion

For a long time, green tea has been a popular beverage in many countries, and epidemiological studies have suggested that its consumption is associated with a lower risk of cancer [20–22]. A great interest is growing in order to understand the molecular mechanisms of the biological effects associated with green tea and its major constituents. Most of the actions associated with green tea are due to catechins, the main polyphenols of green tea [20–24]. It has recently been reported that green tea increased the availability of P-gp substrate in tumor [22]. On the other hand, it has also been reported that some catechins were absorbed faster then others after oral administration of green tea [29]. P-gp is expressed in the intestine and could be in part responsible for limiting the intestinal absorption. Thus, in the present study, we decided to investigate whether GTPs modulate the multidrug resistance transporter P-gp activity and to determine which of the catechins were implicated.

The ability of GTPs to interact at the substrate binding site of P-gp and to modulate its transport activity were first determined. GTPs bind to P-gp, as shown by photoaffinity labeling of P-gp with IAAP, which was previously used to monitor the pharmacological interactions between P-gp and various compounds such as verapamil, cyclosporin A and PSC 833 [30]. Moreover, GTPs inhibit the transport of the P-gp substrates R-123 and VBL, suggesting that a component present in GTPs could be carried by this transporter. Furthermore, the binding of GTPs to P-gp in addition to the data regarding the accumulation and efflux of R-123 support the hypothesis that GTPs modulate P-gp activity rather than a modification of the membrane permeability.

Among the catechins present in GTPs, the three catechin gallate esters EGCG, ECG and CG significantly inhibit both the binding and transport activities of P-gp, where EGCG is the most potent P-gp modulator.

Since EGCG is the principal catechin in green tea as well as that which most strongly affects P-gp transport, we concentrated our study on this catechin. In the presence of EGCG, the growth of CH^{R}C5 cells was less affected than were AuxB1 cells, suggesting that the overexpression of P-gp in CH^{R}C5 cells may play an important role in the protection of these cells against EGCG by transporting this catechin out of the cells. In addition, our results show that EGCG can increase the efficacy of cancer chemotherapeutic drugs. When CH^{R}C5 cells were exposed to the chemotherapeutic agent VBL in combination with EGCG, the IC$_{50}$ of VBL for these cells was the same as the IC$_{50}$ for VBL alone in AuxB1 cells. This result indicates that EGCG potentiates the toxicity of VBL in CH^{R}C5 cells which overexpress P-gp, and sensitizes these cells to anticancer drugs to the level found in the drug sensitive AuxB1 cells. Moreover, P-gp inhibition by GTPs is a rapid reversible process, in contrast to that seen with PSC 833 [31]. Since P-gp has many physiological functions in normal tissues, inhibition of this multidrug transporter during cancer chemotherapy should be rapid and reversible in order to reduce undesirable side effects. Taken together, these results suggest that EGCG could be used to inhibit P-gp in cancer cells, and further in vivo studies are required in order to verify this possibility. Many other biological effects of EGCG have been reported, such as inhibition of tumoral cell growth, induction of apoptosis and inhibition of urokinase and matrix metalloproteinase (MMP-2, MMP-9 and MMP-12) activities [22,32–34]. These actions are important in promoting tumor reduction and reducing metastasis formation. Thus, many beneficial actions are associated with the natural compound EGCG.

EGCG has been reported to be weakly absorbed by the intestine [29]. Since our results suggest that EGCG is a substrate of P-gp, the Caco-2 model was used to verify whether P-gp could be partly responsible for the low intestinal absorption of this catechin. The Caco-2 cell line is a well-established intestinal transport model which forms a polarized epithelial cell monolayer and expresses P-gp at the apical membrane [35]. Apical-to-basolateral transport of VBL in this model allowed us to study the effect of the apical P-gp on the passage of P-gp substrates across the intestinal barrier. Apical-to-basolateral transport of VBL across this intestinal cell monolayer was inhibited by EGCG, suggesting that P-gp transport could explain why EGCG is weakly absorbed by the intestine. In fact, only 0.1% of EGCG was reported to be bioavailable after an intragastric administration of green tea, compared to 13.7% and 31.2% for EGC and EC [29]. These two catechins are not transported by P-gp, as suggested from our binding and transport studies. Thus, P-gp in the apical membranes of intestinal cells may reduce absorption of EGCG by transporting this catechin back into the lumen of the intestine. Furthermore, this result indicates that administration routes other than the oral one seem to be necessary in order to increase its plasma concentration.

Caco-2 cells also express members of the multidrug resistance-associated protein (MRP) family including MRP1, MRP2, MRP3 and MRP5 [36]. These transporters were shown to play a minor part in the basolateral-to-apical efflux of VBL through Caco-2 cells [37]. In addition, it was reported that CsA and PSC 833 are weak inhibitors of MRP1-mediated transport [38]. Thus, in the present apical-to-basolateral transport experiments of VBL, PSC 833 was used at a concentration (10 μM) that does not inhibit MRP-related transport. This flux of VBL, which corresponds to its intestinal absorption, was increased by PSC 833 but not by indomethacin, an inhibitor of MRPs [39]. These results indicate that the apical-to-basolateral passage of VBL across Caco-2 cells was mediated mainly by P-gp and suggests that MRP transporters were not involved. However, we cannot rule out the possibility that EGCG may interact with other MDR-related transporters than P-gp. Along related lines, a recent study has shown that MRP1 transport and ATPase activities were modulated by dietary flavonoids [40] such as quercetin, which have also been reported to inhibit P-gp transport [41].

Other compounds derived from natural products have been reported to inhibit P-gp. Our results show that resveratrol, a polyphenol from red wine, interacts with P-gp. Curcumin, genistein, caffeine, thea-

nine, components of fruit juices, such as quercetin, naringin and furanocoumarins from grapefruit and methoxyflavones from orange, were shown to inhibit drug transport via P-gp [27,41–45]. Taken together, these results suggest that compounds derived from natural dietary products can modulate the activity of this transporter in vivo. Moreover, during oral chemotherapy, the diet of patients may influence the efficacy of therapy using P-gp substrates, suggesting that cancer treatment planning must take into account the diet of each patient.

Our results show that GTPs interact with P-gp and inhibit the transport activity of this enzyme by a reversible manner in $CH^R C5$ cells. Among the catechins present in green tea leaves, EGCG was the most potent inhibitor of P-gp. In addition, we found that intestinal P-gp could be responsible for the low absorption of EGCG. Our results suggest that GTPs, and especially EGCG, could improve the efficacy of cancer treatment by increasing the accumulation of chemotherapeutic drugs in cancer cells by blocking P-gp function. Further studies are required to evaluate in vivo the effect of polyphenols from green tea or the catechin EGCG, on the multidrug resistance associated with P-gp during chemotherapy.

Acknowledgements

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