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Title page

Black tea extract and theaflavin derivatives affect the pharmacokinetics of rosuvastatin by modulating organic anion transporting polypeptide (OATP) 2B1 activity

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Conflicts of interests

The authors declare that there are no conflicts of interest.

Title

Black tea extract and theaflavin derivatives affect the pharmacokinetics of rosuvastatin by modulating organic anion transporting polypeptide (OATP) 2B1 activity

Abstract

Theaflavins (TFs) are derived from black tea, an important source of dietary polyphenols. Although the potential interactions between dietary polyphenols and drugs have been demonstrated through *in vitro* and *in vivo* studies, little information is available concerning the influence of TFs on drug disposition. Organic anion transporting polypeptide 2B1 (OATP2B1) is expressed in human enterocytes and plays a role in the intestinal absorption of numerous drugs. In the current study, we evaluated the effects of black tea extracts on the pharmacokinetics of rosuvastatin in rats, and investigated the effect of four major TFs (theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate) on the transport activity of OATP2B1. Black tea extracts significantly decreased the maximum plasma concentration ($C_{\max}$) and area under the plasma concentration-time curve (AUC_{0-8}) of rosuvastatin by 48% and 37%, respectively ($p < 0.001$ and $p < 0.01$, respectively). Moreover, OATP2B1-mediated rosuvastatin and estrone-3-sulfate uptake was significantly reduced in the presence of TFs. A kinetic study revealed that the uptake efficiency (in terms of $V_{\max}/K_m$) of rosuvastatin was decreased following TF treatment. Black tea extracts also reduced OATP2B1-mediated rosuvastatin uptake. These results suggest that black tea reduces plasma concentrations of rosuvastatin by inhibiting intestinal OATP2B1-mediated transport of rosuvastatin.

Keywords: organic anion-transporting polypeptide 2B1, black tea, theaflavin, rosuvastatin, drug interaction

Introduction

Theaflavins (TFs) are a group polyphenol compounds contained in black tea (*Camellia sinensis*). TFs possess diverse pharmacological activities, including antioxidant, antihyperglycemic, anti-inflammatory, and antiviral effects (Chen et al., 2005; Yang et al., 2008; Miyata et al., 2013; Fu et al., 2018). TFs are prepared commercially and used as dietary supplements owing to the established health benefits. Although TFs and black tea extracts are expected to pass at high concentrations through the small intestine, little is known regarding intestinal transporter-mediated interaction of compounds contained in black tea.

Organic anion-transporting polypeptide (OATP) 2B1 is expressed in various tissues, including the intestine, liver, lung, and placenta (Tamai et al., 2000; St-Pierre et al., 2002). OATP2B1 expressed in the small intestine plays an important role in the absorption of various clinically used therapeutic agents (Nozawa et al., 2004; Satoh et al., 2005; Hirano et al., 2006; Noé et al., 2007; Kitamura et al., 2008; König et al., 2011). Recently, it has been reported that fruit juice, such as orange and apple juice, inhibit the uptake of OATP2B1 substrates *in vitro* (Shirasaka et al., 2013) and decrease the plasma concentration of drugs absorbed by OATP2B1 *in vivo* (Tapaninen et al., 2011). Furthermore, Roth et al. reported that the green tea catechins epicatechin gallate and epigallocatechin gallate inhibit estrone-3-sulfate (E3S) uptake via OATP2B1 (Roth et al., 2011). Thus, there is a possibility that beverages can influence drug absorption through OATP2B1. We previously demonstrated that theaflavin (TF-1) could inhibit OATP2B1-mediated E3S uptake (Kondo et al., 2017). However, the effect of black tea on the pharmacokinetics of substrate

drugs of OATP2B1 remains unknown.

Rosuvastatin is reported to be a substrate for various transporters, such as OATP2B1, that are expressed in the intestine and liver; conversely, it undergoes relatively little metabolism by CYP enzymes (Wang et al., 2017). Thus, rosuvastatin is frequently used as a probe substrate in transporter-mediated drug-drug interaction evaluations, including *in vivo* studies, owing to its unique disposition properties (Johnson et al., 2017). In this study, we determined that black tea extracts and four major TFs (TF-1, theaflavin-3-gallate (TF-2A), theaflavin-3'-gallate (TF-2B), and theaflavin-3,3'-digallate (TF-3)) have an effect on uptake of rosuvastatin *in vitro* and plasma concentration *in vivo*.

Materials and Methods

Chemicals

Commercial black tea leaves, Lipton Extra Quality Ceylon, was purchased from Unilever Japan K.K. (Tokyo, Japan). TF-1 was purchased from Kanto chemical (Tokyo, Japan). TF-2A, TF-2B, TF-3, and rosuvastatin were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical grade.

Preparation of black tea extracts

To prepare black tea extracts, tea leaves were extracted with hot water. Black tea leaves (5 g) were submerged in 200 ml of water and refluxed at 100°C for 20 min. After cooling, the supernatant was separated by filtration. The concentration of black tea extract was adjusted to 10% in Hank's balanced salt solution (HBSS) for further use in uptake experiments. It was revealed that TFs (TF-1, TF-2 and TF-3) were contained in the black tea leaves used this time by measuring with liquid chromatography-mass spectrometry (LC/MS/MS). The concentrations of TF-1, TF-2 (comprising TF-2A and TF-2B), and TF-3 were $3.03 \pm 0.03 \mu\text{M}$, $2.80 \pm 0.70 \mu\text{M}$, and $1.02 \pm 0.04 \mu\text{M}$, respectively.

Uptake experiments in HEK-OATP2B1 cells and HEK-pcDNA cells

The uptake study was carried out as described previously (Ogura et al., 2014). Accumulation of rosuvastatin was analyzed using LC/MS/MS. The radioactivity of [^3H]E3S was measured by liquid

scintillation counting. Uptake values were corrected against protein concentration from each well using bicinchoninic acid (BCA) protein assay.

Pharmacokinetic study in rats

Male Wistar rats were obtained from Hokudo (Sapporo, Japan). The rats (200-250 g, 7 weeks) were housed under standard conditions (23 ± 2 °C), and maintained under a 12-h light/dark cycle. Animals had free access to food and water throughout the experiment. All animal experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of Hokkaido University (approval number 17-0005). Aqueous solution of rosuvastatin (0.02 mg/ml) in the absence or presence of black tea extracts were administered as single oral dose to rats. Control groups received rosuvastatin (0.03 mg/kg) in water (1.67 ml/kg). Comparative groups received rosuvastatin (0.03 mg/kg) + black tea extracts (1.67 ml/kg). The rats sedated with sevoflurane were anesthetized by an intraperitoneal (i.p.) injection of 50 mg/kg sodium pentobarbital. Blood samples were collected from the jugular vein at 0.5, 1, 2, 4, 6, and 8 h after the administration. The samples were centrifuged at $2,000 \times g$ for 20 min at 4°C. Plasma fractions (0.2 ml) were extracted with 0.5 ml of ethyl acetate by vortex-mixing for 60 s. After centrifugation at $20,000 \times g$ for 15 min at 4°C, the supernatant was transferred into 1.5 ml microtubes. These extraction processes were repeated twice. The organic phase was evaporated at room temperature, and the residual fraction was dissolved with 30 μ l of mobile phase.

LC/MS/MS analysis

Quantitation of rosuvastatin was carried out as reported previously with minor modifications (Ogura et al., 2014). A calibration curve was constructed by plotting the peak area versus the nominal concentration and was fit using least-squares regression. Calibration curves were linear in the range of 0.05–100 nM. The lower limit of quantification was 0.05 nM with a relative standard deviation of less than 20%.

Data analysis

Nonlinear regression analysis was performed using SigmaPlot 12.5 (HULINKS). Student's *t*-test was used to determine the significance of the differences between the means of two groups. The statistical significance among the means of more than two groups was evaluated by using one-way analysis of variance (ANOVA) followed by Dunnett's test. Data are shown as the mean with standard deviation (SD) of at least three individual experiments. Statistical significance was defined at $p < 0.05$.

Results

Plasma concentrations of rosuvastatin after oral administration with black tea extract.

In this study, the rats were divided into two groups, each containing eight rats: the control group, administered rosuvastatin alone; and the treatment group, administered rosuvastatin with black tea. Plasma concentrations of rosuvastatin decreased following co-administration of black tea extract when 0.03 mg/kg of rosuvastatin was administered (Figure 1). Furthermore, black tea extracts significantly decreased the AUC_{0-8} and C_{max} values of rosuvastatin (Supplemental Table 1).

Effects of TFs and black tea extracts on the uptake of OATP2B1 substrates

To investigate whether black tea extracts and TF derivatives can influence the transport of OATP2B1 substrates, we next performed uptake analysis of [3H]E3S and rosuvastatin in HEK-OATP2B1 cells. Kinetic analysis of [3H]E3S and rosuvastatin uptake was performed in the presence of each interacting compound or black tea extracts, and results are shown in Table 1. All additives increased the K_m and V_{max} values of [3H]E3S and rosuvastatin. Moreover, a kinetic study revealed that the uptake efficiency (in terms of V_{max}/K_m) for E3S and rosuvastatin were decreased by TFs or black tea extracts.

Discussion

In this study, we showed that black tea extract affects the plasma concentration of rosuvastatin *in vivo*, and TFs affect the transport of OATP2B1 substrates *in vitro*. To the best of our knowledge, this is the first study that describes the potential food-drug interaction between black tea and rosuvastatin.

First, we clarified the effect of black tea on the pharmacokinetics of rosuvastatin *in vivo* (Figure 1). When 0.03 mg/kg rosuvastatin was administered, the $C_{\max}$ and AUC_{0-8} value significantly decreased in the presence of black tea extracts (Supplemental Table 1). Several studies have suggested a role for intestinal OATP2B1 in the absorption of hydrophilic drugs, including rosuvastatin (Nozawa et al., 2004; Varma et al., 2011). Although considerable interspecies differences in OATP expression exist, Oatp2b1 is expressed similarly in humans and rats (Tamai, 2012; Segawa et al., 2013). Human OATP2B1 and rat Oatp2b1 share 77% amino acid identity and transport many common substrates such as taurocholate, E3S, fexofenadine, and BSP (Hagenbuch and Meier, 2003). There are at least three Oatp1a family members in rats, but the only ortholog in humans is OATP1A2 (Tamai, 2012). Oatp1a5 is also expressed in the rat intestinal epithelium (MacLean et al., 2010). In addition, it has been reported that human OATP1A2 and rat Oatp1a5 are capable of rosuvastatin transport. As shown in Supplemental Figure 1, TF-1 had little effect on the OATP1A2-mediated transport of E3S. Given the similarity of the amino acid sequence between human OATP1A2 and rat Oatp1a5, it is unlikely that TFs have a potent inhibitory effect on Oatp1a5. Thus, it is conceivable that black tea extracts inhibit the OATP2B1-mediated intestinal transport of rosuvastatin.

To explain the mechanism of this food-drug interaction, we investigated the effect of TFs and black tea extracts on uptake of OATP2B1 substrates, E3S and rosuvastatin. All TFs and black tea extract significantly inhibited the uptake of OATP2B1 substrates (Supplemental Figure 2). These additives also resulted in a decrease in the transport efficiency for OATP2B1-mediated uptake, due to the greater increase in K_m over V_{max} along with an increase in the values of both K_m and V_{max} (Table 1). The Eadie-Hofstee plot revealed that TFs inhibited OATP2B1-mediated rosuvastatin uptake in a partially competitive manner (Supplemental Figure 3). In addition, the inhibitory effect of black tea extract exceeded the effect of TF derivative alone. We previously reported that TF-1 is a substrate of OATP2B1 (Kondo et al., 2017). In contrast, Supplemental Figure 4 shows TF-2A, TF-2B and TF-3 are not substrates of OATP2B1. TFs competitively interact for the substrate binding site of OATP2B1, while the gallate moiety may interfere with substrate recognition. These results suggest that TFs act as competitive inhibitors of OATP2B1-mediated transport. However, the mechanism leading to increased V_{max} requires further investigation. OATP2B1 is highly expressed in caco-2 cells, an *in vitro* model of human intestinal epithelium, and is localized to the apical membrane (Kobayashi et al., 2003; Gilligan et al., 2017). Several studies using transfected cell lines and caco-2 cells have confirmed the role of OATP2B1-mediated transport on the intestinal absorption of statins (Ho et al., 2006; Varma et al., 2011). Apical to basolateral transport of E3S and rosuvastatin across caco-2 cell monolayers was significantly decreased in the presence of black tea extract (Supplemental Figure 5). These results support the findings that black tea extracts are capable of inhibiting OATP2B1-mediated rosuvastatin and E3S uptake.

Rosuvastatin is reported to be a substrate for various transporters expressed in the liver and kidney (Wang et al., 2017). Thus, the contribution of other transporters to the rosuvastatin-black tea interaction cannot be excluded. However, given the poor oral bioavailability of TFs, hepatic or renal transporter-related drug interactions may not be clinically important (Pereira-Caro et al., 2017).

The present findings suggest an interaction between black tea and rosuvastatin occurs through OATP2B1 mediated transport. Further studies are required to confirm the influence of black tea extracts on OATP2B1-mediated intestinal anionic drug absorption.

Conclusion

We demonstrated an inhibitory effect of black tea and theaflavin derivatives on OATP2B1-mediated transport. The *in vitro* data also supported *in vivo* experiments in which black tea had a significant pharmacokinetic influence on rosuvastatin. Overall, our findings suggest that black tea reduces plasma concentrations of rosuvastatin, in part by inhibiting intestinal OATP2B1-mediated transport of rosuvastatin. Based on our findings, inhibition of intestinal OATP2B1 by black tea could affect disposition of OATP2B1 substrates.

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Figure legends

Figure 1: Mean plasma concentration-time curve of rosuvastatin in Wistar rats after a single dose of rosuvastatin: (A) linear; (B) log-linear. Rosuvastatin (0.03 mg/kg, 0.02 mg/ml in water) was orally administered in the absence (open circles) or presence (filled circles) of black tea extract (1.67 ml/kg). The data shown are the mean $\pm$ S.D. of eight rats. Student's *t*-test was used to determine the significance of differences between the means of two groups. *: significantly different from the control ($p < 0.05$), **: significantly different from the control ($p < 0.01$), ***: significantly different from the control ($p < 0.001$).

Supplemental Figure 1: Influence of TF-1 on OATP1A2-mediated E3S uptake by HEK293T cells.

(A) The uptake of [³H]E3S by HEK293T cells transiently expressing OATP1A2. HEK293T-OATP1A2 and mock cells were exposed to 10 nM [³H]E3S at pH 7.4 for 10 min. Student's *t*-test was used to determine the significance of differences between the means of two groups. *Significantly different to mock cells at $p < 0.05$. (B) The effects of TF-1 on OATP1A2-mediated [³H]E3S uptake. HEK293T-OATP1A2 and mock cells were exposed to 10 nM [³H]E3S in the absence or presence of 10 μ M TF-1 at pH 7.4 for 10 min. The specific uptake by OATP1A2 was estimated by the subtraction of its uptake by mock cells from that by HEK293T cells transiently expressing OATP1A2. Student's *t*-test was used to determine the significance of the differences between the means of two groups. n.s., not significant. All the data are presented as the mean $\pm$ S.E. of at least three independent experiments performed in

triplicate.

The open reading frame (ORF) encoding human OATP1A2 was custom-synthesized by Eurofins Genomics (Tokyo, Japan). The ORF was subcloned into pcDNA3.1(+) (Invitrogen) by using the specific restriction sites NotI and XhoI for expression in HEK293T cells. HEK293T cells were seeded at a density of 2.0×10^5 cells/well on 24-well plastic plates. On the following day, the cells were transiently transfected with pcDNA 3.1 construct expressing human OATP1A2 using Lipofectamine 3000 (Invitrogen) as a transfection reagent, in accordance with the manufacturer's instructions. HEK293T cells, transfected with pcDNA3.1 empty vector, were used as mock cells. Uptake studies were performed at 48 h after transfection.

Supplemental Figure 2: Influence of TFs and black tea extracts on OATP2B1-mediated substrates uptake by HEK293 cells. HEK293-OATP2B1 and HEK293-pcDNA cells were exposed to 5 nM [3 H]E3S (A) or 0.5 μ M rosuvastatin (B) in the absence or presence of 10 μ M TFs, or 10% black tea extracts, at pH 7.4 for 15 s. Specific uptake by OATP2B1 was estimated by subtracting uptake by HEK293-pcDNA cells from that by HEK293-OATP2B1 cells. The uptake measured in the absence of each additive was set at 100% (control). Each column represents the mean $\pm$ S.D. of three independent experiments. Statistical analysis was performed by using ANOVA followed by Dunnett's test. ***: significantly different from the control ($p < 0.001$).

Supplemental Figure 3: Eadie-Hofstee plots of OATP2B1-mediated rosuvastatin uptake by HEK293

cells in the absence (○) and presence (●) of TFs. Eadie–Hofstee transformation of the Michaelis–Menten equation was used to assess the apparent inhibition patterns of TFs on the velocity of rosuvastatin transport. The same data presented in Table 1 are displayed in the Eadie-Hofstee form to illustrate the non-linear kinetic behavior of the transport system.

Supplemental Figure 4: Time course of TF-2A (A), TF-2B (B), and TF-3 (C) uptake by

HEK-OATP2B1 cells (filled circles) and HEK-pcDNA cells (open circles). HEK-OATP2B1 and

HEK-pcDNA cells were exposed to 10 μ M TFs at pH 7.4 for 15 s. TFs were determined by LC/MS/MS

(ACQUITY UPLC® H-Class System connected to Xevo™ TQ-S (Waters, Milford, MA)) method we had

reported (Kondo et al., 2017). In brief, A CAPCELL CORE (150 $\times$ 2.1 mm, 2.7 μ m) (Shiseido, Tokyo,

Japan) and gradient elution was used for separation. The ion transitions monitored were 717 m/z to 139 for

TF-2A, 717 m/z to 139 for TF-2B, and 869 m/z to 139 for TF-3. Data were acquired and analyzed using

Analyst software (version 4.1). Uptake values were corrected against protein concentration from each well.

Each point represents the mean $\pm$ S.D. of three independent experiments.

Supplemental Figure 5: Effect of black tea extract on the transepithelial transport of E3S (A) and

rosuvastatin (B) by caco-2 cells. Caco-2 cells were seeded into collagen-coted 12-well Transwell™

(corning) plates and grown for 21-28 days. Transepithelial electrical resistance (TEER) of caco-2 cell

monolayers was measured using the Millicell ERS volt/ohmmeter from Millipore (Bedford, MA). Only monolayers showing TEER values over $300 \Omega \text{ cm}^2$ were used in experiments. Caco-2 cell monolayers were preincubated with HBSS buffer at pH 6.0 (apical) and pH 7.4 (basal) for 10 min. After pretreatment, caco-2 cell monolayers were exposed to [^3H]E3S (5 nM) or rosuvastatin (5 μM), in the absence or presence of 10% black tea extracts, for 30 min. Samples were collected from the basolateral chamber 30 min after compound addition to the apical chamber. The results are expressed as the apparent permeability coefficient (P_{app}), which is the amount of compound transported per second. P_{app} values were calculated for the apical to basal movement of the compound from the following equation:

$$P_{\text{app}} = (dQ/dt) \times (1/C_0) \times (1/A)$$

where dQ/dt was the permeability rate, C_0 was the initial concentration in the donor chamber, and A was the surface area of the cell monolayer. Each column represents the mean $\pm$ S.D. of three independent experiments. Student's t -test was used to determine the significance of differences between the means of two groups. *,*** Significantly different from the control at $p < 0.05$ and $p < 0.001$, respectively.

Tables

Table 1

Kinetics of OATP2B1-mediated transport in the absence or presence of TF-1, TF-2A, TF-2B, TF-3, and black tea extracts.

Substrate	Treatment	$V_{\max}$ (pmol/mg protein/15 s)	K_m (μ M)	$V_{\max}/K_m$
$[^3\text{H}]\text{E3S}$	None	191 ± 4.6	13.8 ± 1.4	13.8
	TF-1	347 ± 26	60.7 ± 12	5.71
	TF-2A	348 ± 14	52.9 ± 5.9	6.58
	TF-2B	295 ± 13	34.9 ± 4.9	8.45
	TF-3	277 ± 21	37.3 ± 9.0	7.43
	Black tea extracts	296 ± 13	77.4 ± 8.5	3.82
Rosuvastatin	None	12.9 ± 0.69	4.83 ± 0.93	2.67
	TF-1	16.3 ± 0.71	15.6 ± 1.7	1.04
	TF-2A	18.1 ± 0.88	19.2 ± 2.1	0.94
	TF-2B	20.8 ± 0.84	18.0 ± 1.7	1.16
	TF-3	18.4 ± 1.2	17.5 ± 2.6	1.05
	Black tea extracts	41.1 ± 13	81.8 ± 37	0.50

The uptake by HEK-OATP2B1 and HEK-pcDNA was examined in the absence or presence of 10 μ M TFs or 10% black tea extracts at pH 7.4 for 15 s. The specific uptake by OATP2B1 was estimated by subtracting uptake by HEK293-pcDNA cells from that by HEK293-OATP2B1 cells. The kinetic parameters were calculated using the following equation: $V = V_{\max} [S]/(K_m + [S])$, where V represents the uptake rate; $V_{\max}$, the maximum uptake rate; [S], concentration of substrate; K_m , the Michaelis-Menten constant. Each datum represents the mean $\pm$ S.D. of three independent experiments.

Supplemental Table 1

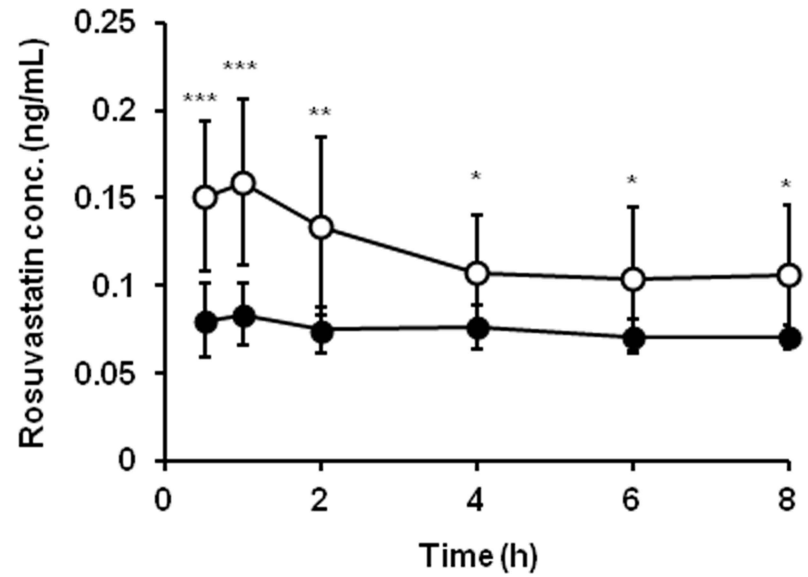
Pharmacokinetic parameters of rosuvastatin after oral administration in the absence or presence of Black tea extracts in Wistar rats.

Parameters	Rosuvastatin	Rosuvastatin
	(Control)	+ Black tea
AUC ₀₋₈ (ng · h/ml)	0.886 ± 0.28	0.561 ± 0.068**
C _{max} (ng/ml)	0.170 ± 0.051	0.0893 ± 0.017***
T _{max} (h)	0.688 ± 0.26	0.750 ± 0.27

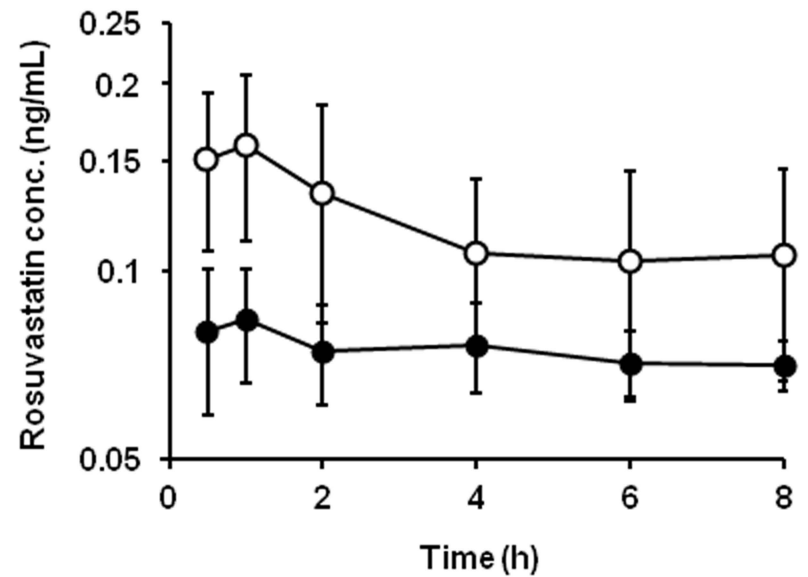
AUC₀₋₈, area under plasma concentration-time curve from 0 h to 8 h; C_{max}, peak plasma drug concentration; T_{max}, time to reach maximum plasma concentration. To analyze the pharmacokinetics of rosuvastatin, the area under the curve (AUC) was calculated using the trapezoidal rule. The data shown are the mean ± S.D. of eight rats. Student's *t*-test was used to determine the significance of differences between the means of two groups. **,*** Significantly different from the values without black tea at *p* < 0.01 and *p* < 0.001, respectively.

Figure 1

(A)

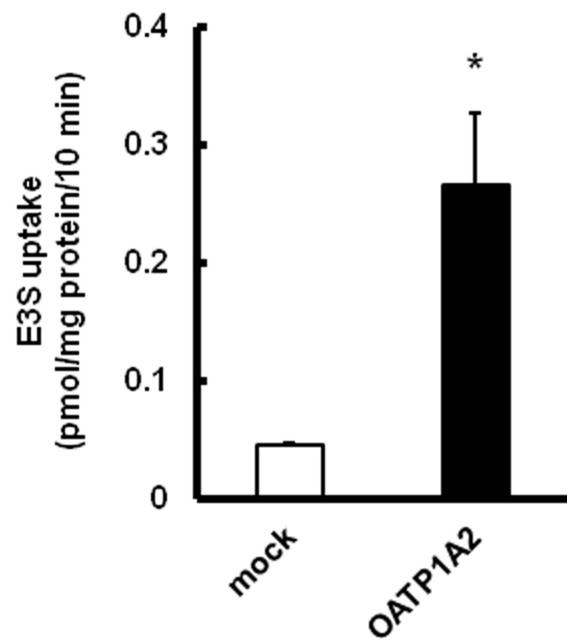


(B)

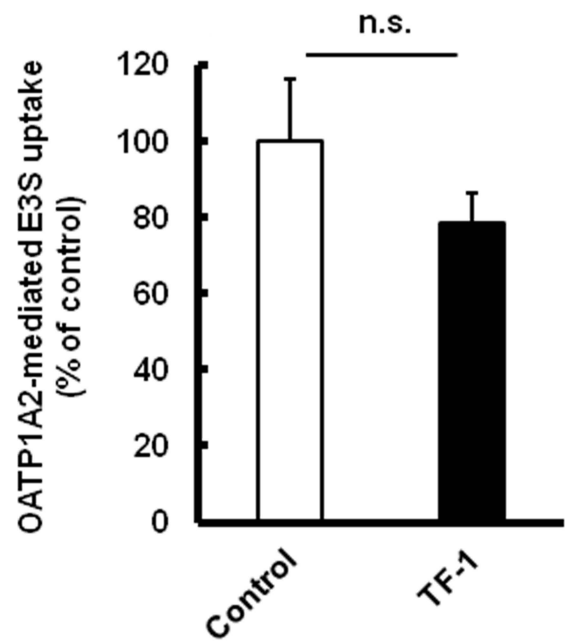


Supplemental Figure 1

(A)

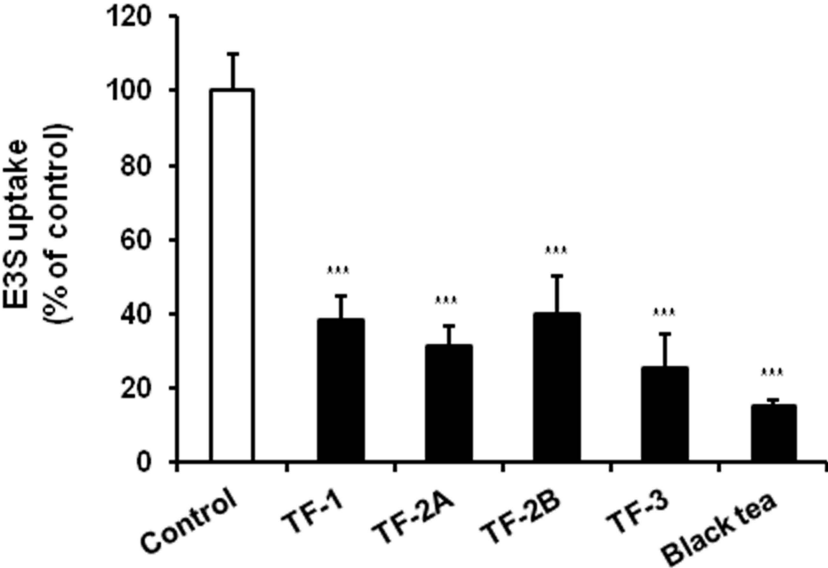


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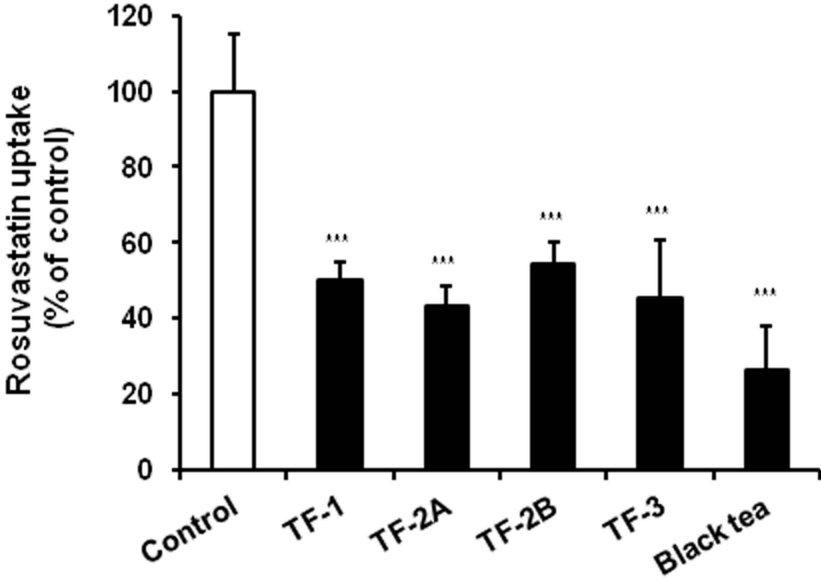


Supplemental Figure 2

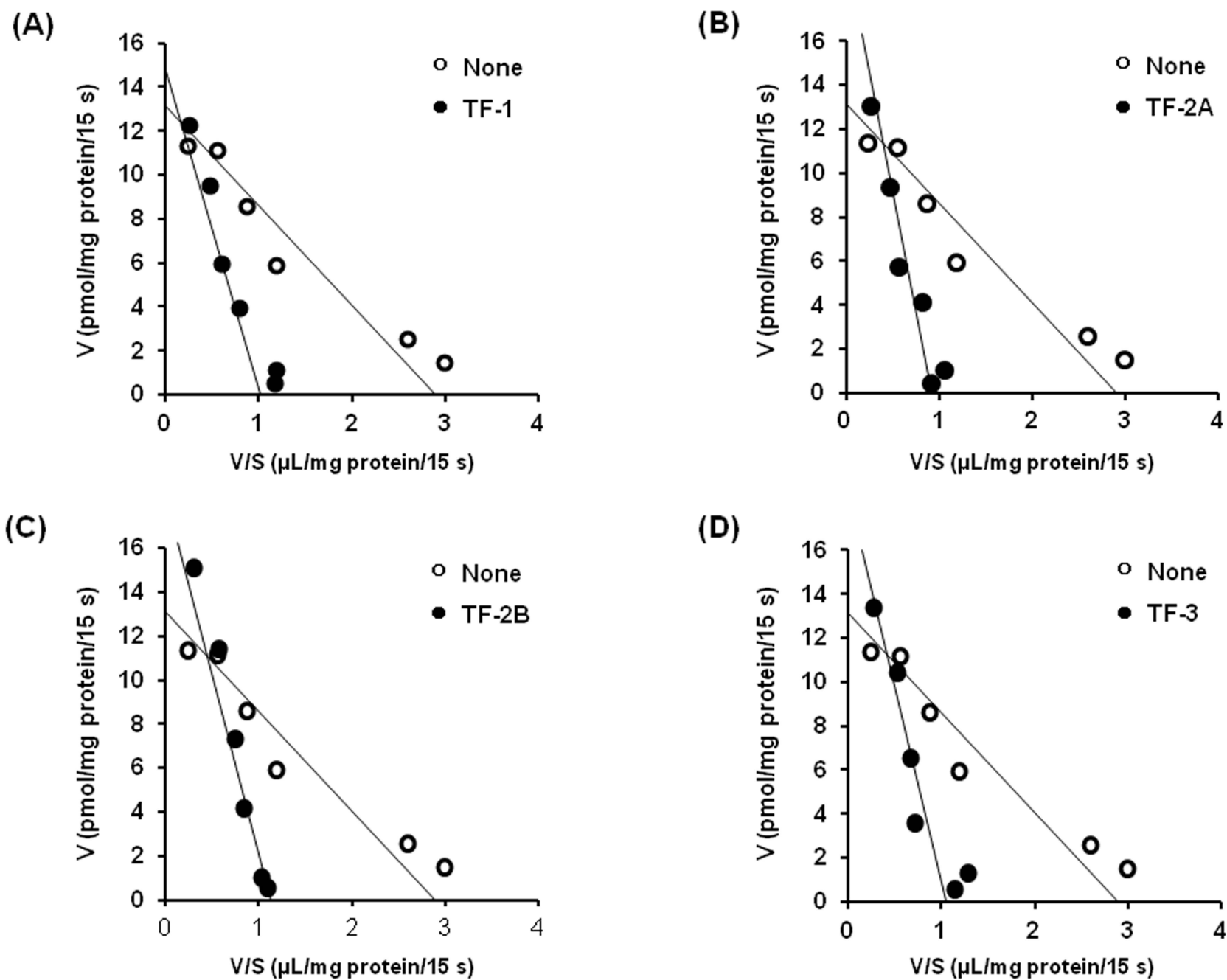
(A)



(B)

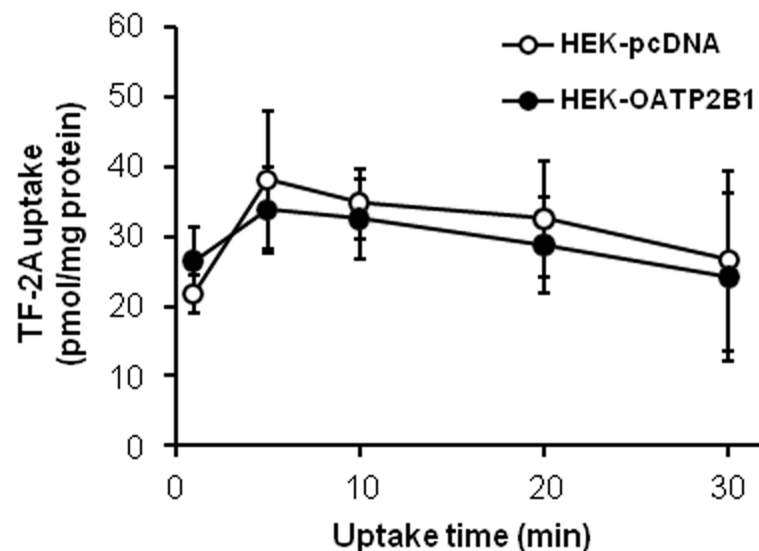


Supplemental Figure 3

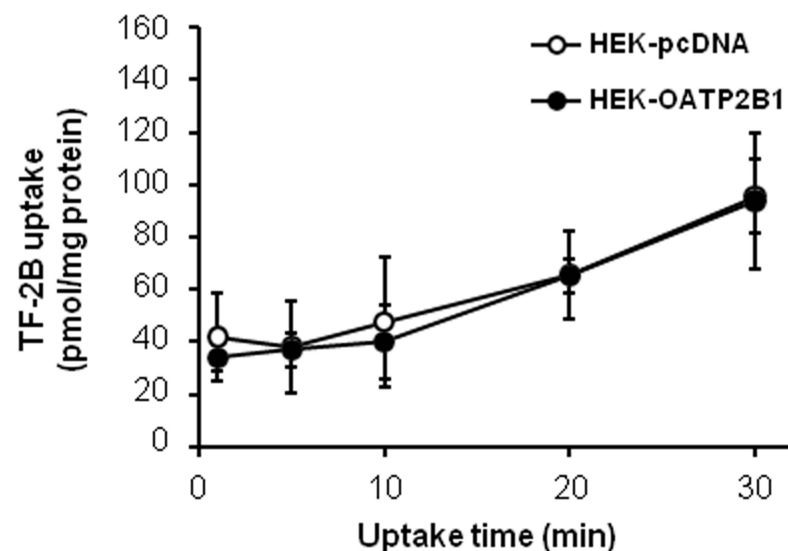


Supplemental Figure 4

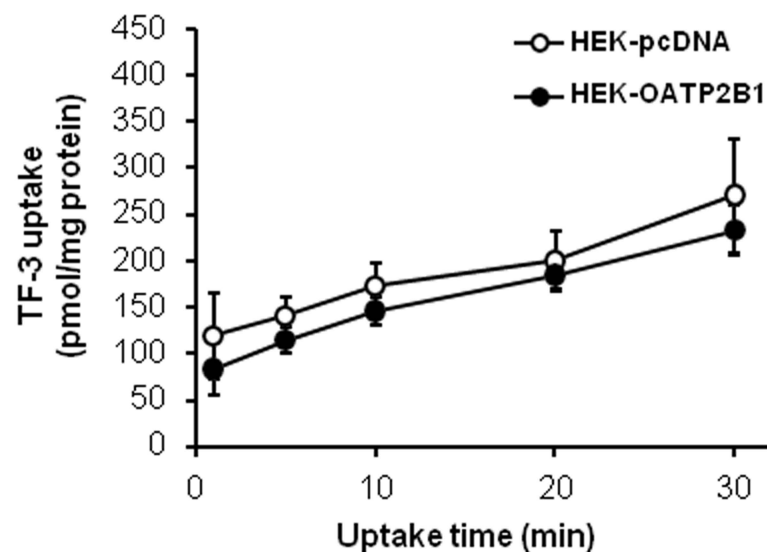
(A)



(B)

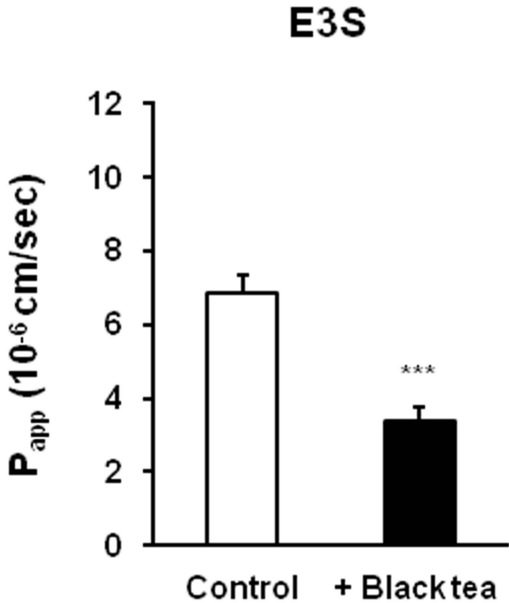


(C)



Supplemental Figure 5

(A)



(B)

