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Inhibition of P-glycoprotein function by tannic acid and pentagalloylglucose

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Abstract

We studied the effects of tannic acid and 1(β),2,3,4,6-penta-*O*-galloyl-D-glucose (pentagalloylglucose), one of the components of tannic acid, on the P-glycoprotein (P-gp) function in multidrug-resistant P-gp over-expressing KB-C2 cells. Both tannic acid and pentagalloylglucose markedly elevated the accumulation of P-gp substrates, rhodamine 123 and daunorubicin, by inhibiting their efflux. A 19-fold increase in cellular rhodamine 123 was observed for tannic acid at 60 μM (85 μg mL^{-1}) and a 21-fold increase was observed for pentagalloylglucose at 100 μM (94 μg mL^{-1}). The increasing effects of these compounds in the accumulation were much larger than that of (–)epigallocatechin-3-*O*-gallate (EGCG), which has been revealed to have a prominent inhibitory effect on P-gp compared with other flavonoids. Analysis of verapamil-stimulated ATPase activity in membrane vesicles expressing human P-gp suggested that inhibition of P-gp function by tannic acid and pentagalloylglucose was at least partly due to ATPase inhibition of P-gp. The findings also suggested that the presence of a large number of galloyl groups in polyphenols strengthens the interaction with regulatory regions in P-gp.

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Introduction

Over-expression of the 170-kDa P-glycoprotein (P-gp), a plasma membrane transporter that extrudes chemotherapeutic agents out of cells, has been associated with the multidrug resistance (MDR) of cancer cells. This ATP-dependent transporter extrudes a wide variety of structurally unrelated compounds, such as vinca alkaloids, podophyllotoxin derivatives, taxenes and anthracyclines (Bosch & Croop 1996; Sharom 1997). P-gp-mediated MDR is also reversed by various compounds, such as verapamil, dihydropyridine analogues, quinidine and ciclosporin A, due to their inhibition of transporter activity (Bosch & Croop 1996; Sharom 1997). Recently, it has been revealed that flavonoids such as quercetin, catechins such as (−)epigallocatechin-3-*O*-gallate (EGCG), and their analogues also modulate P-gp activity (Castro & Altenberg 1997; Shapiro & Ling 1997; Conseil et al 1998; Zhang & Morris 2003; Kitagawa et al 2004). Since flavonoids and their analogues are possible candidates to overcome MDR, structure–activity relationship studies have been developed to find a potent MDR modulator (Boumendjel et al 2002).

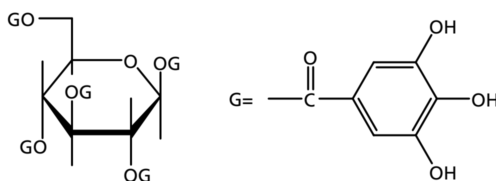
In a previous paper, we clarified that food antioxidants, alkyl gallates with relatively long alkyl chains, such as *n*-octyl gallate and *n*-dodecyl gallate, inhibited P-gp function (Kitagawa et al 2005a) in a human epidermal carcinoma cell line (KB-C2 cells, which over-express P-gp) (Yoshimura et al 1989). In the same paper, we also revealed that lauric acid and *n*-dodecyl-β-D-maltoside had no effect, and that *n*-dodecylresorcinol had a smaller effect than *n*-dodecyl gallate. Taken together with our findings on tea catechins (Kitagawa et al 2004), for non-planar polyphenols both the presence of the hydrophobic moiety and of the gallic acid moiety (pyrogallol group), which have three hydroxyl groups on the benzene ring, seem to strengthen the inhibitory activity on P-gp function (Kitagawa et al 2005b). To further investigate the role of galloyl groups on the interaction of phenolic compounds with P-gp, in this study we examined the effects of tannic acid and one of its components 1(β),2,3,4,6-penta-*O*-galloyl-D-glucose (pentagalloylglucose),

the chemical structure of which is shown in Figure 1 together with that of EGCG, on efflux of P-gp substrates.

The main components of hydrolysable tannins are gallotannins (esters of gallic acid with glucose), also referred to as tannic acid, and ellagitannins (Okuda et al 1995). Tannic acid has also been reported to modify transport activity and ATPase function, such as glucose transport (Liu et al 2005) and gastric H^+, K^+-ATPase (Murakami et al 1992). Tannic acid was also found to exhibit insulin-like activity (Li et al 2005). Tannins have been reported to possess multiple biological actions, including anti-cancer (Okuda et al 1995), antioxidant (Hagerman et al 1998) and antimicrobial activity (Cowan 1999). Pentagalloylglucose, an ester of glucose with five gallic acid molecules, is a common tannic acid component. Pentagalloylglucose has been frequently used in biochemical and biomedical research as a representative expressing the activity of tannic acid or tannin molecules (Li et al 2005), although it is a relatively small molecule among the components of tannic acid (average number of gallic acid is 8.3 in Chinese gallotannin (Nishizawa et al 1982)). One reason is that this compound can be obtained by relatively simple synthesis (Li et al 2005).

For this study, we used fluorescent rhodamine 123 and daunorubicin as the P-gp substrates; these substrates have often been used to study various P-gp transport modulators, including flavonoids (Castro & Altenberg 1997; Wang et al 2001; Zhang & Morris 2003). However, daunorubicin has also been found to be a substrate of MDR-associated protein 1 (MRP1) (Renes et al 1999) and, since MRP 1 is rarely found in KB-C2 cells (Okumura et al 2000), its involvement in substrate efflux is negligible.

A



B

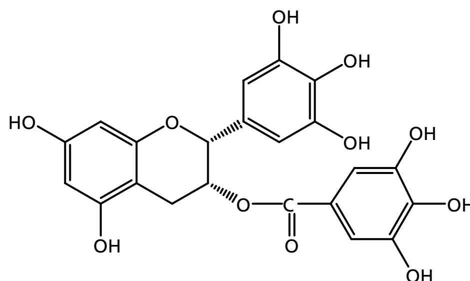


Figure 1 Chemical structures of 1(*β*),2,3,4,6-penta-*O*-galloyl-D-glucose (pentagalloylglucose) (A) and (−)epigallocatechin-3-*O*-gallate (EGCG) (B).

Materials and Methods

Materials

1(*β*),2,3,4,6-penta-*O*-galloyl-D-glucose (pentagalloylglucose) was isolated from Chinese gallotannin (Nishizawa et al 1982). Pentagalloylglucose was separated from the ethyl-acetate-soluble portion of the acetone extract by repeated chromatography on Sephadex LH-20 using a solvent system of ethanol–water–acetone (Nishizawa et al 1982). Tannic acid and (−) epigallocatechin-3-*O*-gallate (EGCG) were purchased from Nacalai 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). The recombinant membrane vesicles derived from the insect cell-baculovirus-expressing human P-gp were purchased from BD-GENTEST (Woburn, MA).

KB-C2 cell culture

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

Cellular accumulation of P-gp substrates

Cellular accumulation of P-gp substrates was measured as described previously (Kitagawa et al 2004, 2005a, b). 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 the addition of tannic acid or pentagalloylglucose, 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) to completely solubilize each substrate. The fluorescence intensity was measured with an F-4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan), and the accumulated amounts of the probes were 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

Following previous reports (Kitagawa et al 2004, 2005a, b), the efflux of rhodamine 123 was also measured using cells incubated with 20 μM rhodamine 123 without tannic acid and pentagalloylglucose for 2 h as described above. Cells were then washed once with D-MEM without serum to remove the fluorescence probe from the medium and incubated again

with the medium in the presence or absence of these polygalloylglucoses. After various incubation times, the cells were washed twice with an excess volume of ice-cold PBS, lysed with 0.1% Triton X-100 and the fluorescence intensity was measured as described above. The amount of rhodamine 123 retained in the cells was recorded.

ATPase activity

The effects of tannic acid and pentagalloylglucose on P-gp-associated ATPase activity were observed by using the membrane vesicles expressing human P-gp. ATPase activity was determined by measuring inorganic phosphate liberation according to the procedure reported by Sarkadi et al (1992), with some modifications, in 96-well microtitre plates (Fujino et al 2005). The membrane vesicles (2 μg of protein) were suspended in 20 μL of the incubation medium containing (in mM) 50 Tris-Mes (pH 6.8), 2 EGTA, 2 dithiothreitol, 50 potassium chloride and 5 sodium azide. This medium was mixed with 10 μL of a test compound containing Tris-Mes solution and 10 μL of 120 μM verapamil, either in the presence or absence of 0.3 mM sodium orthovanadate, and then pre-incubated at 37°C for 5 min. The reaction was initiated by addition of 20 μL of 12 mM MgATP. The final amount of protein in the assay was 0.04 mg. The assay plate was kept at 37°C for 20 min. Incubations were conducted in duplicate and the drug-stimulated ATPase activity was determined as the difference between the amount of inorganic phosphate released from ATP in the absence and presence of ATP. The reaction was terminated by the addition of 30 μL of stopping medium (10% SDS with two drops of antifoam A). The released phosphate and phosphate standards were measured. The SDS-containing samples were supplemented with 200 μL of the detection reagent (5 mL of 35 mM ammonium molybdate in 15 mM zinc acetate, pH 5.0, mixed with 20 mL of 10% ascorbic acid, pH 5.0) at 37°C for 20 min and the reaction product was measured by absorbance at 655 nm.

Statistical analysis

One-way analysis of variance and Bonferroni's post-hoc test were used to analyse differences between the sets of data. $P < 0.05$ was considered significant.

Results

Effect of tannic acid and pentagalloylglucose on the accumulation of P-gp substrates

We first examined the effect of tannic acid and pentagalloylglucose on the accumulation of rhodamine 123 in KB-C2 cells. As shown in Figure 2 regarding the dose-dependent effects, tannic acid and pentagalloylglucose markedly increased the accumulation. A 19-fold increase in cellular rhodamine 123 was observed for tannic acid at 60 μM (85 μg mL^{-1}) when the mean molecular weight of tannic acid was assumed to be about 1400 (Okumura et al 2000) and a 21-fold increase was observed for pentagalloylglucose at 100 μM (94 μg mL^{-1}). The dose-dependent effect of EGCG, which is a compound related to tannins and has one of the most prominent effects among flavonoids (Kitagawa 2006), is also shown in Figure 2, indicating a 5.1-fold increase at 100 μM (46 μg mL^{-1}). Therefore, the increases due to tannic acid and pentagalloylglucose were much larger than that due to EGCG. Although the effect was slightly smaller, a marked increase in accumulation was also observed when daunorubicin was used as a P-gp substrate (Figure 3) for the dose-dependent effect of tannic acid and pentagalloylglucose in comparison with the effect of EGCG.

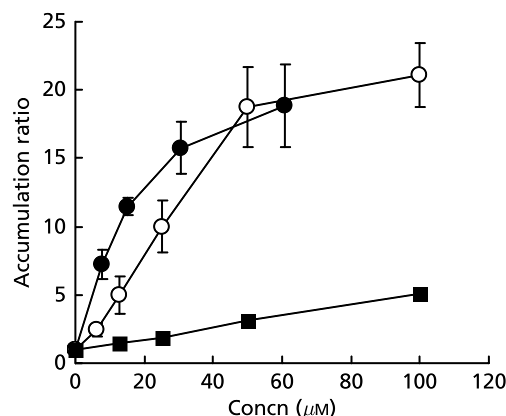


Figure 2 Dose-dependent effects of tannic acid (closed circles), pentagalloylglucose (open circles) and EGCG (closed squares) on the accumulation of rhodamine 123. Data are the means ± s.d. of six experiments. All the values in the presence of tannic acid, pentagalloylglucose and EGCG in the figure are significantly different from the values in their absence ($P < 0.001$).

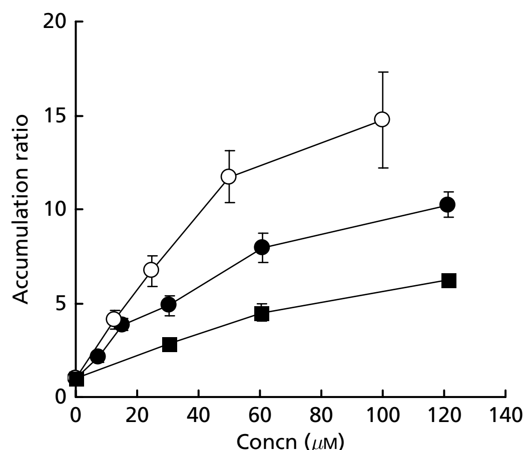


Figure 3 Dose-dependent effects of tannic acid (closed circles), pentagalloylglucose (open circles) and EGCG (closed squares) on the accumulation of daunorubicin. Data are the means ± s.d. of six experiments. All the values in the presence of tannic acid, pentagalloylglucose and EGCG shown in the figure are significantly different from the values in their absence ($P < 0.001$).

Effect of tannic acid and pentagalloylglucose on the efflux of P-gp substrates

The enhanced accumulation of rhodamine 123 and daunorubicin in the presence of tannic acid and pentagalloylglucose, as mentioned above, seemed to be due to the inhibition of the P-gp-mediated efflux of these substrates as already revealed for EGCG (Kitagawa et al 2004). To confirm this, we examined the effects of tannic acid and pentagalloylglucose on the efflux of rhodamine 123. After loading rhodamine 123 and removing the fluorescent substrate from the medium, the amount of substrate remaining in the cells was monitored in the presence or absence of these polygalloylglucoses. Figure 4 shows the effect of 60 μM (85 μg mL^{-1}) tannic acid and that of 50 μM (47 μg mL^{-1}) pentagalloylglucose; the amount of rhodamine 123 remaining in the KB-C2 cells was significantly higher in their presence than in their absence, suggesting that these polygalloylglucoses decreased the efflux of rhodamine 123 by blocking P-gp.

Effect of tannic acid and pentagalloylglucose on the ATPase activity in P-gp membranes

Recently, it was revealed that EGCG inhibited P-gp-associated ATPase activity of recombinant proteins (Mei et al 2004). Flavonoids have been suggested to be modulators with bifunctional interactions at vicinal ATP-binding sites and steroid-interacting regions, which are expected to be in close proximity to the ATP binding site within a cytosolic domain of P-gp (Conseil et al 1998; Boumendjel et al 2002). Therefore, we next examined the effects of tannic acid and pentagalloylglucose on the activity of P-gp ATPase by using recombinant membrane vesicles expressing human P-gp. As shown in Figure 5, indicating the effect of tannic acid at 24 μM (34 μg mL^{-1}) and 60 μM (85 μg mL^{-1}) and that of pentagalloylglucose at 40 μM (37.6 μg mL^{-1}), these polygalloylglucoses significantly inhibited verapamil-induced ATPase activity, the same as ciclosporin A, which has been confirmed to inhibit P-gp ATPase (Ejendal & Hrycyna 2005).

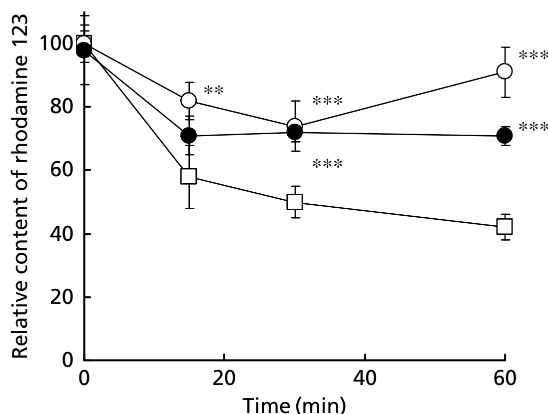


Figure 4 The efflux of rhodamine 123 in the presence of 60 μM (85 μg mL^{-1}) tannic acid (closed circles), in the presence of 50 μM (47 μg mL^{-1}) pentagalloylglucose (open circles) and in their absence (open squares). Data are the means ± s.d. of three experiments; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs absence.

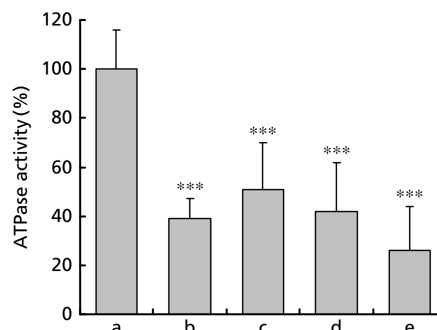


Figure 5 Effects of ciclosporin A, tannic acid and pentagalloylglucose on 20 μM verapamil-induced ATPase activity in membrane vesicles expressing human P-gp: a, control; b, 20 μM ciclosporin A; c, 24 μM (34 μg mL^{-1}) tannic acid; d, 60 μM (85 μg mL^{-1}) tannic acid; e, 40 μM (37.6 μg mL^{-1}) pentagalloylglucose. Data are the means ± s.d. of four experiments; $^{***}P < 0.001$ vs control.

Discussion

Tannic acid consists of esters of gallic acid with glucose, mainly penta-dodeca-galloylglucoses (Nishizawa et al 1982). These findings indicated that tannic acid and pentagalloylglucose, one of the components of tannic acid, markedly increased the cellular accumulation of P-gp substrates by inhibiting their efflux from the cytoplasm. The effects were much larger than that of their related compound EGCG, which has one of the most prominent effects among flavonoids (Kitagawa 2006). These results suggest that the presence of a large number of galloyl groups increases the effects on substrate efflux function of P-gp.

It has been revealed that flavonoids have multiple binding sites in regulatory regions of P-gp (Morris & Zhang 2006), such as substrate binding sites (Castro & Altenberg 1997; Zhang & Morris 2003), an ATP binding site and a vicinal steroid binding site (Conseil et al 1998). Although the interaction mechanism is not clear, our findings also suggest that the inhibition of P-gp function by tannic acid and pentagalloylglucose is at least partly due to the inhibition of P-gp ATPase. These polygalloylglucoses have hydrophobic, as well as hydrophilic, properties. Therefore, they seem to penetrate the cytoplasmic side of the cellular membrane and appear to interact from the cytoplasmic side in which regulatory domains of P-gp function, including ATPase, are present (Bosch & Croop 1996; Sharom 1997). In a previous paper we revealed that hydrophobicity determines the inhibitory activity of flavonoids with planar structures, such as quercetin and kaempferol, on the efflux function of P-gp (Kitagawa et al 2005). On the other hand, previous studies on tea catechins (Kitagawa et al 2004) and alkyl gallates (Kitagawa et al 2005a), and the present findings on tannic acid and pentagalloylglucose, indicated that for non-planar polyphenols, the presence of galloyl groups strengthens the inhibitory activity on P-gp. Hydroxyl groups in galloyl groups seem to be important to strengthen the polar interactions with P-gp, possibly at the ATP-binding site, although the reason why the presence of many galloyl groups strengthens the interaction has not been clarified.

The polyphenols, such as tannic acid and pentagalloylglucose used in this study, are compounds with the potential to overcome MDR, which results from the active efflux of antitumour drugs by P-gp and other efflux transporters, with low toxicity; however, they have a broad spectrum of biological activity, including the inhibition of other ATPases and an anti-estrogen effect (Chan et al 2006). Therefore, at a high dose their side-effects should be considered. Their metabolism in the intestine and liver should also be considered. Further studies are necessary to investigate whether these polygalloylglucoses have sufficiently potent inhibitory effects on P-gp function and increase the pharmacological effects of P-gp substrate antineoplastic drugs in-vivo without inducing severe side-effects.

Conclusions

This study revealed that tannic acid and pentagalloylglucose markedly elevated the accumulation of P-gp substrates, rhodamine 123 and daunorubicin, by inhibiting their efflux in multidrug-resistant P-gp over-expressing KB-C2 cells. The increasing effects of these compounds in the accumulation were much larger than that of EGCG. Analysis of verapamil-stimulated ATPase activity in membrane vesicles expressing human P-gp suggested that inhibition of P-gp function by tannic acid and pentagalloylglucose was at least partly due to ATPase inhibition. The findings also suggested that the presence of a large number of galloyl groups in polyphenols strengthens the interaction with regulatory regions in P-gp.

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