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Uptake of Hydroxy Derivatives of Benzoic Acid and Cinnamic Acid by Caco-2 cells via Monocarboxylic Acid Transporters

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ABSTRACT

We investigated the effect of the hydroxylation of benzoic and cinnamic acids and the effect of cyanating of cinnamic acid at α-position on their uptake by Caco-2 cells via monocarboxylic acid transporters (MCTs). Benzoic and cinnamic acids are typical substrates for MCTs. The mono-hydroxylation of benzoic acid at the *m*- and *p*-positions markedly decreased uptake, whereas that at the *o*-position decreased uptake only marginally, probably due to the decrease in lipophilicity and the formation of internal hydrogen bonds between the carboxyl and hydroxyl groups, respectively. The di-hydroxylation of benzoic acid decreased uptake drastically, and the substitution of the hydroxyl group in the benzoic acid with a methoxy group increased uptake, probably due to the respective decrease and increase in lipophilicity. Similarly, mono-hydroxylation of cinnamic acid led to a marked decrease in uptake, which was decreased even further by di-hydroxylation. The uptake of cinnamic acid derivatives such as α-cyanocinnamic acid, α-cyano-4-hyrdoxycinnamic acid and α-cyano-3-hydroxycinnamic acid was markedly lower than that of cinnamic acid, and the uptake of α-methylcinnamic acid fell between that of cinnamic acid and the α-cyanocinnamic acid derivative. The uptake of benzoic acid was markedly decreased by coincubation with most benzoic and cinnamic acid derivatives, and the cinnamic acids competitively decreased the uptake of benzoic acid. Thus, the hydroxylation of benzoic and cinnamic acids and the cyanating of cinnamic acids at α position could result in decrease of their uptake by Caco-2 cell via MCTs.

Keywords: Benzoic acid, Cinnamic acid, Phenoxyacetic acid, Monocarboxylic acid transporters (MCTs), Caco-2 cells

INTRODUCTION

Monocarboxylic acid transporters (MCTs) are members of the SLC16A gene family, and consist of 14 members, of which only the first four (MCT1-MCT4) are characterized as proton-linked monocarboxylic acid transporters [1,2]. MCTs have an important role in the intestinal absorption of not only short-chain monocarboxylic acids such as lactic acid, propionic acid and pyruvic acid, but also exogenous monocarboxylic acid compounds such as benzoic acid, phenolic acid, and pharmacologically active compounds [3-9].

The human colorectal adenocarcinomal cell line Caco-2 is a useful model with which to study the intestinal absorption of various compounds, since the cells are morphologically and functionally similar to human small intestinal epithelial cells [10]. Caco-2 cells express various MCT subtypes, such as MCT1, MCT3, MCT4, MCT5 and MCT6, with MCT1 being the most abundant [11]. α-Cyanocinnamate analogs are known to inhibit MCT1, MCT2 and MCT4 activities and α-cyano-4-hydroxycinnamic acid (CHC) is used as an inhibitor of those MCTs [1,12]. Benzoic acid and some of its derivatives, fluorescein, and cinnamic acid and some of its derivatives are typical substrates for MCTs and have been used as competitive inhibitors in several studies on MCTs [3,7,9,13,14], although the MCT subtype that mediated their transport has not been elucidated.

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We investigated the uptake mechanisms of phenoxyacetic acid derivatives, such as 4-chlorophenoxyacetic acid (4-CPA), 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and suggested that the uptake of those derivatives from the apical membranes of Caco-2 cells is mediated via the same MCTs as benzoic acid, at least in part, and the increase in lipophilicity due to the chloro-substituent may increase the uptake via the MCTs [15,16]. Furthermore, the uptake of 3,5,6-trichloro-2-pyridinyloxyacetic acid (triclopyr) from the apical membranes of Caco-2 cells appears to occur via MCTs, whereas the uptake of 3,6-dichloro-2-methoxybenzoic acid (dicamba) mainly occurs via passive diffusion, as coincubation with benzoic acid markedly decreased the uptake of triclopyr, in the same manner as the phenoxyacetic acid derivatives, while coincubation with benzoic acid did not decrease the uptake dicamba, and the uptake of dicamba was markedly lower than triclopyr [17]. On the other hand, coincubation with CHC sparingly decreased the uptake of phenoxyacetic acid derivatives [15,16], dicamba [17] and benzoic acid (our unpublished data).

We recently investigated the structural effect of benzoic acid on its uptake into Caco-2 cells via MCTs, with a particular focus on chloro- and methoxy-substituted benzoic acid, and reported that most of the chloro- and methoxy-substitutions at the benzene ring did not so affect initial uptake, but 2,6-disubstitution markedly decreased uptake, probably due to the inhibitory effect of 2,6-disubstitution on the accessibility of carboxylic group to MCTs located on the apical membranes of Caco-2 cells [18]. On the other hand, Konishi et al. [7] investigated the structural effects of benzoic and cinnamic acid derivatives on the transport of fluorescein through Caco-2 cell monolayers, and reported that the most of the derivatives mono-hydroxylated at the benzene ring had an inhibitory effect on fluorescein transport via MCTs, but the di- and tri-hydroxylated derivatives did not.

In the present study, we investigated the structural effect of hydroxylation at the benzene ring of benzoic, cinnamic and phenoxyacetic acids on their uptake into Caco-2 cells via MCTs, and compared our results with the previous results for chloro-substitution [18]. Furthermore, we investigated the inhibitory effects of the α-cyano group of cinnamic acids on MCT activity.

MATERIALS AND METHODS

Materials

Benzoic acid, 2-,3-,4-hydroxybenzoic acids, 2,4-, 2,6- and 3,4-dihydroxybenzoic acids, 2-,3-, 4-methoxybenzoic acids, 2-hydroxy-3-methoxybenzoic acid, 3,4-dimethoxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, α-cyano-3-hydroxycinnamic acid, 3-hydroxycinnamic acid, trans-cinnamic acid, 3,4-dihydroxycinnamic acid, 3-hydroxy-4-methoxycinnamic acid, phenoxyacetic acid, 4-hydroxyphenoxyacetic acid, 2,4-dichlorophenoxyacetic acid (2,4-D) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α-Cyanocinnamic acid and α-methylcinnamic acid were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 2,3-Dihydroxybenzoic acid, 4-hydroxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid and α-cyano-4-hydroxycinnamic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fetal bovine serum (FBS) and nonessential amino acid (NEAA) were obtained from Life Technologies (Carlsbad, CA). All other chemicals used were of the highest purity commercially available.

Cell culture

Caco-2 cells at passage 46 were obtained from RIKEN Cell Bank (Tsukuba, Japan), and cells between passage 60 and 85 were used in this study. As described previously [19], the cells were cultured on 35-mm six-well culture dishes coated with rat collagen type I (Becton Dickinson, Bedford, MA) in DMEM containing FBS (10%), NEAA (1%), streptomycin (100 μg/mL) and penicillin G (70 U/mL) at 37°C in a humidified atmosphere of 5% CO_2-95% air. The culture medium was replaced three times a week, and the confluent Caco-2 cell monolayers, cultured for 10-14 days, were used in this study.

Uptake study using Caco-2 cells

The uptake experiment was performed as described previously [18,19]. The culture medium was removed, and the cells were preincubated at 37°C for 20 min in 1.5 mL of the incubation medium at pH 7.4. After preincubation, the medium was aspirated, and the cells were incubated with 1.5 mL of fresh incubation medium, at pH 6.0, containing benzoic acid with or without the benzoic or cinnamic acid derivatives for 1 min at 37°C. The incubation medium used for the uptake study was Hanks' balanced salt solution containing 25 mM D-glucose and 10 mM MES at pH 6.0 or 10 mM HEPES (pH 7.4).

After the incubation with benzoic or cinnamic acid derivatives, the cell surface was quickly washed three times with ice-cold incubation medium. The cells were suspended in 1.0 mL of extraction solution (1 N H_3PO_4: methanol = 1:1) for 60 min at room temperature, subsequently scraped off, and collected using a cell scraper [18,19]. The suspension was centrifuged at 13,000*g* for 10 min, and a 100-μL aliquot of the supernatant was injected onto the HPLC system.

Determination of benzoic acid derivatives, cinnamic acid derivatives and protein

Benzoic and cinnamic acid derivatives were determined using HPLC system consisting of a Shimadzu LC-10A pump and SPD-10A UV detector (Kyoto, Japan). The column used

was an Inertsil VP-ODS (4mm i.d. x 250 mm; GL Sciences, Tokyo, Japan), the flow rate was 1.0 mL/min, and the wavelength was 230 nm. The mobile phase (50 mM KH_2PO_4 buffer at pH 2.1:acetonitrile) for α-methylcinnamic acid was 55:45 v/v, that for 2,4-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3,4-dihydoroxycinnamic acid and 4-hydroxyphenoxyacetic acid was 90:10 v/v, and that for the other benzoic, phenoxyacetic and cinnamic acid derivatives was 70:30 v/v. The calibration curves of the benzoic and cinnamic acid derivatives were linear over the concentration range of 0.1-20 nmol/mL (r=0.999) and coefficient of variations were lower than 4%.

Protein concentration was determined using a Bio-rad dye reagent (Richmond, CA) with bovine serum albumin as the standard.

Statistical analyses

The data were analyzed by Scheffe's multiple comparison test after the analysis of variance using the Statcel 2 program, and differences with p values < 0.05 were considered to be significant. Data are shown as mean ± standard error (S.E.).

RESULTS

Comparison of the uptake of benzoic acid derivatives and their inhibitory effects on the uptake of benzoic acid

Caco-2 cells were incubated with 50 μM benzoic acid derivatives **(Figure 1)** at pH 6.0 for 1 min, and the uptake of the benzoic acid derivatives was compared with that of benzoic acid (parent compound) **(Figure 2)**. Although mono-hydroxylation of the benzene ring at the ortho (*o*)-, meta (*m*)- and para (*p*)-position (chemical structures are shown in **Figures 1-3** respectively) significantly decreased uptake, the decreases induced by *m*- and *p*-hydroxylation were more profound than that induced by *o*-hydroxylation (The uptake of *m*-, *p*- and *o*-hydroxybenzoic acids was 0.3, 0.8 and 3.7 nmol/mg protein, respectively). The uptake of mono-methoxy benzoic acids at the *m*- and *p*-position (6,7) (5.0 and 4.5 nmol/mg protein) was marginally lower than that of the parent compound (5.8 nmol/mg protein), but that at *o*-substitution (5) was significantly lower (3.5 nmol/mg protein). Di-hydroxylation (8-11) resulted in marked decrease, with the differences in uptake due to substitution position being negligible (0.1-0.3 nmol/mg protein). The methoxylation of one of the hydroxyl groups (12-14) increased uptake.

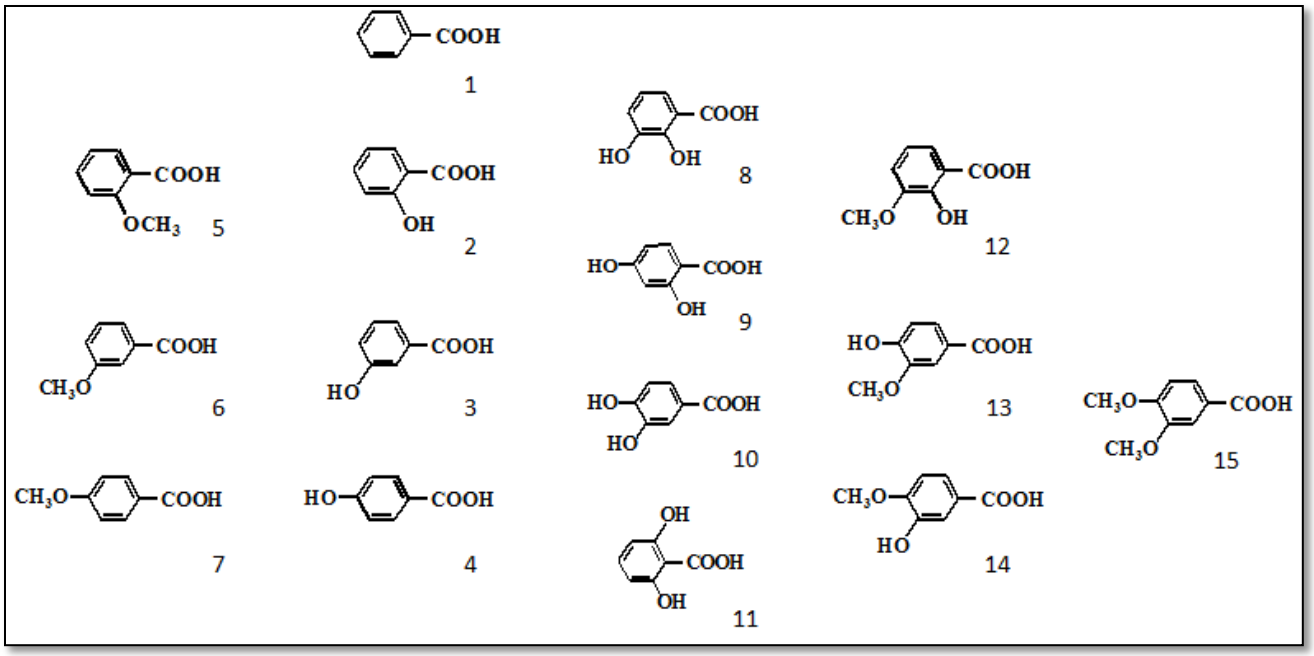

Figure 1. Structures of benzoic acid derivatives.

Caco-2 cells were coincubated with 50 μM benzoic acid and 5 mM benzoic acid derivatives at pH 6.0 for 1 min **(Figure 3)**. Coincubation with mono-hydroxyl benzoic acid at the *o*-position (2) profoundly decreased the uptake of benzoic acid (89%) and that at the *p*-position (4) significantly decreased the uptake of benzoic acid (40%), while that at the *m*-position (3) did not affect uptake at all. Coincubation with di-hydroxyl benzoic acid at different positions (8-11) did not decrease the uptake of benzoic acid. Coincubation with benzoic acid derivatives containing mono-methoxy and mono-hydroxyl groups (12-14) significantly decreased the uptake of benzoic acid (21 to 65%) and that with derivatives

containing di-methoxy groups (15) further decreased uptake (75%).

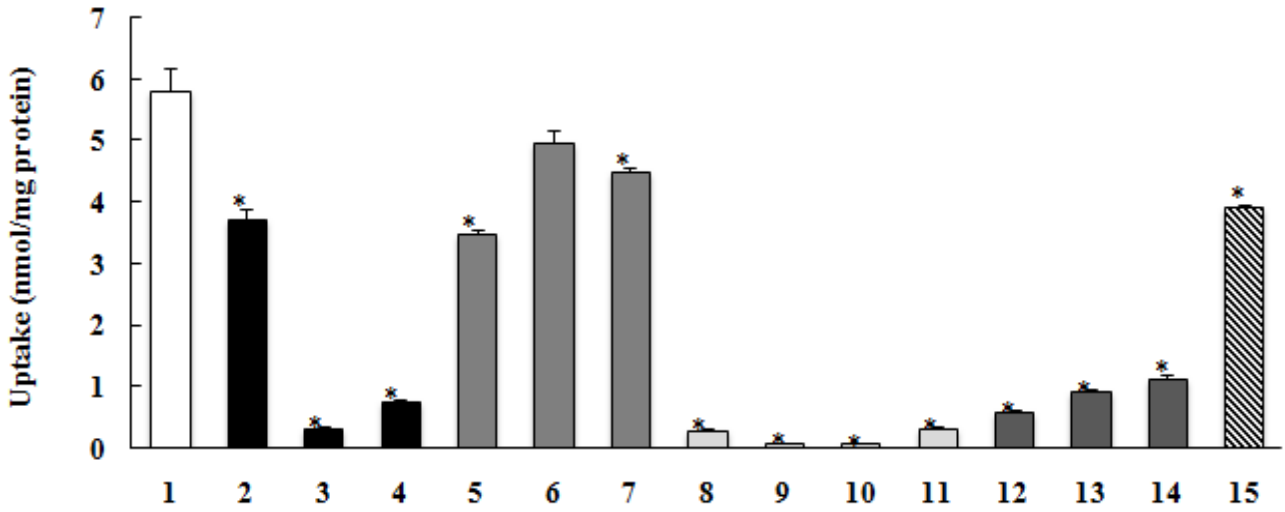

Figure 2. Uptake of benzoic acid derivatives by Caco-2 cells. Caco-2 cells were incubated in a medium at pH 6.0 with 50 μM benzoic acid derivatives at 37°C for 1 min. Each point represents the mean ± S.E. for 4-6 determinations. *Significantly different from uptake of benzoic acid.
1: benzoic acid, 2: 2-hydroxybenzoic acid, 3: 3-hydroxybenzoic acid, 4: 4-hydroxybenzoic acid, 5: 2-methoxybenzoic acid, 6: 3-methoxybenzoic acid, 7: 4-methoxybenzoic acid, 8: 2,3-dihydroxybenzoic acid, 9: 2,4-dihydroxybenzoic acid, 10: 3,4-dihydroxybenzoic acid, 11: 2,6-dihydroxybenzoic acid, 12: 2-hydroxy-3-methoxybenzoic acid, 13: 4-hydroxy-3-methoxybenzoic acid, 14: 3-hydroxy-4-methoxybenzoic acid, 15: 3,4-dimethoxybenzoic acid

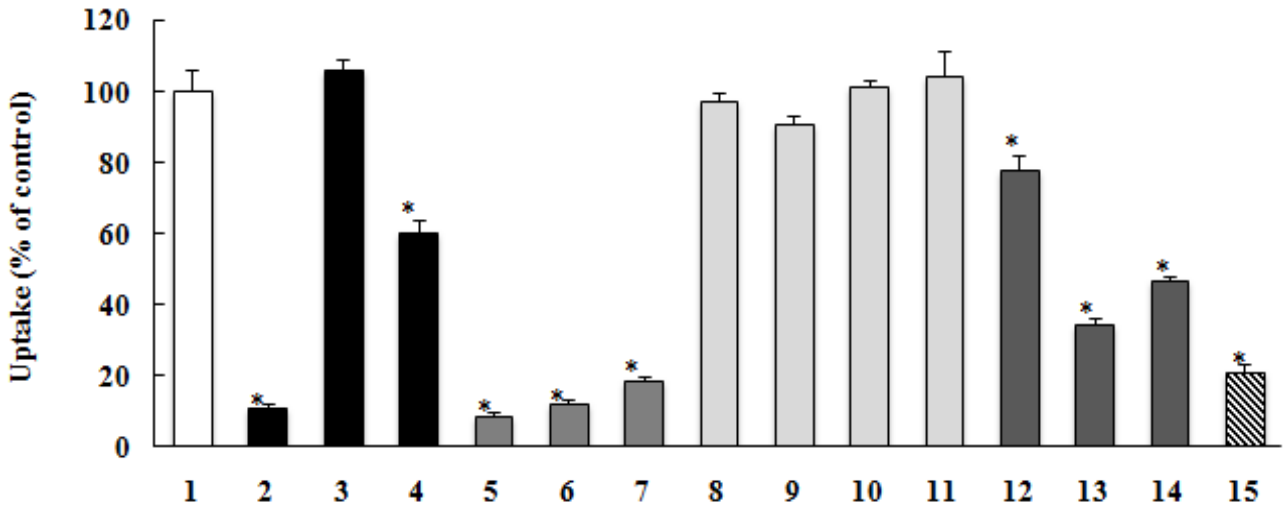

Figure 3. Inhibitory effects of benzoic acid derivatives on the uptake of benzoic acid by Caco-2 cells. Caco-2 cells were incubated in a medium at pH 6.0 with 50 μM benzoic acid in the presence or absence of 5 mM benzoic acid derivatives at 37°C for 1 min. Each point represents the mean ± S.E. for 4-6 determinations. *Significantly different from control.
1: benzoic acid (control), 2: 2-hydroxybenzoic acid, 3: 3-hydroxybenzoic acid, 4: 4-hydroxybenzoic acid, 5: 2-methoxybenzoic acid, 6: 3-methoxybenzoic acid, 7: 4-methoxybenzoic acid, 8: 2,3-dihydroxybenzoic acid, 9: 2,4-dihydroxybenzoic acid, 10: 3,4-dihydroxybenzoic acid, 11: 2,6-dihydroxybenzoic acid, 12: 2-hydroxy-3-methoxybenzoic acid, 13: 4-hydroxy-3-methoxybenzoic acid, 14: 3-hydroxy-4-methoxybenzoic acid, 15: 3,4-dimethoxybenzoic acid

Comparison of the uptake of cinnamic acid and phenoxyacetic acid derivatives and their inhibitory effects on the uptake of benzoic acid

Caco-2 cells were incubated with 50 μM cinnamic acid derivatives **(Figure 4)** at pH 6.0 for 1 min and their uptake was compared with the uptake of cinnamic acid (21) **(Figure 5)**. The uptake of cinnamic acid derivatives containing cyano group, α-cyanocinnamic acid (22), α-cyano-3-hydroxycinnamic acid (23) and α-cyano-4-hydroxycinnamic acid (24), was very low (0.1-0.4 nmol/mg protein). The uptake of α-methylcinnamic acid (25) (1.9 nmol/mg protein) acid was significantly lower than that of cinnamic acid (21) (5.5 nmol/mg protein), but significantly higher than that of α-cyanocinnamic acid (22) and its derivatives (23, 24).

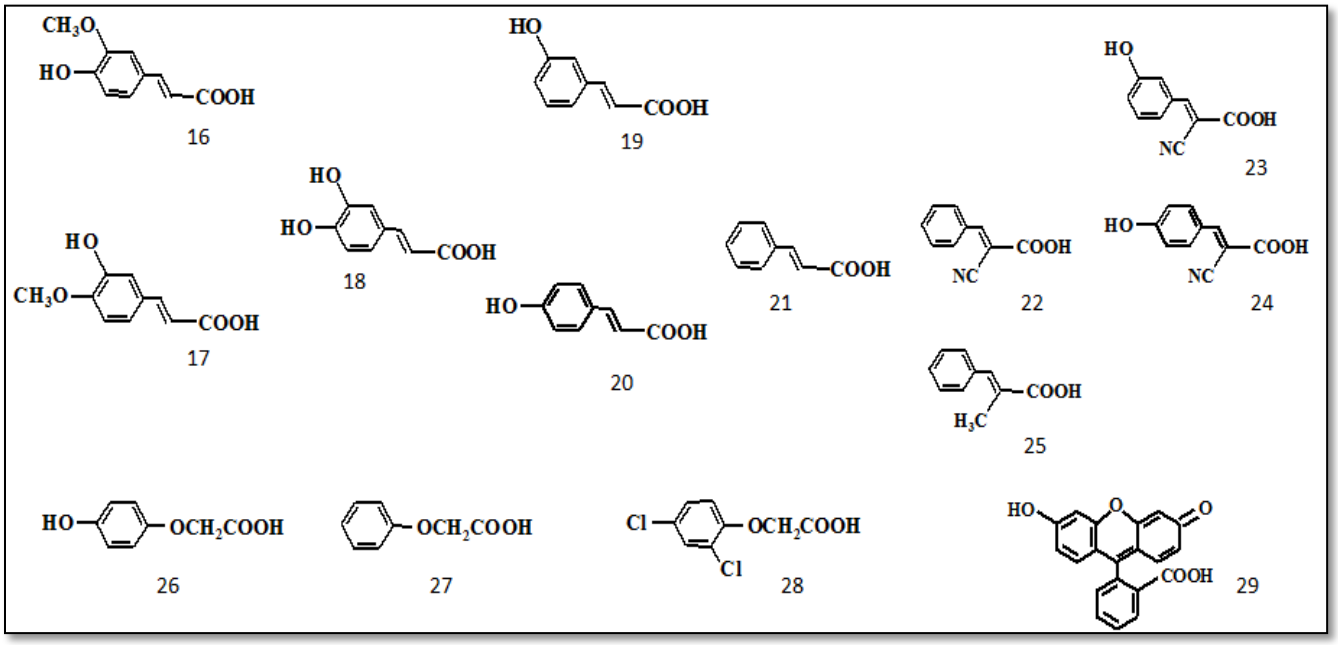

Figure 4. Structures of cinnamic acid derivatives, phenoxyacetic acid derivatives and fluorescein.

The mono-hydroxylation of the benzene ring of cinnamic acid at the *m*- or *p*-position (19, 20) resulted in significant decreased in the uptake (0.7 and 1.7 nmol/mg protein, respectively). Di-hydroxylation at the *m*- and *p*-positions (18) resulted in further decreases, whereas the methoxylation of one of the hydroxy groups at the *m*- or *p*-position (16, 17) increased uptake (the uptake of those were 0.1, 3.5 and 5.2 nmol/mg protein, respectively).

Caco-2 cells were co-incubated with 50 μM benzoic acid and 5 mM cinnamic acid derivatives at pH 6.0 for 1 min **(Figure 6)**. Coincubation with α-cyano-3-hydroxycinnamic acid (23) and α-cyano-4-hydroxycinnamic acid (24) did not decrease the uptake of benzoic acid, while coincubation with α-cyanocinnamic acid (22) significantly decreased the uptake of benzoic acid by 38% and that with α-methylcinnamic acid (25) profoundly decreased the uptake by 83%.

The mono-hydroxylation of the benzene ring at the *m*- and *p*-positions significantly decreased the uptake by 46% (19) and 64% (20), respectively. Di-hydroxylation at the *m*- and *p*-positions marginally decreased uptake by 17% (18) and the methoxylation of the hydroxy group at the *m*- or *p*-position (16, 17) profoundly decreased the uptake of benzoic acid by 86 and 90%, respectively.

The uptake of phenoxyacetic acid (27) was low and that of 4-hydroxyphenoxyacetic acid (26) was even lower **(Figure 5)**. Coincubation with phenoxyacetic acid (27) or 2,4-dichlorophenoxyacetic acid (2,4-D) (28) significantly decreased the uptake of benzoic acid (31 and 86%, respectively), whereas that with 4-hydroxyphenoxyacetic acid (26) marginally decreased uptake (20%) **(Figure 6)**.

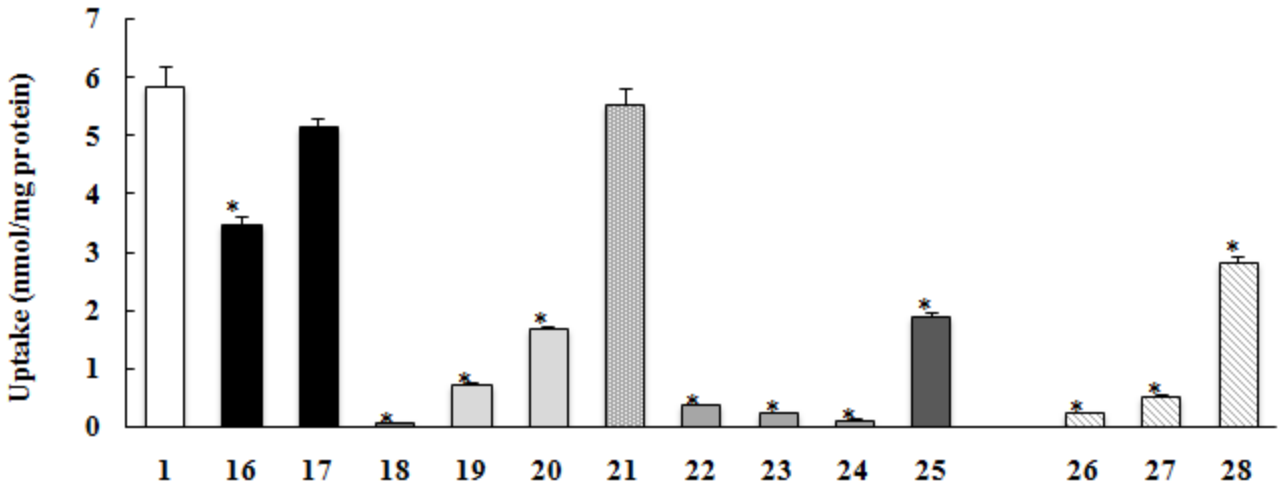

Figure 5. Uptake of cinnamic acid derivatives or phenoxyacetic acid derivatives by Caco-2 cells. Caco-2 cells were incubated in a medium at pH 6.0 with 50 μM cinnamic acid derivatives or phenoxyacetic acid derivatives at 37°C for 1 min. Each point represents the mean ± S.E. for 4-6 determinations. *Significantly different from uptake of benzoic acid.
1: benzoic acid, 16: 4-hydroxy-3-methoxycinnamic acid, 17: 3-hydroxy-4-methoxycinnamic acid, 18: 3,4-dihydroxycinnamic acid, 19: 3-hydroxycinnamic acid, 20: 4-hydroxycinnamic acid, 21: cinnamic acid, 22: α-cyanocinnamic acid, 23: α-cyano-3-hydroxycinnamic acid, 24: α-cyano-4-hydroxycinnamic acid, 25: α-methylcinnamic acid, 26: 4-hydroxyphenoxyacetic acid, 27: phenoxyacetic acid, 28: 2,4-dichlorophenoxyacetic acid

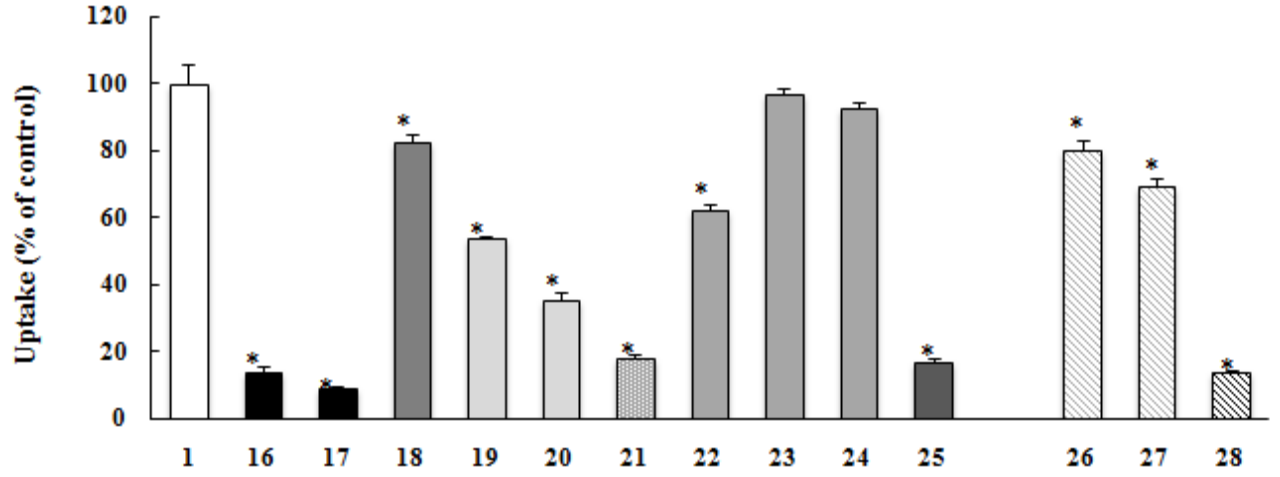

Figure 6. Inhibitory effects of cinnamic acid derivatives and phenoxyacetic acid derivatives on the uptake of benzoic acid by Caco-2 cells. Caco-2 cells were incubated in a medium at pH 6.0 with 50 μM benzoic acid in the presence or absence of 5 mM cinnamic acid derivatives or phenoxyacetic acid derivatives at 37°C for 1 min. Each point represents the mean ± S.E. for 4-6 determinations. *Significantly different from control.
1: benzoic acid (control), 16: 4-hydroxy-3-methoxycinnamic acid, 17: 3-hydroxy-4-methoxycinnamic acid, 18: 3,4-dihydroxycinnamic acid, 19: 3-hydroxycinnamic acid, 20: 4-hydroxycinnamic acid, 21: cinnamic acid, 22: α-cyanocinnamic acid, 23: α-cyano-3-hydroxycinnamic acid, 24: α-cyano-4-hydroxycinnamic acid, 25: α-methylcinnamic acid, 26: 4-hydroxyphenoxyacetic acid, 27: phenoxyacetic acid, 28: 2,4-dichlorophenoxyacetic acid

Competitive inhibition of cinnamic and 4-hydroxycinnamic acid on benzoic acid uptake

Inhibitory effects of cinnamic and 4-hydroxycinnamic acids (20, 21) on benzoic acid uptake by Caco-2 cells were analyzed using Dixon plots **(Figure 7)**. Caco-2 cells were incubated in a medium at pH 6.0 for 1min at 37°C with 25, 50 or 100 μM benzoic acid in the absence or presence of cinnamic acid or 4-hydroxycinnamic acid (1, 2 or 5 mM). Both cinnamic acid and 4-hydroxycinnamic acid inhibited

the uptake of benzoic acid competitively, with apparent K_i values of 0.8 and 1.5 mM, respectively.

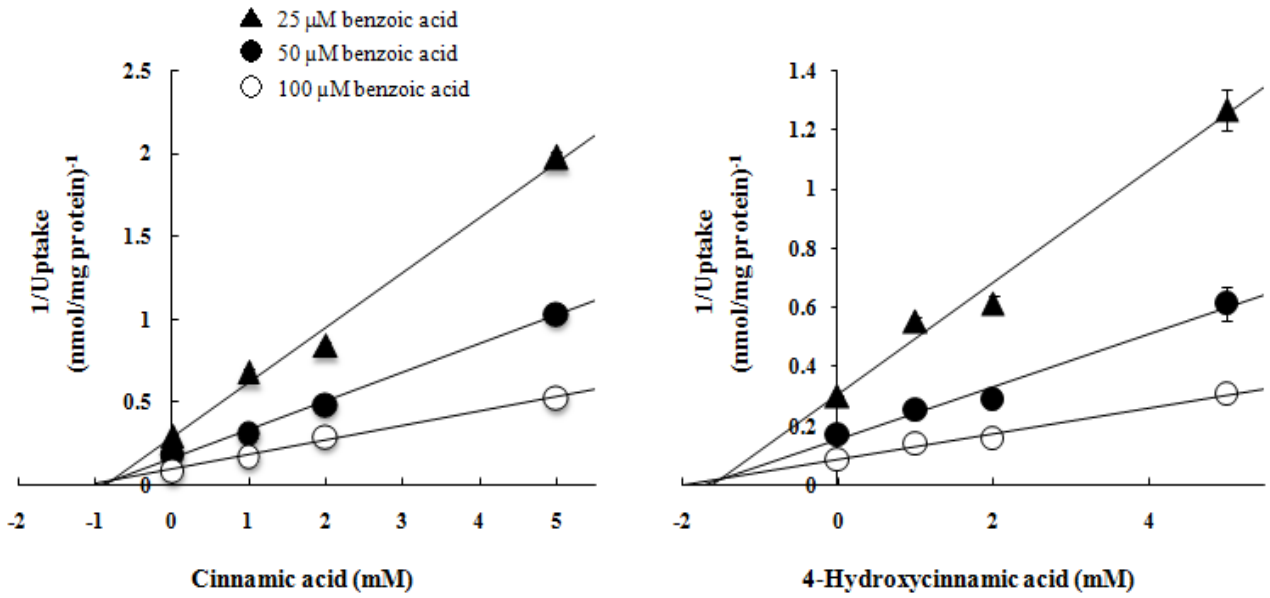

Figure 7. Dixon plots of benzoic acid uptake into Caco-2 cells. Uptake of benzoic acid for 1min at pH 6.0 from the medium containing 25, 50 or 100 μM in the absence or presence of cinnamic acid or 4-hydroxycinnamic acid was measured. Each value represents the mean ± S.E. for 3-6 determinations.

DISCUSSION

The pKa of benzoic acid, and *o*-, *m*- and *p*-hydroxybenzoic acids (2-4) are 4.2, 2.98, 4.08 and 4.57, respectively [20], and the formation of internal hydrogen bonds between the 1-carboxyl and 2-hydroxyl groups contributing to the low pKa of the *o*-hydroxybenzoic acid [21]. The uptake of *o*-hydroxybenzoic acid **(Figure 2)** and its inhibitory effect on benzoic acid uptake **(Figure 3)** were the highest among the *o*-, *m*- and *p*-hydroxybenzoic acids. The formation of internal hydrogen bonds may be a possible reason for the highest uptake and inhibitory effect of *o*-hydroxybenzoic acid. On the other hand, the uptake and inhibitory effect of *m*-hydroxybenzoic acid were the lowest among the three isomers **(Figures 2 and 3)**, which was at variance with the order of the pKa values. A similar order was observed for the inhibitory effects of mono-hydroxybenzoic acids (*m*-<*p*-<*o*-) on the uptake of MCPA (a phenoxyacetic acid derivative) [15] and the inhibitory effects of *m*- and *p*-hydroxybenzoic acids on the transport of salicylic acid [22]. In contrast, Konishi et al. [7] examined the inhibitory effects of *o*-, *m*- and *p*-hydroxycinnamic acids on fluorescein transport, and reported that *m*-hydroxycinnamic acid had a higher inhibitory effect whereas *o*-hydroxycinnamic acid had no inhibitory effect (*o*-<*p*-<*m*-). The reason for this contradiction remains unclear; however, it is speculated that hydroxylation of the benzene ring may change MCT(s) affinity.

The uptake of di-hydroxybenzoic acids (8-11) and di-hydroxycinnamic acid (18) were markedly lower than that of mono-hydroxybenzoic acids (2-4) and mono-hydroxycinnamic acids (19, 20), respectively and the inhibitory effects of di-hydroxybenzoic acids (8-11) and di-hydroxycinnamic acid (18) on benzoic acid uptake were markedly lower than those of the respective mono-hydroxy compounds. Hydroxylation, particularly di-hydroxylation, decreased the affinity for MCTs, probably due to decreased lipophilicity. In agreement with our results, Konishi et al. [7] reported di-hydroxylated benzoic acids (8, 10, 11) and 3,4-dihydroxycinnamic acid (18) did not have an inhibitory effect on fluorescein transport (MCT substrate), while the respective mono-hydroxylated compounds did inhibit fluorescein transport. On the other hand, that mono-chloro- and di-chlorosubstitution of benzoic acids did not affect uptake, whereas coincubation with those chlorobenzoic acids, except for 2,6-dichlorobenzoic acid, markedly decreased the uptake of benzoic acid independent of the chloro-substitution position [18]. Among the dihydroxybenzoic acids (8-11), we expected 2,6-dihydroxybenzoic acid (11) to show the lowest level of uptake based on our previous report of the steric hindrance associated with the 2,6-dichlorosubstitution of the benzoic ring limiting MCT access to the carboxylic group [18]. However, the uptake of dihydroxybenzoic acids investigated was too low to allow us to identify the steric hindrance of 2,6-dihydroxybenzoic acid.

The uptake of 2-hydroxy-3-methoxybenzoic acid (12), 4-hydroxy-3-methoxybenzoic acid (13) and 3-hydroxy-4-methoxybenzoic acid (14) was slightly but significantly higher than that of 2,3-, 2,4 and 3,4-dihydroxybenzoic acid

(8-10), respectively **(Figure 2)**, and the inhibitory effects of those methoxybenzoic acids (12-14) on benzoic acid uptake were significantly higher **(Figure 3)**. A similar tendency due to the presence of a methoxy group was also found in the cinnamic acid derivatives: The uptake of 3-hydroxy-4-methoxycinnamic acid (17) and 4-hydroxy-3-methoxycinnamic acid (16) was higher than that of 3,4-dihydroxycinnamic acid (18) **(Figure 5)** and the inhibitory effect of these methoxycinnamic acids (16,17) was higher than that of 3,4-dihydroxycinnamic acid (18) **(Figure 6).** The substitution of the hydroxy group with a methoxy group could increase the uptake via MCTs.

The uptake of 3,4-dihydroxycinnamic acid (18) was markedly lower than that of 3- and 4-monohydroxycinnnamic acid (19,20) as well as 4-hydroxy-3-methoxycinnamic acid (16) and 3-hydroxy-4-methoxycinnamic acid (17) and the inhibitory effect of 3,4-dihydroxcinnamic acid (18) was significantly lower than that of the other acids (16-20). Saito et al. [9] compared the inhibitory effects of 4-hydroxycinnnamic acid (20), 3,4-dihydroxycinnamic acid (18) and 4-hydroxy-3-methoxycinnamic acid (16) on the uptake of nateglinide (a possible substrate for MCTs or MCT-like transporters) by Caco-2 cells, and found that the non-competitive inhibition of 3,4-dihydroxycinnamic acid (18) with a higher K_i value, in comparison with the competitive inhibition of 4-hydroxycinnnamic acid (20) and 4-hydroxy-3-methoxycinnamic acid (16). The internal hydrogen bonds between the 3- and 4-hydroxyl groups on the benzene ring may be one reason for these phenomena [23]. Further study of 2,3-,2,4-,2,5- and 3,5-dihydroxylcinnamic acids is necessary to confirm the effect of internal hydrogen bounds.

The uptake of 4-hydroxyphenoxyacetic acid (26) was lower than that of phenoxyacetic acid (27: PA) and 2,4-dichlorophenoxyacetic acid (28: 2,4-D) **(Figure 5)** and the inhibitory effect of 4-hydroxyphenoxyacetic acid on benzoic acid uptake was also lower **(Figure 6)**. Like benzoic acid and cinnamic acid, the hydroxylation of benzene ring could decrease the MCT(s) affinity. The pKa of 4-hydroxyphenoxyacetic acid was 4.5 and that of phenoxyacetic acid and 2,4-D were 4.28 and 2.64, respectively. According to our previous report [16], the uptake of phenoxyacetic acids increased in the order of chloride substituent and lipophilicity (PA<4-CPA<2,4-D=MCPA<2,4,5-T), with the pKa values of those compounds being 4.28, 4.19, 2.64, 3.07 and 2.88, respectively. The uptake of 4-hydroxyphenoxyacetic acid and its pKa are in good agreement with this order. These results strengthen our hypothesis that the uptake of phenoxyacetic acid derivatives is mediated by the same MCTs as benzoic acid, at least in part, and the affinity of those derivatives for MCTs is determined by their lipophilicity and pKa values [16].

The uptake of α-cyanocinnamic acid derivatives was trace (22-24) **(Figure 5)** and these derivatives decreased slightly or not at all the uptake of benzoic acid **(Figure 6)**. In contrast, the uptake of α-methylcinnamic acid (25) was significantly higher than that of the α-cyanocinnamic acid derivatives (22-24) **(Figure 5)** and this compound significantly decreased the uptake of benzoic acid **(Figure 6)**. The α-cyano group of cinnamic acids may inhibit the accessibility of the carboxyl group to the MCTs.

The uptake of α-cyano-4-hydroxycinnamic acid (24) and α-cyano-3-hydroxycinnamic acid (23) was lower than that of α-cyanocinnamic acid (22), although the uptake of all of those compounds was low **(Figure 5)**. The inhibitory effects of α-cyano-4-hydroxycinnamic acid (24) and α-cyano-3-hydroxycinnamic acid (23) on the uptake of benzoic acid were slight, whereas that α-cyanocinnamic acid (22) was significant **(Figure 6)**. Hydroxylation of cinnamic acids at the benzene ring appears to slightly weaken their affinity for the MCTs that mediate benzoic acid uptake. In agreement, the Dixon plot **(Figure 7)** suggests that 4-hydroxycinnamic acid (24) has a higher K_i value than cinnamic acid (21) in terms of benzoic acid uptake.

Available information on MCT subtypes is somewhat controversial. MCT1-4 are characterized as proton-linked MCTs among 14 MCT subtypes [1] and Caco-2 cells expressed MCT1, MCT3, MCT4 and MCT6 with MCT1 being the most abundant [11]. α-Cyano-cinnamic acid analogs are the inhibitors of MCT1, MCT2 and MCT4, and the inhibitory effect of α-cyano-4-hydroxycinnamic acid (CHC) on MCT1 activity is about ten times higher than that of other α-cyano-cinnamic acids [1]. However, the uptake of benzoic acid and phenoxyacetic acid derivatives could be mediated via proton-linked MCTs and co-incubation with CHC hardly decreased their uptake. Kuwayama et al. [24] reported the similar results that fluorescein uptake by Caco-2 cells could be mediated via proton-lined MCTs which were distinct from MCT1-4. Further study is necessary to clarify the contradicted information in the MCT subtypes and elucidated the MCT subtype mediated the benzoic acid, phenoxylic acid derivatives and fluorescein.

In conclusion, the hydroxylation of benzoic acid, cinnamic acid and phenoxyacetic acid decreased their uptake via Caco-2 cells, and the uptake of those compounds was mediated via common MCTs at least in part. Further, the α-cyano group of cinnamic acid decreased the affinity of their carboxyl groups to MCT(s).

DECLARATION OF INTEREST

The authors declare no conflict of interest.

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