Short Communication

Functional characterization of carrier-mediated transport of pravastatin across the blood-retinal barrier in rats

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Carrier-mediated transport of pravastatin across the BRB

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List of abbreviations

ABC, ATP-binding cassette; BBB, blood-brain barrier; Bcrp, breast cancer resistance protein; BRB, blood-retinal barrier; CNS, central nervous system; E17βG, estradiol 17-β glucuronide; Mrp, multidrug resistance-associated protein; oat, organic anion transporter; oatp, organic anion transporting polypeptides; RPE, retinal pigment epithelium.

ABSTRACT

Systemically administered pravastatin effectively treats diabetic retinopathy without central nervous system side effects. The efflux transport mechanism of pravastatin from the brain has already been clarified. In this study, the influx of pravastatin across the blood-retinal and blood-brain barriers (BRB and BBB), as well as the efflux of pravastatin from the retina, were investigated using rats. Pravastatin influx (blood-to-tissues) was assessed using the retinal and brain uptake index (RUI and BUI) methods, and microdialysis was performed to investigate the efflux (retina-to-blood) transport of pravastatin. The RUI and BUI values for [^{3}H]pravastatin were lower than those expected based on its lipophilicity, suggesting that the influx transport across the BRB and BBB was less than the reverse direction transport. The RUI and BUI values for [^{3}H]pravastatin were significantly decreased by pravastatin, digoxin, and probenecid, indicating that pravastatin undergoes carrier-mediated influx transport in the blood-to-tissues direction across the BRB and BBB. Following intravitreal injection, [^{3}H]pravastatin and the bulk flow marker [^{14}C]D-mannitol were found to be eliminated bi-exponentially from the vitreous humor. The elimination rate constant of [^{3}H]pravastatin during the terminal phase was 1.66-fold greater than that of [^{14}C]D-mannitol. Efflux transport was reduced in the retinal presence of pravastatin, digoxin, and benzylpenicillin, suggesting that pravastatin is transported via efflux transporters. In conclusion, pravastatin is transported across the BRB via uptake and efflux transporters in both the blood-to-retina and retina-to-blood directions, and the retina-to-blood transporters are dominant, based on the lower values of the RUI compared with the values expected from the lipophilicity.

Introduction

Although some statins have pharamacological effects on diabetic retinopathy as well as central nervous system (CNS) side effects, systemically administrated pravastatin reduces signs of diabetic retinopathy in diabetic patients and does not produce CNS side effects such as sleep disturbances (Gordon et al., 1991; Saheki et al., 1994).

Generally, the permeability of drugs into the retina from the blood is strictly regulated by the blood-retinal barrier (BRB), which is analogous to the blood-brain barrier (BBB). The BRB consists of both retinal capillary endothelial cells (inner BRB) and retinal pigment epithelial (RPE) cells (outer BRB), which together form the complex tight junctions that restrict paracellular solute transport. The control of the intraocular environment and the maintenance of neuroretinal homeostasis are mediated, in part, by the various transporters expressed at the BRB (Hosoya and Tomi, 2005).

Pravastatin is a substrate of the organic anion transporting polypeptides1a4 (oatp2; slco1a4) and the organic anion-transporter 3 (oat3; slc22a8) (Kikuchi et al., 2004). Oatp1a4 is localized to both the luminal and abluminal membranes of the inner BRB and on the apical membrane of RPE cells of rats (Akanuma et al., 2013), whereas oat3 is expressed on only the abluminal side of the rat inner BRB and is not found on RPE cells (Hosoya et al., 2009). The expression pattern of these transporters at the BBB is similar to that at the BRB; specifically, oatp1a4 is found on both the luminal and abluminal sides of the rat brain capillary endothelium while oat3 is found on only the abluminal side. Previously, transporters, including oatp1a4 and oat3, have been implicated in the brain-to-blood transport of pravastatin, and the efflux of pravastatin across the BBB has been found to be more than 3-fold greater than its uptake (Kikuchi et al., 2004). However, the transport mechanism of

pravastatin across the BRB remains unclear.

The aim of the present study was to clarify the characteristics of pravastatin transport across the BRB in both the blood-to-retina and retina-to-blood directions and to obtain further evidence to support the clinical effects of pravastatin on diabetic retinopathy. Pravastatin influx (blood-to-tissues) was assessed using the retinal uptake index (RUI) and compared with that of the BBB, and microdialysis was performed to investigate efflux (retina-to-blood) transport of pravastatin. In addition, E17βG influx was evaluated as a model substrate of oatp1a4 and oat3 because the efflux mechanism of E17βG has already been clarified, that is, E17βG is transported from the vitreous humor to the blood via at least oatp1a4 (Katayama et al., 2006) and from the brain to blood via the oat family (mainly oat3) and oatp1a4 (Sugiyama et al., 2001).

Materials and Methods

Animals and Reagents. Male Sprague-Dawley *Mdr1a* knockout rats (6–7 weeks) and wild-type Sprague-Dawley rats (6–8 weeks) were obtained from Sage Labs (St. Louis, MO) and Charles River Laboratories (Yokohama, Japan), respectively. All experiments were performed according to the Ethical Guidelines for Animal Experiments of Santen Pharmaceutical Company (Ikoma, Japan). Pravastatin [^{3}H(G)] sodium salt ([^{3}H] pravastatin, 5 Ci/mmol) and *n*-[1-^{14}C]butanol ([^{14}C]*n*-butanol, 2 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). [Estradiol-6,7-^{3}H(N)]-estradiol 17β-D-glucuronide ([^{3}H]E17βG, 36.6 Ci/mmol) and D-[1-^{14}C]mannitol ([^{14}C]D-mannitol, 55 mCi/mmol) were purchased from PerkinElmer (Waltham, MA). All other chemicals were commercially available and of reagent grade.

Uptake Index Method and Microdialysis Study. The RUI and brain uptake index (BUI) were calculated according to previously reported uptake index methods. A microdialysis study was performed as previously described. Each experiment was performed once. The details are provided in the Supplemental Data.

Data Analysis. All data, which were obtained from independent biological samples, are expressed as the mean ± SE (n = 3 or 4 rats). An unpaired, two-tailed Student's *t*-test was used to assess the significance of the differences between the means of the two groups. The statistical significance of the differences in the means of the groups was determined using Bartlett's test followed by Dunnett's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

Results and Discussion

The RUI and BUI of [^{3}H]pravastatin and [^{3}H]E17βG in wild-type rats were almost the same as those in *Mdr1a* knockout rats and were lower than the lipophilicity trend line (Fujii et al., 2014) (Fig. 1A, 1B). The results indicate that neither of these compounds is recognized by P-glycoprotein and that influx transport into tissues across the BRB and BBB is less than efflux transport. The RUI and BUI of [^{3}H]pravastatin were significantly reduced by pravastatin (40 mM), digoxin (0.01 mM)—which has a very high affinity for oatp1a4—and probenecid (1 mM), an inhibitor of both the oatp and oat families (Table 1). Also, [^{3}H]E17βG influx across the BRB was significantly reduced by administration of digoxin and probenecid, although the BUI values were unchanged (Supplemental Fig. 1). These results suggested that oatp1a4 plays a role in the blood-to-retina transport of organic anions and that oatp1a4 is involved in both BRB- and BBB-mediated pravastatin influx. Further study is needed to elucidate the reason why no change was observed in the BUI for E17βG.

To investigate the elimination of pravastatin across the BRB, a microdialysis study was performed. The concentration of [^{3}H]pravastatin and [^{14}C]D-mannitol, co-injected as a bulk flow marker, in the dialysate after intravitreous injection decreased bi-exponentially (Fig. 1C). The apparent elimination rate constant (β) during the terminal phase of [^{3}H]pravastatin (0.0179 ± 0.001 min^{-1}) was 1.66-fold greater than that of [^{14}C]D-mannitol (0.0107 ± 0.0005 min^{-1}) (Supplemental Fig. 2). The differences in the β value between [^{3}H]pravastatin and [^{14}C]D-mannitol co-administered with digoxin (0.01 mM) and benzylpenicillin (20 mM), an inhibitor of oat3, were both significantly lower than that of the control (Fig. 1D), whereas choline had no effect. These findings suggest that transporters, including oatp1a4 and oat3, were involved in the retinal clearance of pravastatin, in addition to its elimination from the vitreous

humor via bulk flow and passive diffusion. Thus, the efflux mechanism of pravastatin across the BRB is similar to that across the BBB (Kikuchi et al., 2004).

Moreover, pravastatin is a substrate of ABCC2 (Mrp2), ABCC4 (Mrp4), and ABCG2 (Bcrp) (Yamazaki et al., 1997, Hirano et al., 2005; Uchida et al., 2007). Mrp2 protein is expressed in human retina and RPE (Pelis et al., 2009), but Mrp2 cannot be detected in the mouse inner BRB (Tachikawa et al., 2008) or in rat brain (Ohtsuki et al., 2007) at the mRNA level. Mrp4 is found on the luminal side of the mouse inner BRB (Tagami et al., 2009) and both sides of the BBB (Kusuhara and Sugiyama, 2005). Bcrp is localized to the luminal side of the mouse inner BRB (Asashima et al., 2006) as well as the BBB. Taken together, the ATP-binding cassette (ABC) transporters may be involved in the transport of pravastatin across the BRB and BBB. Based on our results and previous reports, asymmetrical pravastatin transport across both barriers would be expected (Supplemental Fig. 3). It will be important to reveal whether the ABC transporters are involved in the efflux transport of pravastatin across the BRB and BBB in future experiments.

Recently, in a comparison between diabetic and normal rats, increased expression of oatp1a4 mRNA and decreased expression of MRP2 mRNA were found in the liver, as well as a markedly altered pravastatin disposition (Hasegawa et al., 2010). Decreases in renal oat3 function and expression have also been reported in diabetic rats (Phatchawan et al., 2014). It is likely that there are changes in BRB transporters in diabetic rats. The functional and expressional differences in transporters between normal and pathological conditions might be exploitable when treating diabetic retinopathy.

Oatp1a4 falls into the same subfamily as human OATP1A2 (Hagenbuch and Meier, 2004). OATP1A2 and OAT3 are expressed in human retina/choroid at the

mRNA level (Chan et al., 2015) as well as at the BBB (Gao et al., 2015; Cha et al., 2001). In addition, both transporters recognize pravastatin (Takeda et al., 2004; Shirasaka et al., 2010). Therefore, our results support the clinical effects of pravastatin on diabetic retinopathy without the CNS side effects. Pravastatin was transported into the target tissues, retinal endothelial cells, via uptake transporters and showed pharmacological effects, whereas pravastatin taken into brain endothelial cells was transported via efflux transporters prior to reaching the CNS.

In conclusion, pravastatin is transported across the BRB and BBB via uptake and efflux transporters in both directions, and the efflux transporters are dominant, based on the lower values of the RUI and BUI than those expected from the lipophilicity. These findings provide useful information for the development of new approaches to the design of precision pharmaceuticals for the systemic delivery of drugs to retinal vessels without CNS side effects.

Authorship Contributions

Participated in research design: Fujii, Kawazu, Hosoya

Conducted experiments: Fujii, Setoguchi

Contributed new reagents or analytic tools: Fujii

Performed data analysis: Fujii

Wrote or contributed to the writing of the manuscript: Fujii, Kawazu, Hosoya

References

Akanuma SI, Hirose S, Tachikawa M, and Hosoya KI (2013) Localization of organic anion transporting polypeptide (Oatp) 1a4 and Oatp1c1 at the rat blood-retinal barrier. *Fluids Barriers CNS* **10**:29.

Asashima T, Hori S, Ohtsuki S, Tachikawa M, Watanabe M, Mukai C, Kitagaki S, Miyakoshi N, and Terasaki T (2006) ATP-binding cassette transporter G2 mediates the efflux of phototoxins on the luminal membrane of retinal capillary endothelial cells. *Pharm Res* **23**:1235–1242.

Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T, Endou H (2001) Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59:**1277–1286.

Chan T, Zhu L, Madigan MC, Wang K, Shen W, Gillies MC, and Zhou F (2015) Human organic anion transporting polypeptide 1A2 (OATP1A2) mediates cellular uptake of all-trans-retinol in human retinal pigmented epithelial cells. *Br J Pharmacol* **172:**2343–2353.

Fujii S, Setoguchi C, Kawazu K, and Hosoya K (2014) Impact of P-glycoprotein on blood-retinal barrier permeability: comparison of blood-aqueous humor and blood-brain barrier using mdr1a knockout rats. *Invest Ophthalmol Vis Sci* **55**:4650–4658.

Gao B, Vavricka SR, Meier PJ, and Stieger B (2015) Differential cellular expression of organic anion transporting peptides OATP1A2 and OATP2B1 in the human retina and brain: implications for carrier-mediated transport of neuropeptides and neurosteriods in the CNS. *Pflugers Arch* **467**:1481–1493.

Gordon B, Chang S, Kavanagh M, Berrocal M, Yannuzzi L, Robertson C, and Drexler A (1991) The effects of lipid lowering on diabetic retinopathy. *Am J Ophthalmol*

112:385–391.

Hagenbuch B and Meier PJ (2004) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* **447**:653–665.

Hasegawa Y, Kishimoto S, Shibatani N, Inotsume N, Takeuchi Y, and Fukushima S (2010) The disposition of pravastatin in a rat model of streptozotocin-induced diabetes and organic anion transporting polypeptide 2 and multidrug resistance-associated protein 2 expression in the liver. *Biol Pharm Bull* **33**:153–156.

Hirano M, Maeda K, Matsushima S, Nozaki Y, Kusuhara H, and Sugiyama Y (2005) Involvement of BCRP (ABCG2) in the biliary excretion of pitavastatin. *Mol Pharmacol* **68**:800–807.

Hosoya K, Makihara A, Tsujikawa Y, Yoneyama D, Mori S, Terasaki T, Akanuma S, Tomi M, and Tachikawa M (2009) Roles of inner blood-retinal barrier organic anion transporter 3 in the vitreous/retina-to-blood efflux transport of p-aminohippuric acid, benzylpenicillin, and 6-mercaptopurine. *J Pharmacol Exp Ther* **329**:87–93.

Hosoya K and Tomi M (2005) Advances in the cell biology of transport via the inner blood-retinal barrier: establishment of cell lines and transport functions. *Biol Pharm Bull* **28**:1–8.

Katayama K, Ohshima Y, Tomi M, and Hosoya K (2006) Application of microdialysis to evaluate the efflux transport of estradiol 17-beta glucuronide across the rat blood-retinal barrier. J Neurosci Methods 156:249–256.

Kikuchi R, Kusuhara H, Abe T, Endou H, and Sugiyama Y (2004) Involvement of

multiple transporters in the efflux of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors across the blood-brain barrier. *J Pharmacol Exp Ther* **311**:1147–1153.

Kusuhara H and Sugiyama Y (2005) Active efflux across the blood-brain barrier: role of the solute carrier family. *NeuroRx* **2:**73–85

Ohtsuki S, Yamaguchi H, Asashima T, and Terasaki T (2007) Establishing a method to isolate rat brain capillary endothelial cells by magnetic cell sorting and dominant mRNA expression of multidrug resistance-associated protein 1 and 4 in highly purified rat brain capillary endothelial cells. *Pharm Res* **24**:688–694.

Pelis RM, Shahidullah M, Ghosh S, Coca-Prados M, Wright SH, and Delamere NA (2009) Localization of multidrug resistance-associated protein 2 in the nonpigmented ciliary epithelium of the eye. *J Pharmacol Exp Ther* **329**:479–485.

Phatchawan A, Chutima S, Varanuj C, and Anusorn L (2014) Decreased renal organic anion transporter 3 expression in type 1 diabetic rats. *Am J Med Sci* **347**:221–227.

Saheki A, Terasaki T, Tamai I, and Tsuji A (1994) In vivo and in vitro blood-brain barrier transport of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. *Pharm Res* **11**:305–311.

Shirasaka Y, Suzuki K, Nakanishi T, and Tamai I (2010) Intestinal absorption of HMG-CoA reductase inhibitor pravastatin mediated by organic anion transporting polypeptide. *Pharm Res* **27**:2141–2149.

Sugiyama D, Kusuhara H, Shitara Y, Abe T, Meier PJ, Sekine T, Endou H, Suzuki H, and Sugiyama Y (2001) Characterization of the efflux transport of

17beta-estradiol-D-17beta-glucuronide from the brain across the blood-brain barrier. *J Pharmacol Exp Ther* **298:**316–322.

Tachikawa M, Toki H, Tomi M, and Hosoya K (2008) Gene expression profiles of ATP-binding cassette transporter A and C subfamilies in mouse retinal vascular endothelial cells. *Microvasc Res* **75**:68–72.

Tagami M, Kusuhara S, Honda S, Tsukahara Y, and Negi A (2009) Expression of ATP-binding cassette transporters at the inner blood-retinal barrier in a neonatal mouse model of oxygen-induced retinopathy. *Brain Res* **1283**:186–193.

Takeda M, Noshiro R, Onozato ML, Tojo A, Hasannejad H, Huang X-L, Narikawa S, and Endou H (2004) Evidence for a role of human organic anion transporters in the muscular side effects of HMG-CoA reductase inhibitors. *Eur J Pharmacol* **483**:133–138.

Uchida Y, Kamiie J, Ohtsuki S, and Terasaki T (2007) Multichannel liquid chromatography-tandem mass spectrometry cocktail method for comprehensive substrate characterization of multidrug resistance-associated protein 4 transporter. *Pharm Res* **24:**2281–2296.

Yamazaki M, Akiyama S, Ni'inuma K, Nishigaki R, and Sugiyama Y (1997) Biliary excretion of pravastatin in rats: contribution of the excretion pathway mediated by canalicular multispecific organic anion transporter. *Drug Metab Dispos* **25**:1123–1129.

Figure Legend

Fig. 1. A and B, Correlation of the retinal uptake index (RUI, A) and brain uptake index (BUI, B) with the Log $D_{7.4}$ of pravastatin (Log $D_{7.4}$: −0.67) and E17βG (Log $D_{7.4}$: −2.03) in wild-type (closed symbols) and *Mdr1a* knockout (open symbols) rats. The Log $D_{7.4}$ value of each compound was calculated using ACD/Percepta (Advanced Chemistry Development). The line represents the lipophilicity trend line in wild-type rats using the data of D-mannitol, thiourea, and progesterone, which are expected to permeate by passive diffusion. The line represents the linear regression curve according to the linear least-squares methods for the three compounds in wild-type rats. RUI = 69.4 × exp (0.493 × Log $D_{7.4}$) (r^2 = 0.993) (A) and BUI = 16.9 × exp (0.496 × Log $D_{7.4}$) (r^2 = 0.998) (B) (Fujii et al., 2014).

C, Time-profile of [^{3}H]pravastatin and [^{14}C]D-mannitol in the vitreous humor after their intravitreous injection into rats. Closed squares and open squares represent the concentrations of [^{3}H]pravastatin and [^{14}C]D-mannitol in the dialysate, respectively.

D, Inhibitory effect on the difference in the elimination rate constants (β) between [^{3}H]pravastatin and [^{14}C]D-mannitol during the terminal phase. Percentage of control was calculated as follows: {β value of [^{3}H]pravastatin − β value of [^{14}C]D-mannitol in the presence of inhibitor}/{β value of [^{3}H]pravastatin − β value of [^{14}C]D-mannitol in the absence of inhibitor} × 100. The statistical significance of the differences in the means of the groups was determined using Bartlett's test followed by Dunnett's multiple comparison test. *P < 0.05 and **P < 0.01, significantly different from control.

Each column or symbol represents the mean ± SE (n = 3 or 4 rats).

Table

TABLE 1

The effect of compounds on the retinal and brain uptake index for [^{3}H]pravastatin in wild-type rats

	Uptake index (%)		Percentage of control (%)	
Inhibitor	Retina	Brain	Retina	Brain
Pravastatin				
Control	2.58 ± 0.52	0.485 ± 0.031	100.0 ± 20.3	100.0 ± 6.3
40 mM pravastatin	1.38 ± 0.22*	0.263 ± 0.024**	53.4 ± 8.4	54.2 ± 5.0
0.01 mM digoxin	1.20 ± 0.24*	0.224 ± 0.024**	46.6 ± 9.2	46.1 ± 4.9
0.3 mM E17βG	1.21 ± 0.16*	0.361 ± 0.079	46.8 ± 6.1	74.5 ± 16.2
1 mM probenecid	1.36 ± 0.14*	0.204 ± 0.025**	52.8 ± 5.4	42.1 ± 5.2

[^{3}H]Pravastatin (10 μCi/rat), with [^{14}C]*n*-butanol (0.1 μCi/rat) as the highly diffusible reference, injected into the carotid artery in the absence (control) and presence of inhibitors. Values represent means ± SE (n = 3 or 4 rats). *P < 0.05 and **P < 0.01, significantly different from the control. E17βG, estradiol 17-β glucuronide.

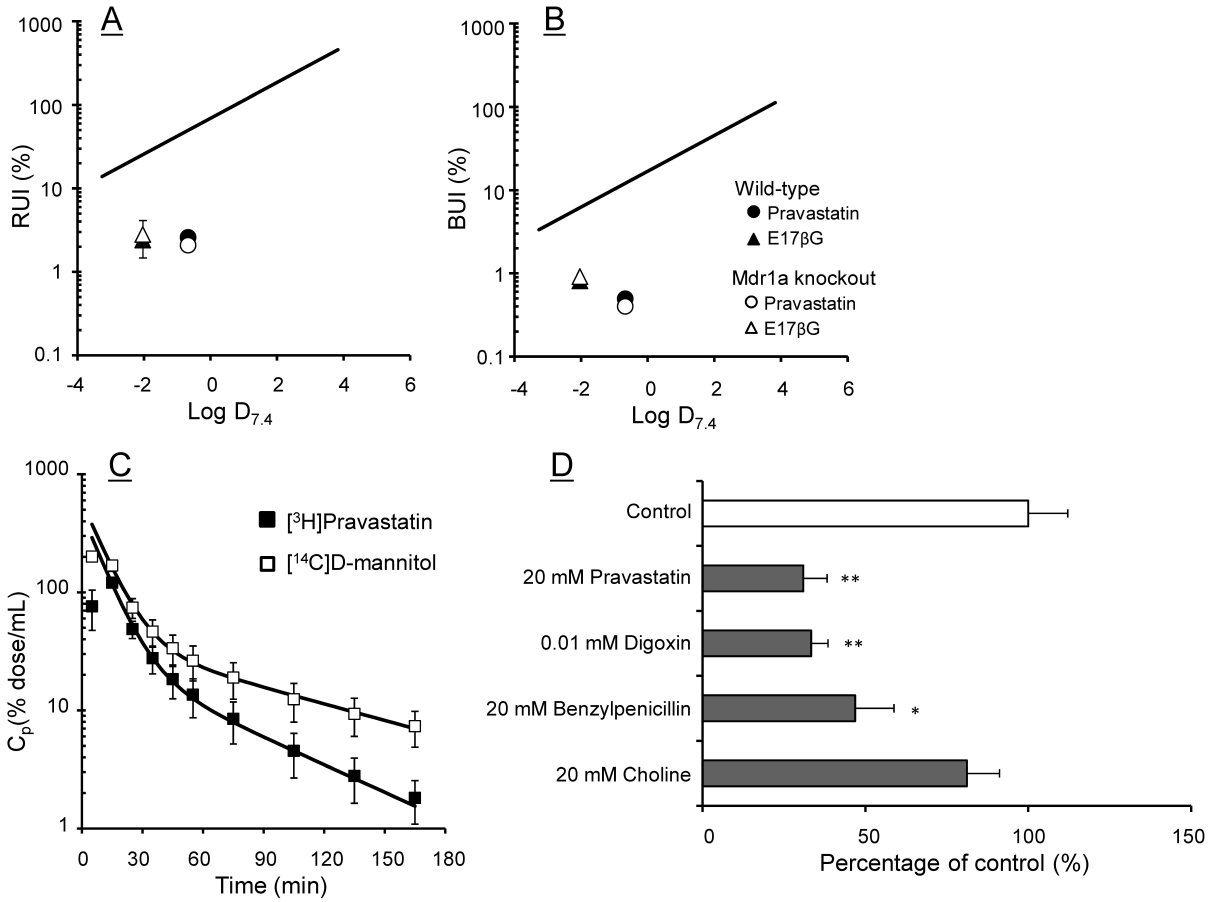
A
RUI (%)
1000
100
10
1
0.1
-4
-2
0
2
4
6
Log D7.4
B
BUI (%)
Wild-type
Pravastatin
E17βG
Mdr1a knockout
Pravastatin
E17βG
C
Cp(% dose/mL)
[3H]Pravastatin
[14C]D-mannitol
0
30
60
90
120
150
180
Time (min)
D
Control
20 mM Pravastatin
0.01 mM Digoxin
20 mM Benzylpenicillin
20 mM Choline
**
**
*
0
50
100
150
Percentage of control (%)

Figure 1