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Effects of alkyl gallates on P-glycoprotein function

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Abstract

In this study, we examined the effects of the food antioxidants, alkyl gallates, on the function of P-glycoprotein (P-gp) and elucidated the importance of alkyl chains and gallic acid moieties on the activity of P-gp. We examined the effects of three alkyl (*n*-butyl, *n*-octyl and *n*-dodecyl) gallates and their related compounds on the cellular accumulation and efflux of rhodamine 123 and daunorubicin in P-gp overexpressing KB-C2 cells. Alkyl gallates increased the cellular accumulation of these P-gp substrates dependent on their alkyl chain lengths by inhibiting the efflux of the substrates. *n*-Dodecylresorcinol also increased the accumulation, but its effect was less than that of *n*-dodecyl gallate. However, either lauric acid or *n*-dodecyl-β-D-maltoside, which does not have a phenol group, did not increase the accumulation. The results indicated that both the gallic acid moiety and a long alkyl chain play important roles in the modification of P-gp function. The cytotoxicity of daunorubicin was recovered in the presence of alkyl gallates possibly due to their inhibition of P-gp function.

Keywords: Alkyl gallates; P-glycoprotein; Polyphenols; KB-C2 cells; MDR

1. Introduction

Overexpression of P-glycoprotein (P-gp), a plasma membrane transporter which extrudes chemotherapeutic agents out of cells, has been associated with the multidrug resistance (MDR) of cancer cells. Compounds such as verapamil, dihydropyridine analogs, quinidine and cyclosporine A reversed the P-gp-mediated MDR due to their inhibition of the transporter activity of P-gp [1,2]. In addition to these compounds, polyphenols have also been demonstrated to modulate P-gp activity [3–7]. Among polyphenols, we recently demonstrated that tea catechins inhibited P-gp function dependent on their chemical structure in multidrug-resistant human epidermal carcinoma cell line KB-C2 cells [8], which overexpress P-gp [9]. We found the maximal activity in the (−)epigallocatechin gallate (EGCG), and clarified that the presence of the galloyl moiety on the C-ring, and the presence of the trihydric pyrogallol group as the B-ring instead of the dihydric catechol group, markedly increased its activity on P-gp, although their effects on the partition coefficients between *n*-octanol and phosphate-buffered saline (PBS) were opposite to each other [8].

Alkyl gallates (3,4,5-trihydroxybenzoic acid alkyl esters), propyl, octyl and dodecyl gallates are widely used as food antioxidants in oils and butters [10,11]. They have also been shown to possess antibacterial activities [12]. In this study, we tried to clarify the effects of three alkyl gallates and their related compounds (Fig. 1) on P-gp function. In relation to the effects of tea catechins, we tried to elucidate the role of both the hydrophobic moiety (alkyl chain) and gallic acid moiety on their modifying effects on P-gp function. We used fluorescent rhodamine 123 and daunorubicin as the P-gp substrates; these substrates have often been used for the study of various P-gp transport modulators including flavonoids [3,6].

Abbreviations: D-MEM, Dulbecco's modified Eagle medium; EGCG, (−)epigallocatechin gallate; MDR, multidrug resistance; MRP, multidrug resistance protein; PBS, phosphate-buffered saline; P-gp, P-glycoprotein

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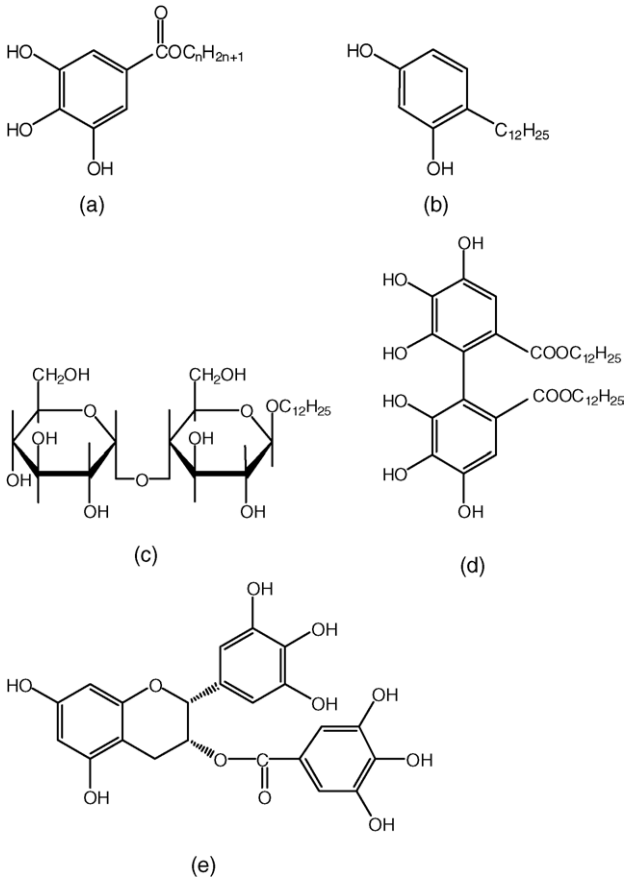

Fig. 1. Chemical structures of alkyl gallates and related compounds tested in this study: (a) alkyl (*n*-butyl ($n = 4$), *n*-octyl ($n = 8$) and *n*-dodecyl ($n = 12$)) gallates; (b) *n*-dodecylresorcinol; (c) *n*-dodecyl-β-D-maltoside; (d) *n*-didodecyl-(2,2′,3,3′,4,4′-hexahydroxy)-biphenyl-6,6′-dicarboxylate; (e) (−)epigallocatechin gallate (EGCG).

Although daunorubicin has also been revealed to be a substrate of multidrug resistance protein 1 (MRP1) [13], since MRP1 is rarely found in KB-C2 cells [14], its involvement in the substrate efflux is negligible.

2. Materials and methods

2.1. Materials

Alkyl gallates were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Rhodamine 123 was from Molecular Probe, Inc. (Eugene, OR, USA). Dulbecco's modified Eagle medium (D-MEM) and fetal bovine serum were from Invitrogen Co. (Carlsbad, CA, USA). Dodecyl-β-D-maltoside and the Cell Counting Kit-8 were purchased from Dojindo Laboratories (Kumamoto, Japan). *n*-Dodecylresorcinol was from the Aldrich Chemical Co. (Milwaukee, WI). *n*-Didodecyl-(2,2′,3,3′,4,4′-hexahydroxy)-biphenyl-6,6′-dicarboxylate was synthesized from ellagic acid according to the method described previously [15]. Daunorubicin hydrochloride and all other reagents were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). KB-C2 cells and drug sensitive cell line KB-3-1 cells were kindly provided by Prof. Shin-ichi Akiyama (Kagoshima University, Japan).

2.2. KB-C2 cell culture

KB-C2 cells were cultured in D-MEM culture medium supplemented with 10% fetal bovine serum and 2 μg/ml colchicine. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2/95% air. KB-3-1 cells were cultured similarly in the absence of colchicine.

2.3. Cellular accumulation of P-gp substrates

Cellular accumulation of P-gp substrates was measured as described previously [8]. 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 alkyl gallates, either 20 μM rhodamine 123 or 50 μM daunorubicin was added, and the cells were incubated for another 2 h in a CO_2 incubator. Cells were then washed twice with an excess volume of ice-cold 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.

2.4. Efflux of daunorubicin

Following previous reports [8], the efflux of daunorubicin was also measured using cells incubated with 50 μM daunorubicin without alkyl gallates for 2 h as described above. Cells were then washed twice 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 alkyl gallates. After various incubation times, the cells were washed twice with an excess volume of ice-cold PBS, lysed with 1% SDS, and the fluorescence intensity was measured as described above. The amount of daunorubicin retained in the cells was recorded.

2.5. Cytotoxicity of daunorubicin

The resistance of KB-C2 cells to daunorubicin cytotoxicity was determined as described previously [7] using the water-soluble 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8) (Cell Counting Kit-8) performed in 96-well plates. 5×10^3 cells in 100 μl of culture medium in the presence or absence of alkyl gallates were inoculated into each well.

After 24 h incubation in a CO_2 incubator at 37 °C, daunorubicin was added to a concentration of 12.5 μM and the plates were incubated for 48 h. Thereafter, 10 μl of the cell counting kit solution (5 mM WST-8) was added to each cell. The plates were incubated for further 3 h, and the absorbance at 450 nm was measured using a microplate reader (model 550, Bio-Rad Laboratories, Hercules, CA).

2.6. Statistic analysis

One-way analysis of variance and Bonferroni's post hoc test were used to analyze differences between the sets of data. A p-value less than 0.05 was considered significant.

3. Results

3.1. Effects of alkyl gallates on the accumulation of P-gp substrates

We first examined the effects of three alkyl gallates, *n*-butyl, *n*-octyl and *n*-dodecyl gallates, whose numbers of carbon atoms in their alkyl chains are 4, 8 and 12, respectively, on the accumulation of rhodamine 123 in KB-C2 cells. As shown in Fig. 2 for the results at 100 μM, *n*-butyl gallate and gallic acid had no effects, but *n*-octyl and *n*-dodecyl gallates, which have longer alkyl chains, increased the cellular accumulations of the fluorescent substrate about 3.5-fold and 5.2-fold, respectively. The effect of *n*-dodecyl gallate was similar to that of EGCG and about twice as large as that of verapamil. There was no effect of the nonionic detergent *n*-dodecyl-β-D-maltoside, which also has an *n*-dodecyl group but no phenol group.

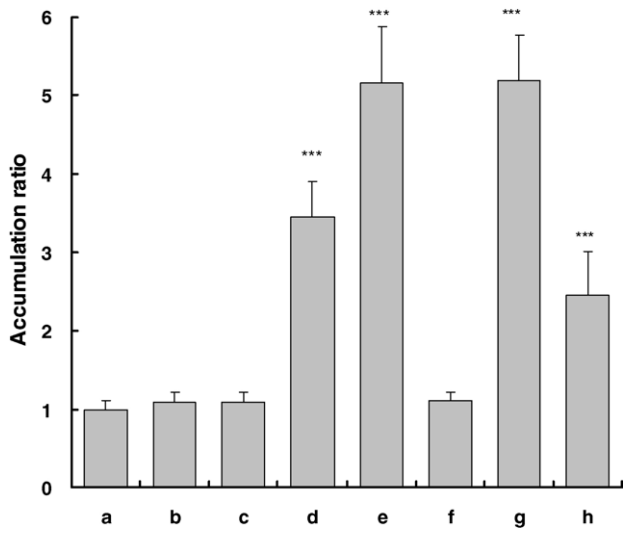

Fig. 2. Effects of 100 μM gallic acid, *n*-butyl gallate, *n*-octyl gallate, *n*-dodecyl gallate, *n*-dodecyl-β-D-maltoside, EGCG and verapamil on the relative accumulative amounts of rhodamine 123: (a) control; (b) gallic acid; (c) *n*-butyl gallate; (d) *n*-octyl gallate; (e) *n*-dodecyl gallate; (f) *n*-dodecyl-β-D-maltoside; (g) EGCG; (h) verapamil. Data are means ± S.D. of four experiments. The control value of accumulation, which was $4.09 \pm 0.51 \times 10^8$ molecules/cell for 14 experiments, was defined as 1.00; $^{***}p < 0.001$.

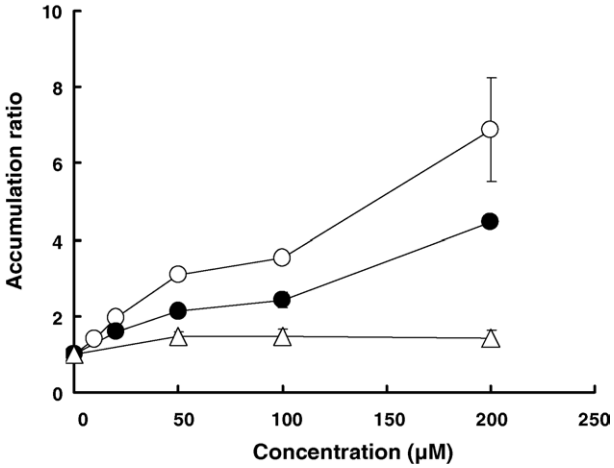

Fig. 3. Dose-dependent effects of *n*-butyl (△), *n*-octyl (●) and *n*-dodecyl (○) gallates on the accumulation of daunorubicin. Data are means ± S.D. of four experiments. The control value of accumulation, which was $3.02 \pm 0.14 \times 10^9$ molecules/cell for 10 experiments, was defined as 1.00.

We also examined the effects of alkyl gallates on the intracellular accumulation of daunorubicin, which is more sensitive to inhibition than rhodamine 123 [16]. As shown in Fig. 3 on the dose-dependent effects, *n*-butyl gallate had no significant effect, and marked increasing effects were observed for the other two alkyl gallates (significant effects were observed above 50 μM for *n*-octyl gallate and above 20 μM for *n*-dodecyl gallate). Similar to the results using rhodamine 123 as a substrate (Fig. 2), maximum effects were observed for *n*-dodecyl gallate (significantly different at $p < 0.05$ and <0.001 compared with the effects of *n*-octyl gallate at 100 and 200 μM, respectively). As shown in Fig. 4, there was no effect of lauric acid, which has 12 carbon atoms but no phenol group. On the other hand, no effects for these alkyl gallates were observed on the

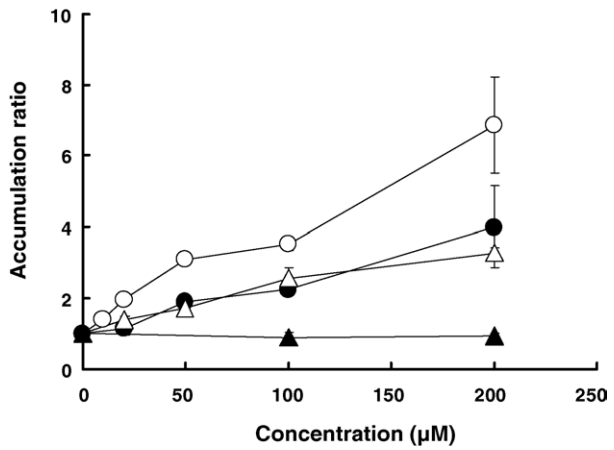

Fig. 4. Dose-dependent effects of *n*-dodecylresorcinol (●), *n*-didodecyl-(2,2′,3,3′,4,4′-hexahydroxy)-biphenyl-6,6′-dicarboxylate (△) and lauric acid (▲) on the accumulation of daunorubicin. Data for *n*-dodecyl gallate (○) are also shown for comparison. Data are means ± S.D. of four experiments. The control value of accumulation, which was $3.02 \pm 0.14 \times 10^9$ molecules/cell for 10 experiments, was defined as 1.00.

cellular accumulation of daunorubicin in the drug sensitive KB-3-1 cells (data not shown).

Furthermore, we examined the effect of *n*-dodecylresorcinol, which has an *n*-dodecyl group as its alkyl chain on the resorcinol ring, using KB-C2 cells. As also shown in Fig. 4, *n*-dodecylresorcinol increased the accumulation of daunorubicin (significantly different at $p < 0.01$ and <0.001 compared with control values at 100 and 200 μM, respectively). However, the effect was smaller than that of *n*-dodecyl gallate. The effect of *n*-didodecyl-(2,2′,3,3′,4,4′-hexahydroxy)-biphenyl-6,6′-dicarboxylate, which has an *n*-dodecyl gallate dimeric structure, was also smaller than that of *n*-dodecyl gallate (the effects of these two compounds were significantly different from those of *n*-dodecyl gallate at $p < 0.05$ at 50 and 100 μM and at $p < 0.001$ at 200 μM).

3.2. Effects of alkyl gallates on the efflux of P-gp substrates

The enhanced accumulations of rhodamine 123 and daunorubicin in the presence of alkyl gallates, as mentioned above, seemed to be due to the inhibition of the P-gp-mediated efflux of these substrates. To confirm this, we examined the effect of *n*-octyl gallate on the efflux of daunorubicin. After loading daunorubicin and removing the fluorescent substrate from the medium, the amount of substrate remaining in the cells was monitored in the presence or absence of *n*-octyl gallate. As shown in Fig. 5 on the effect of *n*-octyl gallate at 50 μM, the amount of daunorubicin remaining in the KB-C2 cells was higher in its presence than in its absence, suggesting that the *n*-octyl gallate decreased the efflux of daunorubicin by blocking P-gp. Similar inhibitory effect on daunorubicin efflux was also observed for *n*-dodecyl gallate (data not shown).

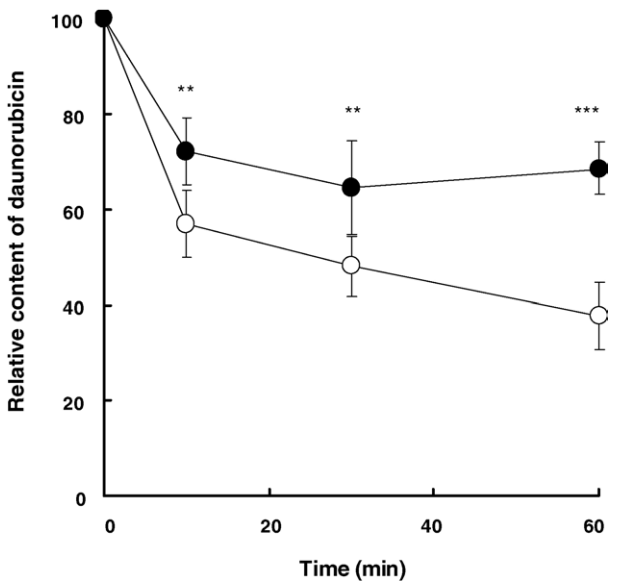

Fig. 5. The efflux of daunorubicin in the presence (●) or absence (○) of 50 μM *n*-octyl gallate. Data are means ± S.D. of six experiments; $^{**}p < 0.01$ and $^{***}p < 0.001$.

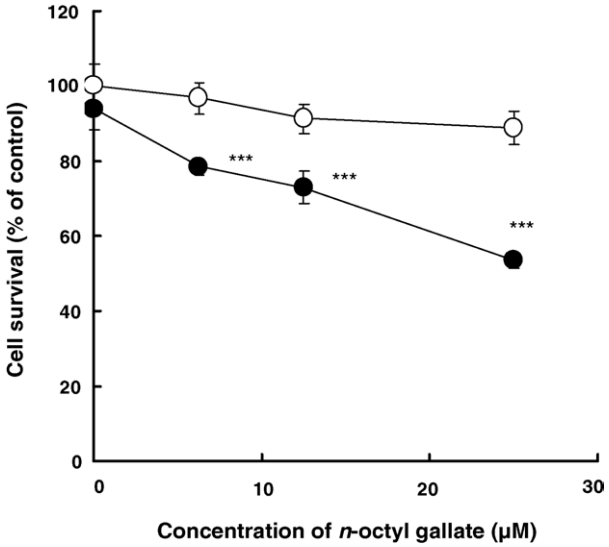

Fig. 6. Effect of *n*-octyl gallate concentration on the cytotoxicity of daunorubicin in KB-C2 cells: (○) control (in the absence of daunorubicin) and (●) in the presence of 12.5 μM daunorubicin. Data are means ± S.D. of four experiments; $^{***}p < 0.001$.

3.3. Effects of alkyl gallates on the cytotoxicity of daunorubicin

To demonstrate the effect of alkyl gallates on the cytotoxicity of P-gp substrate anticancer drugs, we next examined the effect of *n*-octyl gallate on the cytotoxicity of daunorubicin. The addition of *n*-octyl gallate (6.25, 12.5 and 25 μM) recovered the cytotoxicity of daunorubicin (Fig. 6). A relatively high concentration of *n*-octyl gallate itself induced slight cytotoxicity ($p < 0.05$ at 25 μM). A similar effect on the recovery of daunorubicin cytotoxicity was also observed in the presence of 6.25 and 12.5 μM *n*-dodecyl gallate (data not shown). Although the effective concentrations of alkyl gallates were different from those in the accumulation study due to differences in cell numbers, the trend of daunorubicin cytotoxicity enhancement by alkyl gallates was consistent with the results described above, whereby alkyl gallate increased the cellular accumulation of daunorubicin due to their inhibition of P-gp-mediated drug efflux.

4. Discussion

The present findings revealed that alkyl gallates with relatively long alkyl chains inhibited P-gp function. Since lauric acid and *n*-dodecyl-β-D-maltoside had no effects, and *n*-dodecylresorcinol had a smaller effect than *n*-dodecyl gallate, both the gallic acid moieties, which have three hydroxyl groups on the benzene ring, and long alkyl chains seem to play important roles in the modification of P-gp

function. Taken together with the previous results shown for tea catechins [8], both the presence of the hydrophobic moiety and the gallic acid moiety (pyrogallol group) were important for the modification of the P-gp function. These findings are consistent with previous findings for hydroxylchalcones, whose hydrophobicity of the B-ring has been suggested to be important in the modification of P-gp function [17]. These characteristic structures are possibly important for the inhibition of ATPase activity or the modification of substrate-binding sites on the transporter, as suggested for the inhibitory mechanisms of polyphenols [3,6,18]. Hydrophobic moiety may be important for interaction at the steroid-interacting hydrophobic sequence of P-gp [5,17]. Hydroxyl groups in the gallic acid moieties may be important in polar interactions with P-gp [19], possibly at the ATP-binding site [5,17]. It is probably due to the steric hindrance that the compound with *n*-dodecyl gallate dimeric structure had a smaller activity than *n*-dodecyl gallate.

Alkyl gallates enhanced the cytotoxicity of daunorubicin. This indicates that alkyl gallates could reverse MDR in KB-C2 cells due to their possible effect of increasing the intracellular accumulation of the anti-tumor drug due to their inhibition of P-gp function, although there is another possibility to reverse MDR by down-regulating MDR1 gene expression, as has been suggested for the effect of green tea polyphenols [18].

It has been demonstrated that polyphenols also inhibit other ATP-binding cassette transporters such as breast cancer resistance protein [20] and MRP1 and MRP2 [21]. Since the high concentration of *n*-octyl gallate and *n*-dodecyl gallate examined in this study is not expected in vivo due to the limited amount of these compounds used as food additives [11,22], the inhibition of P-pg function is not likely to occur. However, synergistic effects with other dietary polyphenols on P-gp function might be considered. Polyphenols have the possibility to become compounds for overcoming MDR that results from the active efflux of anti-tumor drugs by P-gp and other efflux transporters without inducing severe side-effects. Further studies are currently in progress to clarify the structural requirements for the inhibitory effects of polyphenols on P-gp function.

Acknowledgment

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