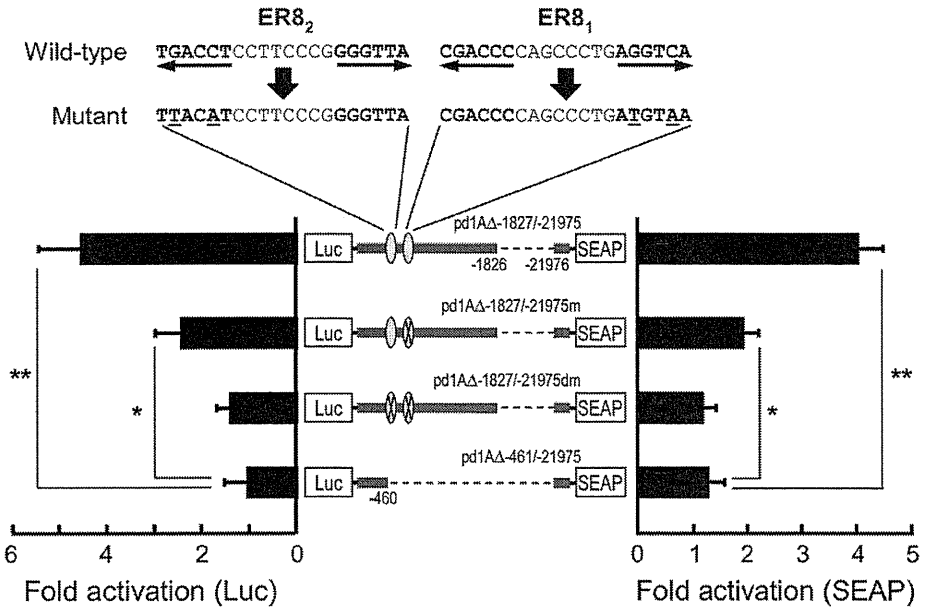

Fig. 5. Role of two ER8 motifs in the hLXRα-mediated transcription of h*CYP1A* genes. Schematic structures of the reporter plasmids used are shown in the middle. X indicates mutation. Wild-type and mutated sequences of ER8$_1$ and ER8$_2$ are also shown above. Reporter assays were performed as in Figs. 1 and 3. Data are shown as the ratio of reporter activities of hLXRα-transfected/24HC-treated cells against those of mock-transfected/vehicle-treated cells for each reporter construct (the mean ± S.D. (n = 3–4)). *P < 0.05, **P < 0.01 versus pd1AΔ-461/-21975 (one-way ANOVA followed by Dunnett's post hoc test).

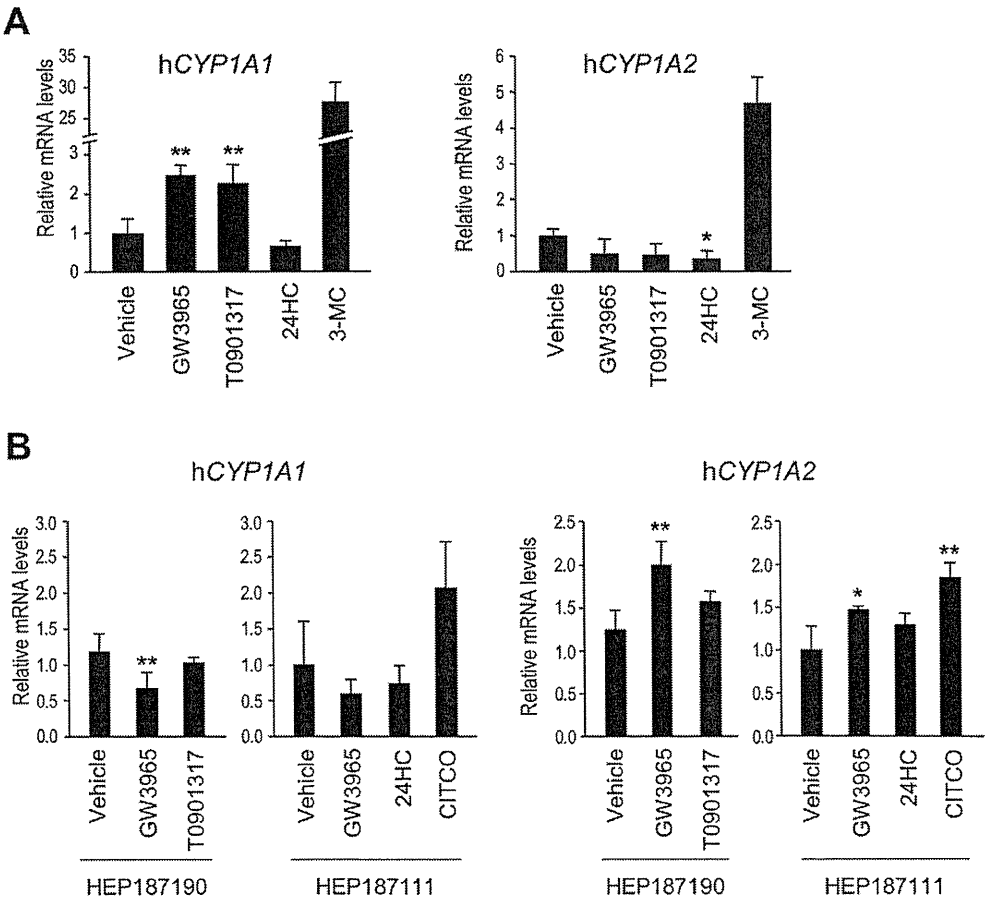

Fig. 6. Changes in mRNA levels of h*CYP1A1* and h*CYP1A2* in HuH-7 cells and primary human hepatocytes. Relative mRNA levels of h*CYP1A1*, h*CYP1A2* and *GAPDH* in HuH-7 cells (A) and primary human hepatocytes (B) were determined in duplicate or triplicate as described in Section 2.5. h*CYP1A1* and h*CYP1A2* mRNA levels were normalized with those of *GAPDH*. In A, statistical analyses were performed only among the cells treated with vehicle or LXRα ligands. *P < 0.05, **P < 0.01 versus vehicle-treated group (one-way ANOVA followed by Dunnett's post hoc test).

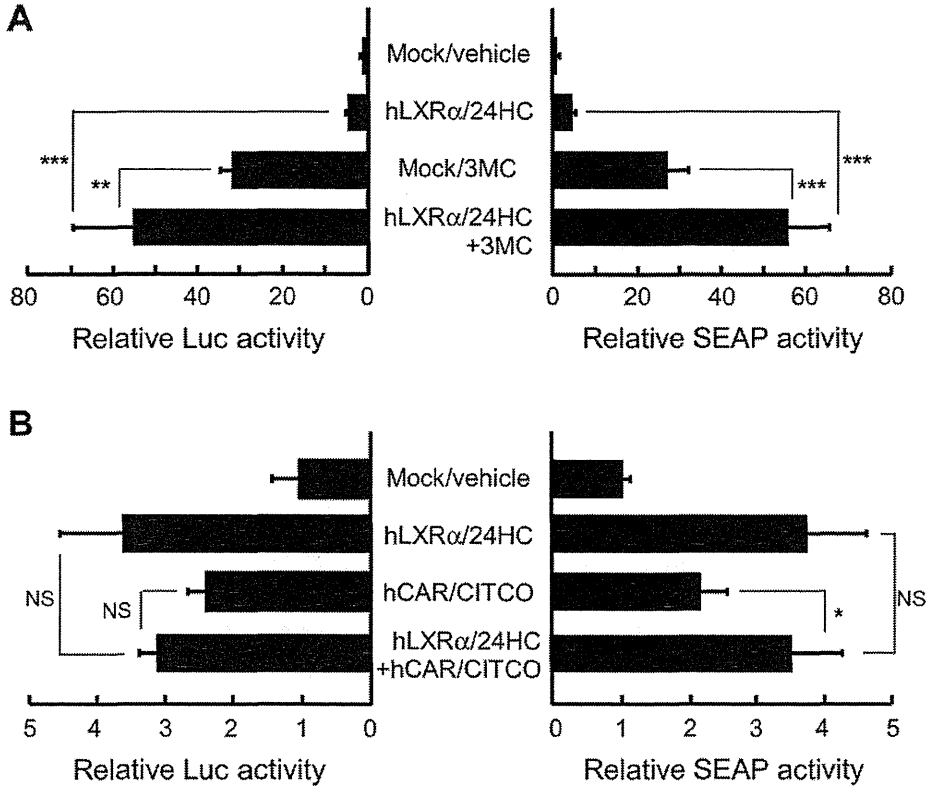

Fig. 7. Co-regulation of h*CYP1A* genes by LXRα, AhR and CAR. (A) HuH-7 cells (3×10^4 cells in 48-well plate) were transfected with pd-1A1/1A2 (300 ng), phRL-tk (10 ng) and either control or hLXRα expression plasmid (20 ng). Eight hours after transfection, cells were treated with 10 μM 24HC, 1 μM 3MC or vehicle (0.2% DMSO) for 40 h. (B) HuH-7 cells (3×10^4 cells in 48-well plate) were transfected with pd1AΔ-1827/-21975 (300 ng), phRL-tk (10 ng) and either control, hLXRα or hCAR expression plasmid (20 ng). Total amount of expression plasmid was adjusted to 40 ng with control plasmid. Eight hours after transfection, cells were treated with vehicle (0.2% DMSO), 10 μM 24HC, 0.3 μM CITCO or both ligands for 40 h. Reporter activities were determined and relative reporter activities are shown as in Fig. 1. Data are shown as the mean ± S.D. ($n = 4$). The significance of differences between the groups indicated was analyzed with one-way ANOVA followed by Bonferroni's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. NS, not significant.

for the hLXRα-mediated expression of both h*CYP1A1* and h*CYP1A2*. Yet, difference is observed for the hLXRα binding sequence between their and our studies. We have identified two ER8 motifs as LXRα binding sites while Shibahara et al. demonstrated that LXRα binds to a DR4 motif located in a region from −452 to −467 in EMSAs (Shibahara et al., 2011). Because the DR4 motif and $ER8_2$ share a common AGGTCA nuclear receptor binding half-site, we cannot rule out a possibility that both motifs function as LXRα-responsive elements in hepatocytes. In the meantime, the mutation of the DR4 motif did not completely eliminate LXRα-response of the h*CYP1A1* reporter construct (Shibahara et al., 2011). Because this mutated reporter construct still contained $ER8_1$, $ER8_1$ might mediate the residual LXRα-responsiveness.

LXRα is expected as a potential target of anti-arteriosclerotic drugs because it regulates the transcription of several genes involved in reverse cholesterol transport (Repa and Mangelsdorf, 2002). Thus, combinational use of these LXRα-activating drugs with drugs that are metabolized by CYP1A1 and/or CYP1A2 such as clozapine and theophilline could lead to drug-drug interactions. Recently, a proton pump inhibitor lansoprazole has been reported as an LXRα ligand (Cronican et al., 2010). In our dual-reporter system, this drug transactivated both h*CYP1A1* and h*CYP1A2* expression (Yoshinari et al., 2008). Since lansoprazole is long known to induce CYP1A1 and CYP1A2 through the activation of AhR probably without direct binding to the receptor (Curi-Pedrosa et al., 1994; Kikuchi et al., 1996; Krusekopf et al., 2003), this drug might be a dual activator of AhR and LXRα. Nonetheless, it is worth considering a possibility of LXRα-associated drug-drug interactions through inducing CYP1A enzymes in future studies and drug development processes.

Physiological relevance of the LXRα-mediated regulation of h*CYP1A1* and h*CYP1A2* expression remains unclear. Recombinant hCYP1A2 has been reported to catalyze the conversion of cholesterol into 4β-hydroxycholesterol, an LXRα ligand (Honda et al., 2011). Both CYP1A1 and CYP1A2 metabolize endogenous cholesterol-derived compounds such as estrogens (Badawi et al., 2001; Lee et al., 2003). Intriguingly, LXRα is reported to regulate the expression of human SLUT1E1 (Gong et al., 2007), which metabolizes estrogens into the corresponding sulfates. Thus, the LXRα-mediated regulation of h*CYP1A1* and h*CYP1A2* might be associated with homeostasis of cholesterol and its related compounds as in the case of estrogens and SULT1E1.

Conflict of interest statement

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2012.09.021.

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Green Tea Ingestion Greatly Reduces Plasma Concentrations of Nadolol in Healthy Subjects

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This study aimed to evaluate the effects of green tea on the pharmacokinetics and pharmacodynamics of the β-blocker nadolol. Ten healthy volunteers received a single oral dose of 30 mg nadolol with green tea or water after repeated consumption of green tea (700 ml/day) or water for 14 days. Catechin concentrations in green tea and plasma were determined. Green tea markedly decreased the maximum plasma concentration (C_{max}) and area under the plasma concentration–time curve (AUC_{0-48}) of nadolol by 85.3% and 85.0%, respectively ($P < 0.01$), without altering renal clearance of nadolol. The effects of nadolol on systolic blood pressure were significantly reduced by green tea. [^{3}H]-Nadolol uptake assays in human embryonic kidney 293 cells stably expressing the organic anion–transporting polypeptides OATP1A2 and OATP2B1 revealed that nadolol is a substrate of OATP1A2 (Michaelis constant (K_m) = 84.3 μmol/l) but not of OATP2B1. Moreover, green tea significantly inhibited OATP1A2-mediated nadolol uptake (half-maximal inhibitory concentration, IC_{50} = 1.36%). These results suggest that green tea reduces plasma concentrations of nadolol possibly in part by inhibition of OATP1A2-mediated uptake of nadolol in the intestine.

Green tea catechins, flavonoids mainly contained in green tea (*Camellia sinensis*), are reported to have diverse effects on health, such as prevention of cancer or cardiovascular diseases.[1,2] Because green tea is consumed worldwide and its extract is one of the most common herbal supplements,[3] the likelihood of concomitant use of green tea and medicines is thought to be increasing. Most dietary food vs. drug interactions can be attributed to the inhibition or induction of cytochrome P450 (CYP) 3A enzymes and/or drug transporters such as P-glycoprotein (P-gp, *ABCB1*) and organic anion–transporting polypeptides (OATPs) expressed in enterocytes.[4,5] With respect to CYP-mediated interactions, green tea has been considered to cause negligible or minor interactions with coadministered drugs in humans.[6,7] With regard to the possibility of drug transporter–mediated interactions, previous *in vitro* studies suggested that catechins, including (−)-epigallocatechin-3-gallate (EGCG), inhibit P-gp.[8,9] Moreover, Roth *et al.* showed that EGCG and (−)-epicatechin-3-gallate (ECG) inhibit OATP1A2- and OATP2B1-mediated uptake of estrone-3-sulfate in cells expressing these transporters.[10] However, whether green tea affects the pharmacokinetics of substrate drugs of P-gp and/or OATPs *in vivo* remains to be elucidated.

Nadolol, a nonselective β-adrenoceptor blocker, is not metabolized by drug-metabolizing enzymes such as CYP3A, and the absorbed fraction of nadolol is predominantly excreted unchanged into the urine.[11] In line with a previous *in vitro* study suggesting that nadolol is transported out of cells by P-gp,[12] we recently found that the coadministration of itraconazole, a potent P-gp inhibitor, significantly increased nadolol plasma concentrations in healthy subjects.[13] In addition, nadolol has been reported to be an OATP1A2 substrate *in vitro*,[14] and because of poor permeability of nadolol across the cellular membrane,[15] influx carriers may contribute to the intestinal absorption of nadolol. A previous clinical study showed that single ingestion of grapefruit juice slightly decreased the area under the plasma concentration–time curve (AUC) of nadolol,[13] although the concentration of naringin, a major constituent of grapefruit juice responsible for the *in vivo* inhibition of OATP1A2 and OATP2B1, was about one-third lower than that in other studies.[16,17] These results suggest that consumption of green tea may lead to changes in the pharmacokinetics of nadolol by inhibition of P-gp- or OATP-mediated nadolol transport in humans.

The current study was conducted to evaluate the effects of green tea on the pharmacokinetic and pharmacodynamic (PD) actions

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of nadolol in healthy volunteers and to gain insight about possible mechanisms. Considering that green tea is a habitual beverage, and that most clinical studies investigating PD effects of green tea have been carried out under chronic exposure to green tea, subjects received nadolol with green tea or water after repeated ingestion of green tea or water for 14 days in a randomized two-way crossover design. Plasma concentrations and urinary excretion of nadolol were determined. Moreover, PD effects were measured by changes in systolic (SBP) and diastolic (DBP) blood pressures and pulse rate (PR). In addition, *in vitro* experiments were undertaken to clarify the involvement of OATP1A2 and OATP2B1 in the cellular accumulation of nadolol and the effects of EGCG and green tea on OATP-mediated nadolol transport.

RESULTS

Pharmacokinetics of nadolol and catechins

All participants completed the study without adverse events. The green tea used in this study contained (−)-epicatechin (EC), (−)-epigallocatechin (EGC), ECG, and EGCG at ~80, 240, 130, and 460 μg/ml, respectively. Therefore, during the green tea phase, the daily intakes of EC, EGC, ECG, and EGCG were 56, 168, 91, and 322 mg, respectively, brought about by drinking green tea (700 ml/day).

Green tea markedly reduced the plasma concentrations of nadolol (**Figure 1** and **Table 1**). The maximum plasma concentration (C_{max}) and AUC_{0-48} of nadolol were decreased by 85.3% (range: 4.6–50.4% of water phase, $P = 0.007$) and 85.0% (range: 6.8–37.6% of water phase, $P < 0.001$), respectively. The C_{max} of nadolol occurred at an earlier time point during the green tea phase (median: 2.0 h) than during the water phase (median: 3.0 h, $P = 0.013$). The individual AUC_{0-48} value (water phase) was significantly correlated with the decrease in nadolol AUC_{0-48} ($r = 0.977$, $P < 0.0001$). The amount of nadolol excreted in urine in 48 h (A_e) was reduced by 81.6% (range: 5.7–43.1% of water phase, $P < 0.001$) in the green tea phase (**Figure 2**).

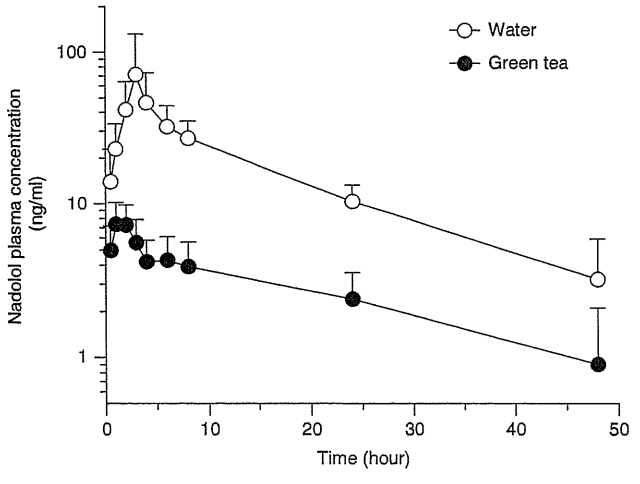

Figure 1 Plasma concentrations (arithmetic means ± SD) of nadolol after the oral administration of 30 mg nadolol with 700 ml (350 ml × 2 times at 0 and 0.5 h) of green tea or water following pretreatment with green tea (filled circles) or water (open circles) for 14 days in 10 healthy volunteers.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ Green tea catechins have been reported to inhibit several drug transporters such as OATPs and P-glycoprotein *in vitro*. Nadolol, a nonmetabolized β-adrenoceptor blocker, is a possible substrate of OATP1A2 and P-glycoprotein.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ This study evaluated whether green tea affects the pharmacokinetics and pharmacodynamics of nadolol in healthy subjects. The effects of green tea and green tea catechins on OATP-mediated nadolol uptake were also investigated.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ Green tea decreased plasma concentrations, urinary elimination, and blood pressure–lowering effects of nadolol without affecting its renal clearance. Nadolol is a substrate of OATP1A2 but not of OATP2B1. The green tea catechin EGCG and green tea itself inhibited OATP1A2-mediated nadolol uptake into cells.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ These findings suggest that concomitant use of OATP1A2 substrate drugs with green tea or green tea catechins may cause significant pharmacokinetic interactions, possibly mediated by an inhibition of OATP1A2 in humans. Patients treated with nadolol should avoid taking green tea.

Green tea had no significant effect on the renal clearance (CL_R) of nadolol (**Table 1**).

In the green tea phase, plasma concentrations of EGCG, ECG, EC, and EGC were measured after the coadministration of nadolol and green tea (**Figure 3**). The geometric means (90% confidence intervals, CIs) of the C_{max} of EGCG and ECG were 141.2 (111.5–171.0) and 49.4 (30.0–68.7) ng/ml, with $AUC_{0-\infty}$ values

Table 1 Pharmacokinetic parameters of nadolol after oral administration of 30 mg of nadolol with 700 ml (350 ml × 2 times at 0 and 0.5 h) of green tea or water following pretreatment with green tea (700 ml/day) or water for 14 days in 10 healthy volunteers

	Water	Green tea	% Of water phase
C_{max} (ng/ml)	55.7 (24.8–86.5)	8.2 (6.7–9.6)**	14.7 (8.2–21.1)
t_{max} (h)	3.0 (2.0–4.0)	2.0 (0.5–3.0)*	—
AUC_{0-48} (h·ng/ml)	708.9 (569.8–848.0)	106.6 (67.8–145.5)***	15.0 (10.5–19.6)
A_e (% of dose)	11.4 (8.5–14.3)	2.1 (1.5–2.6)***	18.4 (13.1–23.8)
CL_R (l/h)	4.9 (4.4–5.4)	5.8 (4.8–6.8)	118.5 (91.9–145.2)

Values are expressed as the geometric means (90% confidence intervals), except for t_{max} (the median and range).

A_e, amount excreted into urine; AUC, area under the plasma concentration–time curve; C_{max}, maximum plasma concentration; CL_R, renal clearance; t_{max}, time to reach C_{max}. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

of 467.0 (316.0–618.0) and 160.6 (124.0–197.1) h·ng/ml, respectively. C_{max} of EGC was 37.8 (34.7–40.9) ng/ml and AUC_{0-8} was 106.6 (75.3–137.8) h·ng/ml. The apparent elimination half-life ($t_{1/2}$) values of EGCG and ECG were 1.6 (1.3–1.8) and 1.5 (1.3–1.8) h, respectively. Plasma concentrations of EC were below the lower limit of quantitation in most of the plasma samples.

PDs of nadolol

The baseline (predose) values of PR, SBP, and DBP were 67 ± 9 beats/min, 114 ± 7 mmHg, and 71 ± 8 mmHg (arithmetic means ± SD), respectively. No statistically significant differences were observed in the baseline values of these parameters between the green tea and water phases. Changes in PR, SBP, and DBP after nadolol administration were expressed as percentage decrease from baseline, as shown in **Figure 4**. In the water phase, nadolol lowered PR, SBP, and DBP, with maximum decreases of 20, 12, and 11%, respectively, based on arithmetic means. Coadministration of green tea tended to reduce the PD responses to nadolol in all parameters. In particular, green tea significantly suppressed the SBP-lowering effect of nadolol as evaluated by the area under the effect-time curve ($AUEC_{0-48}$) for SBP ($P = 0.042$), although large interindividual variability was also observed.

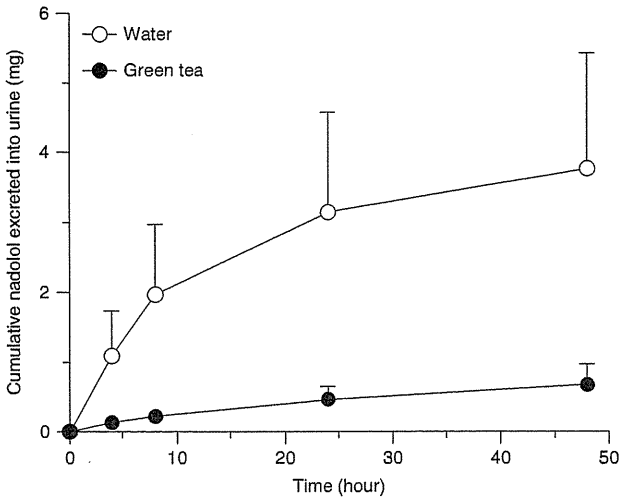

Figure 2 Urinary excretion of nadolol after oral administration of 30 mg nadolol with 700 ml (350 ml × 2 times at 0 and 0.5 h) of green tea or water following pretreatment with green tea or water for 14 days in 10 healthy volunteers (arithmetic means + SD). Mean cumulative amount of nadolol excreted in urine is shown during 48 h in green tea phase (filled circles) or water phase (open circles).

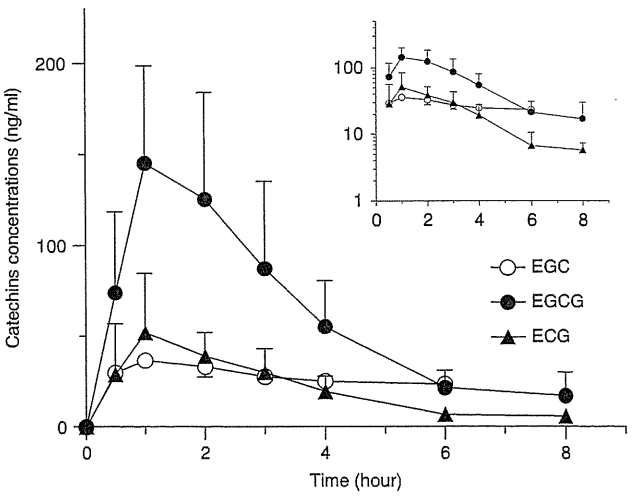

Figure 3 Plasma concentrations (arithmetic means ± SD) of (−)-epigallocatechin (EGC, open circles), (−)-epigallocatechin gallate (EGCG, filled circles), and (−)-epicatechin gallate (ECG, filled triangles) after oral administration of 30 mg nadolol with 700 ml (350 ml × 2 times at 0 and 0.5 h) of green tea following pretreatment with green tea for 14 days in 10 healthy volunteers. Insert depicts the same data on a semilogarithmic scale.

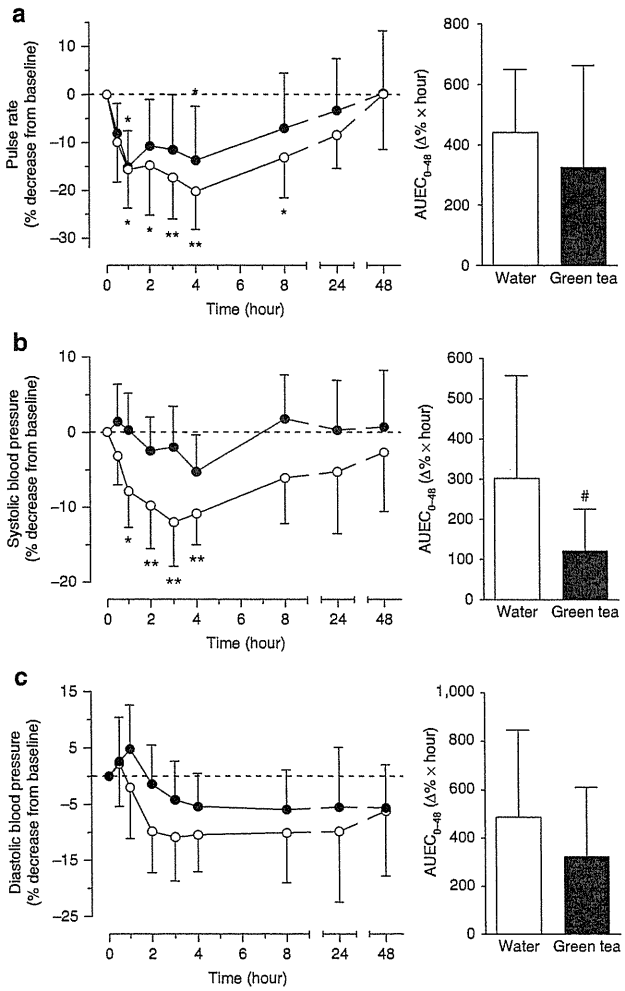

Figure 4 Pharmacodynamic responses and area under the effect-time curve ($AUEC_{0-48}$) of nadolol in the green tea phase (filled circles) or water phase (open circles) in 10 healthy volunteers. Percentage changes in (**a**) pulse rate, (**b**) systolic blood pressure, and (**c**) diastolic blood pressure from baseline are represented as arithmetic means ± SD after the oral administration of 30 mg nadolol with 700 ml (350 ml × 2 times at 0 and 0.5 h) of green tea or water following pretreatment with green tea or water for 14 days. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. baseline. $^{\#}P < 0.05$ vs. water phase.

Cellular uptake of nadolol by OATP1A2 and OATP2B1

To address whether nadolol is a substrate for OATP1A2 or OATP2B1, the uptake of nadolol was investigated using OATP1A2- and OATP2B1-expressing human embryonic kidney (HEK) 293 cells and vector-transfected cells (vector control, VC). The uptake of the prototypical substrate [^{3}H]-sulfobromophthalein (BSP) was significantly higher in OATP1A2-expressing (2.0-fold increase, $P = 0.001$) and OATP2B1-expressing (6.3-fold increase, $P = 0.014$) cells as compared with the respective VC cells based on the arithmetic mean values (**Figure 5**). The uptake of [^{3}H]-nadolol in OATP1A2-expressing cells was 51.7- (10 μmol/l) and 11.5-fold (100 μmol/l) higher than that in VC cells ($P < 0.01$, **Figure 5**). Kinetic analysis revealed that the OATP1A2-mediated uptake of nadolol was saturable, with Michaelis constant (K_m) and maximum rate (V_{max}) values of 84.3 ± 1.0 μmol/l and 332.8 ± 125.5 pmol/min/mg protein (arithmetic mean ± SEM), respectively (data not shown). By contrast, OATP2B1-mediated uptake of [^{3}H]-nadolol was not observed.

Inhibition of nadolol uptake by green tea catechins

[^{3}H]-Nadolol accumulation in OATP1A2-expressing cells was significantly reduced by coincubation with known OATP1A2 inhibitors, including naringin, BSP, and verapamil, at concentrations of 100 μmol/l (**Figure 6a**). In addition, EGCG inhibited OATP1A2-mediated nadolol uptake in a concentration-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) value of 37.3 ± 5.9 μmol/l (**Figure 6b**). Furthermore, coincubation with the green tea used in the current clinical study also showed concentration-dependent inhibition of OATP1A2-mediated nadolol uptake, with an IC_{50} value of 1.36 ± 0.04% (i.e., 50% inhibition at a 1.36:100 dilution of the green tea; **Figure 6c**).

DISCUSSION

In this study, concomitant administration of nadolol with green tea after repeated ingestion of green tea for 14 days resulted in significant changes in pharmacokinetics and the blood pressure–lowering effect of nadolol in healthy volunteers. Plasma concentrations and urinary excretion of nadolol were markedly reduced by 85.0% and 81.6%, respectively, in the green tea phase as compared with the water phase, whereas no significant difference was observed in the mean CL_R of nadolol between the two study phases. Subsequent *in vitro* experiments using [^{3}H]-nadolol revealed that OATP1A2, but not OATP2B1, transported nadolol into cells in a time- and concentration-dependent manner. Furthermore, EGCG and the green tea used in this clinical study potently inhibited OATP1A2-mediated uptake of nadolol. These findings show that green tea ingestion greatly decreases plasma concentrations of nadolol in humans,

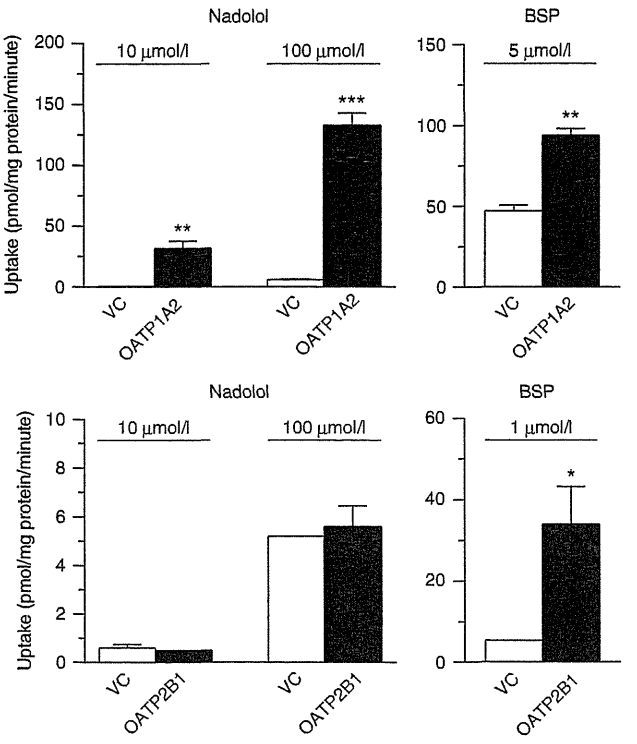

Figure 5 *In vitro* uptake assays of [^{3}H]-nadolol and [^{3}H]-sulfobromophthalein (BSP) in organic anion–transporting polypeptide (OATP)1A2- and OATP2B1-stably expressing human embryonic kidney (HEK) cells. Data are presented as arithmetic means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vector control (VC).

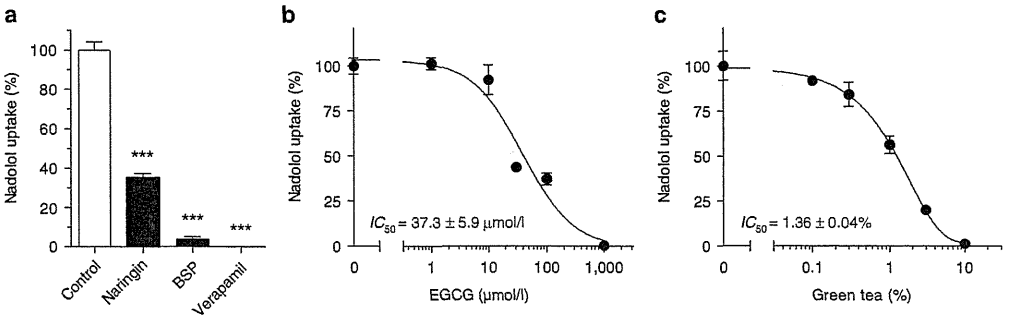

Figure 6 Inhibition of organic anion–transporting polypeptide (OATP)1A2-mediated [^{3}H]-nadolol uptake by OATP1A2 inhibitors, the green tea catechin (–)-epigallocatechin gallate (EGCG), and green tea in OATP1A2-stably expressing human embryonic kidney (HEK) cells. (**a**) The inhibitory effects of naringin, [^{3}H]-sulfobromophthalein (BSP), and verapamil at concentrations of 100 μmol/l and concentration-dependent inhibition by (**b**) EGCG and (**c**) green tea on OATP1A2-mediated nadolol uptake are shown (100 μmol/l, 5 min). The data are expressed as percentage of control (uptake without inhibitors) and presented as arithmetic means ± SEM. ***$P < 0.001$ vs. control.

presumably in part by inhibition of OATP1A2-mediated intestinal absorption of nadolol.

Previous investigations showed that fruit juices, including apple, grapefruit, and orange juice, affect disposition of OATP substrates such as fexofenadine, talinolol, etoposide, and aliskiren.[5,11,16,18,19] Our results are similar to these OATP-mediated drug–fruit juice interactions. For instance, in the case of talinolol, which is reported to be a substrate of OATP1A2, OATP2B1, and P-gp,[20] ingestion of grapefruit juice resulted in a significant decrease in talinolol bioavailability, suggesting that the inhibition of influx transporters such as OATPs by grapefruit juice is the relevant underlying mechanism.[16] In line with this, we recently found that a single oral administration of green tea extract significantly decreased plasma concentration of nadolol in rats, suggesting an acute effect of green tea on oral bioavailability of nadolol.[21] Although an enhanced efflux of nadolol to the intestinal lumen as a result of the induction of the efflux pump in enterocytes by repeated exposure to green tea cannot be excluded, no studies have indicated, to our knowledge, an inductive effect of green tea catechins on efflux transporters. The current study demonstrated the effect of repeated intake of green tea on nadolol kinetics. Whether a single ingestion of green tea affects nadolol pharmacokinetics in humans will need to be addressed.

Although a recent study by quantitative analysis based on liquid chromatography–tandem mass spectrometry failed to detect the expression of OATP1A2 in human jejunum and ileum,[22] other studies have reported that OATP1A2 and OATP2B1 are expressed in the intestinal epithelium.[23,24] Our *in vitro* experiments revealed that nadolol is a substrate of OATP1A2, with a K_m value of 84.3 μmol/l, which extends a previous report using OATP1A2-expressing *Xenopus* oocytes and a single concentration of nadolol (50 μmol/l).[14] In addition, typical OATP1A2 inhibitors such as BSP, naringin, and verapamil significantly inhibited the cellular accumulation of nadolol by OATP1A2-expressing cells in our study. By contrast, it is of interest that nadolol was not transported by OATP2B1. These properties of nadolol are comparable with those observed in recent results for aliskiren and mirabegron, which have been reported to be substrates of OATP1A2 but not of OATP2B1.[25,26] Assuming that OATP1A2 indeed functions as an influx transporter in enterocytes, it could contribute to the intestinal absorption of nadolol.

Our recent study in rats showed that concomitant administration of nadolol with EGCG significantly decreases plasma concentrations of nadolol.[21] In line with this, we observed that EGCG and the green tea used in the current clinical study inhibited OATP1A2-mediated nadolol uptake in a dose-dependent manner. The IC_{50} of EGCG (37.3 μmol/l) for inhibition of OATP1A2-mediated nadolol uptake was comparable with a previously reported value (54.8 μmol/l) using [^{3}H]-estrone-3-sulfate as an OATP1A2 substrate.[10] The concentration of EGCG in green tea was found to be 1,000 μmol/l (460 μg/ml). Therefore, it is possible that EGCG contained in the green tea could inhibit OATP1A2-mediated nadolol uptake in enterocytes, even if the green tea is diluted by up to 27-fold by the gastrointestinal fluid. Considering the fact that the green tea additionally contained ~300 μmol/l of ECG, which inhibits OATP1A2-mediated [^{3}H]-estrone-3-sulfate uptake more potently than EGCG,[10] the IC_{50} value of green tea (1.36%, i.e., containing 13.6 μmol/l of EGCG and 4.1 μmol/l of ECG) appears to be sufficient for inhibition of OATP1A2-mediated nadolol uptake in humans. Taken together, these findings suggest that the inhibition of OATP1A2 by green tea catechins in enterocytes is one of the possible mechanisms underlying the green tea–nadolol interaction in humans.

Due to the potential preventive effect on cancer and cardiovascular diseases, green tea and its polyphenolic components, the catechins, have attracted increasing attention in recent years.[1,2] Green tea catechins are generally taken as brewed green tea or supplements such as green tea extract or EGCG capsules. A typical cup of green tea, brewed with 2.5 g of tea leaves in 250 ml of hot water, contains 207–293 mg of catechins,[2] and as a dietary supplement, EGCG is administered at a daily dose ranging from 400–800 mg in clinical settings.[6,7,27] In our study, the content of EGCG in 700 ml of green tea was calculated to be 322 mg. Thus, the dose appears to be clinically relevant. To better understand OATP-mediated green tea–drug interactions, further pharmacokinetic studies investigating the interaction between green tea and other OATP substrates are required. In particular, because EGCG and ECG have also been reported to inhibit OATP2B1-mediated transport,[10] pharmacokinetics of dual substrates of both OATP1A2 and OATP2B1, such as fexofenadine, may be more profoundly affected by green tea ingestion.

In conclusion, we observe that green tea ingestion leads to a significant reduction in plasma concentrations and blood pressure–lowering effects of nadolol in healthy volunteers. Although further studies are required, e.g., those investigating effects after a single administration of green tea and those in patients, the current data suggest that patients treated with nadolol should avoid drinking green tea. Inhibition of intestinal OATP1A2 may contribute to this interaction, although the involvement of other mechanisms, such as upregulation of intestinal apical efflux transporters, e.g., P-gp, cannot be excluded.

METHODS

Subjects. Ten healthy Japanese volunteers (eight males, two females; age: 20–30 years; body mass index: 18.3–23.9 kg/m^2) participated in the study after giving written informed consent. The volunteers were ascertained to be healthy by evaluation of medical history, physical examination, and routine laboratory tests before entering the study. None of the volunteers used continuous medication, nor was any volunteer a smoker. The study procedures were in accordance with the ethical standards of the Declaration of Helsinki. The number of volunteers was estimated to be sufficient to detect a 50% change in the $AUC_{0-\infty}$ of nadolol with a power of 80% (α-level 5%) on the basis of a previous study.[13] The study protocol was approved by the ethics committee of the Fukushima Medical University, and this trial was registered at the UMIN Clinical Trials Registry as UMIN000006064.

Study design. A randomized crossover study was carried out in two phases, separated by washout periods of 2 weeks. After a 14-day pretreatment with a commercial green tea beverage (Healthya; Kao, Tokyo, Japan) with a daily intake of 700 ml, a single oral dose of 30 mg nadolol (NADIC; Dainippon Sumitomo Pharma, Osaka, Japan) was administered in the morning of the study day with 350 ml green tea or water. Subjects consumed a further 350 ml of green tea or water 30 min after nadolol administration. Subjects fasted overnight before

nadolol administration and had a standardized meal 1 and 4 h later. In each study period, 5-ml venous blood samples were collected from an indwelling catheter placed in an antecubital vein or by direct venipuncture into sodium EDTA–treated tubes at predose (0 h) and at 0.5, 1, 2, 3, 4, 6, 8, 24, and 48 h after nadolol administration. Blood samples were centrifuged for 10 min at ~2,000*g* at 4 °C. Urine was collected during periods of 0–8, 8–24, and 24–48 h after the administration of nadolol. The volume of each urine sample was recorded. Plasma and urine samples were stored at −80 °C until analysis. Subjects were instructed to refrain from consuming green tea and fruit products, including apple, cranberry, grapefruit, and orange juices, for 2 weeks before and for 48 h after nadolol administration. As PD tests, PR, SBP, and DBP were recorded after 10 min rest in a sitting position at predose and at 0.5, 1, 2, 3, 4, 8, 24, and 48 h after nadolol administration using an automatic blood pressure monitor (HEM-7051-HP, Omron, Kyoto, Japan). The mean values for the three PD parameters were obtained from triplicate measurements and given as percentage changes from the individual predose values.

Determinations of drug concentration. The plasma concentrations and the urinary excretion of nadolol were determined up to 48 h using high-performance liquid chromatography with fluorometric detection (JASCO, Tokyo, Japan) according to the method used in a previous report.[13]

The plasma concentrations of EC, ECG, EGC, and EGCG over 48 h after concomitant green tea and nadolol administration were measured using ultra-performance chromatography/electrospray ionization–single mass spectrometry (Waters, Milford, MA) according to the method used in a previous study.[28] The contents of catechins in the green tea used in the clinical study were also determined using the same method with some modifications. The lower limit of quantitation of all catechins was 10 ng/ml. The accuracies were within 100 ± 15%, with corresponding precision of <15% of the coefficient of variation.

***In vitro* assays.** The uptake assays were carried out as described previously.[29] Briefly, 7.0×10^5 HEK-OATP cells (stably expressing OATP1A2 or OATP2B1 protein) or the respective HEK-VC cells (HEK-VC/418; control cell line transfected with the empty vectors pQCXIN and pcDNA3.1(+)) were seeded in poly-D-lysine hydrobromide (0.1 mg/ml)-coated 12-well plates and cultured for 2 days. Before uptake experiments, cells were washed with prewarmed uptake buffer (142 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l K_2HPO_4, 1.2 mmol/l $MgSO_4$, 1.5 mmol/l $CaCl_2$, 5 mmol/l glucose, and 12.5 mmol/l HEPES, pH 7.3; all from Carl Roth KG, Karlsruhe, Germany). [^{3}H]-Nadolol (25 Ci/mmol, >99% purity; American Radiolabeled Chemicals, St Louis, MO) and [^{3}H]-BSP (14 Ci/mmol; Hartmann Analytic, Braunschweig, Germany) were dissolved in uptake buffer. Unlabeled nadolol (Sigma–Aldrich Chemie, Munich, Germany) and BSP (Applichem, Darmstadt, Germany) were added to reach the final concentrations. OATP1A2-mediated nadolol transport was linear for at least 10 min (data not shown), and an uptake time of 5 min was used in subsequent experiments. The cells were incubated with the uptake solution (at 37 °C) for 5 and 10 min in the OATP1A2 and OATP2B1 assays, respectively, and subsequently washed three times with ice-cold uptake buffer to remove radioactivity bound to the cell membrane. After the cells were lysed with 0.2% sodium dodecylsulfate, the intracellular accumulation of radioactivity was determined by liquid scintillation counting (TriCarb 2800; PerkinElmer Life and Analytical Sciences, Rodgau, Germany), and the appropriate protein concentrations were determined by a bicinchoninic acid assay (BCA Protein Assay Kit; Thermo Fisher Scientific, Bonn, Germany). All experiments were carried out in triplicate on at least 2–3 separate experimental days.

Data analysis. Pharmacokinetic and statistical analyses were performed using WinNonlin (version 5.0, Pharsight, Mountain View, CA) and Prism (version 5.4, GraphPad software, San Diego, CA). The data are expressed as geometric means and 90% CIs unless otherwise noted. The C_{max} and t_{max} (time to reach C_{max})were obtained by inspection. AUC was calculated by the trapezoidal method for the observed values. The renal clearance was obtained from the equation $CL_R = A_e/AUC_{0-48}$, in which A_e is the amount of nadolol excreted in urine for up to 48 h. For the catechins, apparent $t_{1/2}$ during the log-linear terminal phase was calculated from the elimination rate constant determined by a linear regression analysis, and $AUC_{0-\infty}$ was determined by extrapolation to infinity. The $AUEC_{0-48}$ for PR, SBP, and DBP was calculated using the trapezoidal rule (Microsoft Excel). The OATP1A2- and OATP2B1-mediated net uptake was obtained by subtracting the uptake in VC cells from that in OATP1A2- and OATP2B1-expressing cells. The percentage of uptake inhibition was calculated from control experiments in the absence of inhibitors (100% uptake). The corresponding IC_{50} values for inhibition of OATP1A2-mediated nadolol uptake were calculated by fitting the data to a sigmoid dose–response regression curve. C_{max}, AUC_{0-48}, A_e, CL_R, and AUEC for PR, SBP, and DBP were analyzed by paired *t*-test for the comparison between the green tea and water phases. Difference in t_{max} was analyzed by Wilcoxon matched-pairs signed-rank test. The OATP1A2- or OATP2B1-mediated uptakes of BSP and nadolol were analyzed using an unpaired *t*-test with Welch's correction. Differences in PR, SBP, and DBP between baseline value and values in each time point were analyzed by one-way analysis of variance, followed by Dunnett's test. A value of $P \leq 0.05$ was considered statistically significant.

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CONFLICT OF INTEREST

F.M. received research funding from Sanofi-Aventis Deutschland. J.P.W. received fees for lectures from AstraZeneca and Merck Sharp and Dohme. M.F.F. reported receiving personal compensation for expert testimony from Boehringer Ingelheim Pharma and payments for lectures from Bayer-Schering Pharma and Sanofi-Aventis Deutschland. The Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander-Universität Erlangen-Nürnberg, received compensation for commissioned research by M.F.F.'s group from Merck KGaA and Sanofi-Aventis Deutschland and for supplying materials to Gilead. The other authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

S.M., F.M., H.G., J.P.W., M.F.F., and J.K. wrote the manuscript; S.M., J.Y., F.M., H.G., S.O., J.P.W., H.W., S.Y., M.F.F., and J.K. designed the research; S.M., J.Y., K.T., K.K., and M.S.Y. performed the research; S.M., F.M., K.K., H.G., M.F.F., and J.K. analyzed the data.

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Note

Green Tea Extract Affects the Cytochrome P450 3A Activity and Pharmacokinetics of Simvastatin in Rats

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Summary: Effects of green tea extract (GTE) on the activity of cytochrome P450 (CYP) enzymes and pharmacokinetics of simvastatin (SIM) were investigated in rats. Inhibitory effects of GTE on CYP3A activity were investigated in rat hepatic microsomes (RHM) using midazolam (MDZ) 1′-hydroxylation as a probe reaction. SD female rats received a single oral dose of GTE (400 mg/kg) or troleandomycin (TAO, a CYP3A selective inhibitor, 500 mg/kg), followed 30 min later by SIM (20 mg/kg). Plasma concentrations of SIM and its active metabolite, simvastatin acid, were determined up to 6 h after the SIM administration using LC/MS/MS. In RHM, GTE inhibited MDZ 1′-hydroxylation with IC_{50} and K_i^{app} values of 12.5 and 18.8 μg/mL, respectively, in a noncompetitive manner. Area under plasma concentration-time curves for SIM in the GTE and TAO groups were increased by 3.4- and 10.2-fold, respectively, compared with the control. The maximum concentrations of SIM were higher in the GTE (3.3-fold) and TAO (9.5-fold) groups. GTE alters the pharmacokinetics of SIM, probably by inhibiting intestinal CYP3A.

Keywords: CYP3A; green tea extract; midazolam; pharmacokinetics; rat; simvastatin

Introduction

Over recent decades, consumption of green tea (*Camellia sinensis*) has been growing worldwide in accordance with an increasing number of publications reporting beneficial effects such as chemoprevention, anti-oxidation and anti-infection.[1–3] Catechins, the major polyphenol constituents of freshly brewed green tea, are thought to exert those effects.[4] Naturally occurring catechins in green tea comprise (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC) and (−)-epicatechin-3-gallate (ECG). Previously, Muto *et al.* demonstrated that green tea catechins, especially galloylated catechins, inhibited drug oxidation catalyzed by cytochrome P450 (CYP) enzymes *in vitro*.[5] In addition, green tea catechins, especially EGCG, have been reported to exhibit pharmacokinetic interactions with various drugs in animal experiments.[6–10] For instance in rats, repeated oral administration of green tea extract (GTE) for one week significantly increased the maximum plasma concentration (C_{max}) and area under the time-concentration curve (AUC) of midazolam, which is metabolized extensively by CYP3A, suggesting that catechins in GTE inhibited CYP3A activity in the intestine.[11] Recently, our group reported that the consumption of green tea containing large amounts of catechins increased the bioavailability of simvastatin in a hypercholesterolemia patient.[12] The effect on CYP3A activity of drinking green tea may contribute to the alteration of simvastatin pharmacokinetics; however, to the best of our knowledge, there are no reports concerning the interaction of simvastatin with green tea catechins in rodents.

Simvastatin is clinically used as a lactone prodrug and is hydrolyzed by esterase in the liver to yield an active metabolite,

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simvastatin acid.[13] Both simvastatin and simvastatin acid are further metabolized to several inactive metabolites including 6′-hydroxy or 3′,5′-dihydrodiol forms by CYP3A in rats and humans.[14–16] A previous study demonstrated that the metabolism of simvastatin in female Sprague-Dawley (SD) rats is more similar than in male rats to that observed in humans,[15] suggesting that female rats could represent a proper model to clarify the underlying mechanism of herb-drug interaction reported in our previous human case. As an example, Ishigami *et al.* showed that the co-administration of simvastatin and itraconazole resulted in significant increases in the C_{max} and AUC of simvastatin due to the inhibition of CYP3A in female rats.[15] Concerning the pharmacokinetic interaction between simvastatin and dietary components, Butterweck *et al.* reported that repeated intake of grapefruit juice elevated the plasma concentrations of simvastatin and simvastatin acid in rats.[17] Given that green tea catechins actually inhibit CYP3A activity *in vivo*, plasma concentrations of simvastatin and simvastatin acid may be altered.

The objective of the present study was to assess the effect of GTE on the metabolism of simvastatin in rats. Using rat hepatic microsomes, the *in vitro* inhibitory effect of GTE on CYP3A activity was tested with midazolam as a specific probe substrate of CYP3A. To investigate the pharmacokinetic interaction, rats were given a single dose of simvastatin 30 min after receiving GTE or troleandomycin, a CYP3A selective inhibitor, by oral gavage, and plasma concentrations of simvastatin and simvastatin acid were measured.

Methods

Reagents: Simvastatin, simvastatin acid, lovastatin, lovastatin acid and 1′-hydroxy midazolam were purchased from Toronto Research Chemicals (North York, ON, Canada). Midazolam and troleandomycin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diazepam, carboxymethylcellulose (CMC) and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), were purchased from Sigma Aldrich (St. Louis, MO). As a GTE, Sunphenon BG3™ was provided by Taiyo Kagaku (Yokkaichi, Japan), containing EGCG, EGC, EC and ECG at 43.4, 24.8, 9.7 and 1.7% with a total catechin content of 86.5% (w/w).

Animal experiments: Female SD rats (11 weeks, 250–300 g) (Japan SLC, Hamamatsu, Japan) were maintained at 24 ± 2°C with free access to food and water in a 12-h light-dark cycle. After overnight fasting, rats received single oral doses of 400 mg/kg GTE, 500 mg/kg troleandomycin or saline (control) by gavage. GTE was dissolved in distilled water, and troleandomycin was suspended in saline (pH 4.0). Thirty minutes later, simvastatin suspended in 0.5% CMC was administered by gavage at a dose of 20 mg/kg. Blood samples were taken periodically from a tail vein up to 6 h after simvastatin administration. Plasma samples were stored at −80°C prior to analysis. The experimental procedure was approved by the Animal Ethical Committee of the University of Shizuoka.

Assay of midazolam 1′-hydroxylation in rat hepatic microsomes: Rat hepatic microsomes were prepared according to a previous report.[18] Protein concentrations of microsomal samples were determined by Lowry's method, and samples were stored at −80°C before use. The midazolam 1′-hydroxylation assay was performed according to a previous study with some modifications.[19] The incubation mixture contained 100 mM potassium phosphate buffer (pH 7.4), rat hepatic microsomes (0.5 mg/mL protein) and midazolam (1, 5, 10, 25 and 50 μM) in a final volume of 100 μL. The reaction was initiated by the addition of 1 mM NADPH. Incubations were carried out for 5 min at 37°C with increasingly higher concentrations of GTE (from 0 to 100 μM), and terminated by adding 100 μL of 10 μM diazepam, an internal standard, in ice-cold methanol. The samples were then centrifuged at 10,000×*g* for 10 min at 4°C, and filtered through 0.2 μm membrane filters (Millex-LG, Millipore, Bedford, MA).

Determination of simvastatin and simvastatin acid concentrations: Simvastatin and simvastatin acid were extracted from plasma using a solid phase extraction system (Oasis HLB, Waters, Milford, MA), and their concentrations were quantified using a high-performance liquid chromatography (HPLC)/electrospray ionization (ESI) tandem mass spectrometry (MS/MS) system (Finnigan TSQ4000, Thermo Scientific, Waltham, MA). Lovastatin and lovastatin acid were used as internal standards for simvastatin and simvastatin acid, respectively. Chromatographic separation was carried out with the Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) using an Inertsil ODS-3 C18 analytical column (2.1 × 50 mm i.d., 2.0 μm; GL Science Inc., Tokyo, Japan) at 40°C. The mobile phases were 5 mM ammonium acetate with pH 4.3 (A) and acetonitrile (B) with a flow rate of 0.3 mL/min. The gradient of the mobile phase was 0–5.5 min, 58% (B); 5.5–6.0 min, 55–95% (B); and 6.0–8.5 min, 95% (B), followed by 4 min of column equilibration. The mass spectrometer was operated in the positive ionization mode for the detection of lactone compounds, and in the negative ionization mode for the detection of acid compounds. The monitored fragmentation reactions were from 441 *m/z* to 325 *m/z* for simvastatin, from 427 *m/z* to 325 *m/z* for lovastatin, from 435 *m/z* to 319 *m/z* for simvastatin acid, and from 421 *m/z* to 101 *m/z* for lovastatin acid. The lower limits of quantification were 0.1 ng/mL for simvastatin and 0.5 ng/mL for simvastatin acid. The interday coefficients of variation (CV) for simvastatin and simvastatin acid were 7% and 4%, respectively, at plasma concentrations of 10 ng/mL.

Determination of 1′-hydroxy midazolam concentrations: The concentrations of 1′-hydroxy midazolam in microsomal samples were quantified by ultra-performance liquid chromatography (UPLC)/ESI-MS using the Waters ACQUITY UPLC system. Chromatography was performed on an ACQUITY BEH C_{18} column (2.1 × 50 mm i.d., 1.7 μm; Waters) with a column oven temperature of 40°C. The mobile phases were 5 mM ammonium acetate (A) and acetonitrile (B) with a flow rate of 0.25 mL/min. The gradient of the mobile phase was 0–2.0 min, 40–60% (B), followed by 1.5 min of column equilibration. Selective ion monitoring was employed to detect $[M+H]^+$ ions for 1′-hydroxy midazolam and diazepam, and the monitored *m/z* of 1′-hydroxy midazolam and diazepam was 342 and 326, respectively. The lower limit of quantification for 1′-hydroxy midazolam was 10 ng/mL with an interday CV of less than 15%.

Data analysis: Data are presented as the mean ± standard error mean (SEM). Enzyme kinetic analyses were performed using SigmaPlot 12.0 (Systat Software, San Jose, CA). IC_{50} was defined as the concentration of inhibitor showing a 50% reduction in metabolite formation rate, and the value was estimated through nonlinear regression of relative reaction velocities at a single substrate concentration in the presence of varying inhibitor concentrations. The pharmacokinetic parameters of simvastatin and simvastatin acid were calculated using a non-compartmental analysis by WinNonlin (Pharsight, Mountain View, CA). Statistical

analyses were performed with a one-way ANOVA and Dunett's test as a post-hoc test using Prism (GraphPad Software, San Diego, CA) with a criterion for significance of $p < 0.05$.

Results and Discussion

Our group previously reported that the consumption of green tea increased the bioavailability of simvastatin in a hypercholesterolemic patient, which poses the intriguing question about the mechanics that explain the pharmacokinetic interaction between green tea and simvastatin.[12] Since simvastatin is extensively metabolized by the CYP3A subfamily, the present study was aimed to investigate the effects of an extract of green tea (Sunphenon BG3) on CYP3A activity *in vitro* and on simvastatin pharmacokinetics *in vivo* in rats.

The inhibitory effect of GTE on CYP3A activity was investigated in rat hepatic microsomes using midazolam as a CYP3A probe. The K_m and V_{max} values for midazolam 1′-hydroxylation were 1.3 ± 0.1 μM and 4.4 ± 0.1 pmol/mg protein/min, and the intrinsic clearance was 3.4 μL/min/mg protein. When GTE was added to the incubation mixture at concentrations ranging from 1 to 100 μg/mL, midazolam 1′-hydroxylation was dose-dependently inhibited with an IC_{50} of 12.5 ± 1.4 μg/mL (**Fig. 1A**). This value is in good agreement with a previous study showing the IC_{50} of GTE of 12.6 μg/mL for CYP3A-mediated testosterone 6β-hydroxylation in human hepatic microsomes, although the contents of catechins in the GTE were different.[11] GTE also had an apparent K_i of 18.8 ± 2.7 μg/mL in a noncompetitive manner (**Fig. 1B**). Xia *et al.* suggested that if the IC_{50} value of an inhibitor for CYP3A activity was less than 4 μg/mL, the inhibition would be moderate in rat hepatic microsomes.[20] Accordingly, GTE used in this study was found to a be weak to moderate inhibitor for CYP3A.

To clarify the effects of GTE on the pharmacokinetics of simvastatin and simvastatin acid, a single oral dose of GTE (400 mg/kg) was administered to rats 30 min before the oral administration of simvastatin. Plasma concentration-time profiles and pharmacokinetic parameters of simvastatin and simvastatin acid are shown in **Figure 2** and **Table 1**. Compared with the control, pretreatment with GTE resulted in a significant increase in the AUC_{0-6} values of simvastatin by 3.4-fold. The C_{max} of simvastatin was also increased, by 3.3-fold, with little change in the time at the maximum plasma concentration (t_{max}). The terminal half-life ($t_{1/2}$) of simvastatin was not changed by GTE, suggesting that GTE did not affect the elimination of simvastatin from blood. For simvastatin acid, the GTE increased the C_{max} and AUC_{0-6} by 2.8- and 2.0-fold, respectively. However, these values did not reach

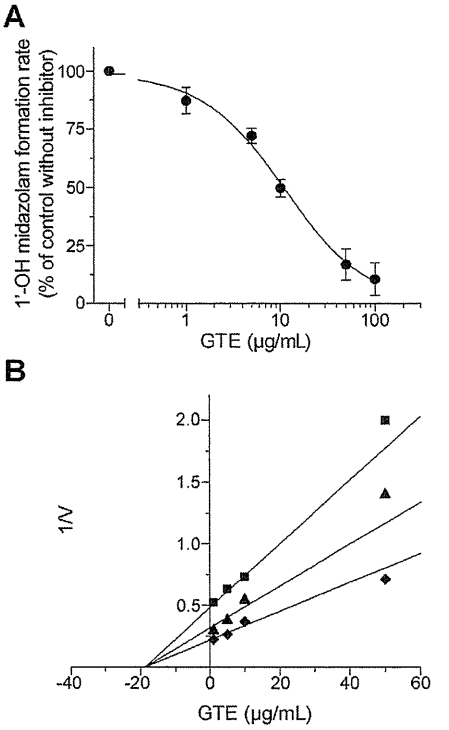

Fig. 1. Inhibitory effect of green tea extract (GTE) on midazolam 1′-hydroxylation in rat hepatic microsomes
(A) Concentration-dependent effects of GTE on CYP3A-mediated midazolam 1′-hydroxylation. Rat hepatic microsomes were incubated with 5 μM of midazolam and various concentrations of GTE (0 to 100 μg/mL) to determine the IC_{50}. Reaction velocities are expressed as a ratio (in percent) relative to the velocity without GTE. (B) Dixon plots of inhibition of CYP3A-mediated midazolam 1′-hydroxylation by GTE. For the estimation of apparent K_i values, midazolam at doses of 1.0 μM (■), 2.0 μM (▲) and 5.0 μM (◆), and a range of concentrations of GTE (0 to 50 μg/mL) were used to construct Dixon plots. Data represent the mean ± SEM for three independent experiments.

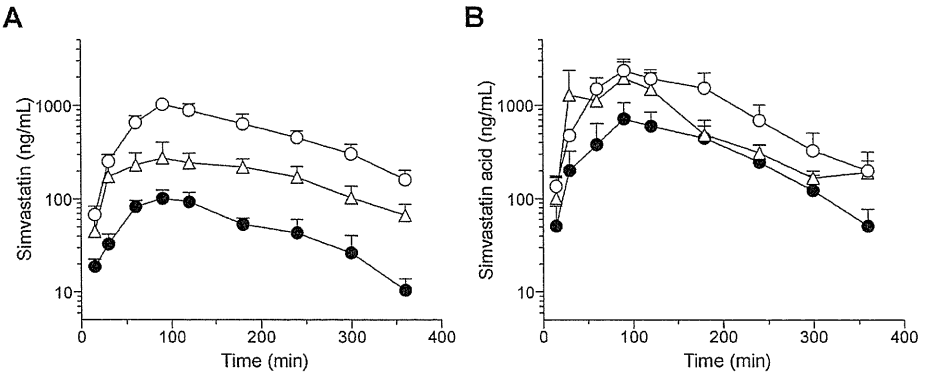

Fig. 2. Effects of a single administration of green tea extract (GTE) on the pharmacokinetics of simvastatin in rats
Plasma concentration-time profiles of simvastatin (A) and simvastatin acid (B) up to 6 h after an oral administration of simvastatin (20 mg/kg) in female SD rats pretreated 30 min before with a single oral dose of GTE (400 mg/kg, △), troleandomycin (500 mg/kg, ○) or saline (control, ●). Data represent the mean ± SEM ($n = 5$).

Table 1. Pharmacokinetic parameters of simvastatin and simvastatin acid after single oral administration of simvastatin (20 mg/kg) in rats pretreated with saline (control), green tea extract (GTE, 400 mg/kg) or troleandomycin (500 mg/kg)

	Control	GTE	Troleandomycin
Simvastatin			
C_{max} (ng/mL)	115 ± 23	378 ± 102	1,096 ± 113***
t_{max} (h)[a]	1.5 (1.0–3.0)	4.0 (1.5–4.0)	1.5 (1.5–2.0)
$t_{1/2}$ (h)	1.6 ± 0.6	1.2 ± 0.2	1.8 ± 0.3
AUC_{0-6} (h·ng/mL)	301 ± 51	1,032 ± 134*	3,069 ± 304***
Simvastatin acid			
C_{max} (ng/mL)	655 ± 273	2,062 ± 1,123	2,424 ± 613
AUC_{0-6} (h·ng/mL)	1,918 ± 880	4,319 ± 1,730	6,201 ± 1,586

[a]Median values with range.
C_{max}, maximum plasma concentration; t_{max}, time to maximum concentration; $t_{1/2}$, elimination half-life; AUC_{0-6}, area under the plasma concentration-time curve from time 0 to 6 h.
*$p < 0.05$, significant difference from the control. Data expressed as the mean ± SEM ($n = 5$).

statistical significance due to large individual differences. Single pretreatment with troleandomycin, a specific CYP3A inhibitor, markedly elevated the C_{max} and AUC_{0-6} of simvastatin by 9.5- and 10.2-fold, respectively, as compared to the control. Pretreatment with troleandomycin also led to increases in the C_{max} and AUC_{0-6} of simvastatin acid by 3.3- and 3.0-fold, respectively, although we did not observe significant differences between the control and troleandomycin groups. Previous studies in rats showed that the coadministration of EGCG or GTE significantly increased the plasma concentrations of CYP3A substrate drugs including, diltiazem, midazolam, nicardipine, and tamoxifen.[8–11] In accordance with these reports, our results indicate that GTE elevates the plasma concentration of simvastatin. We have recently demonstrated that after a single oral administration of GTE (400 mg/kg) to rats, C_{max} values of EC, ECG, EGC, and EGCG were 755, 110, 1,399 and 1,217 ng/mL, respectively.[21] The total concentration of these four catechins was approximately 3.5 μg/mL, lower than the K_i^{app} value estimated in this study. This could support the hypothesis that the inhibition of hepatic CYP3A activity was weak in the case of a single oral administration of GTE. On the other hand, intestinal concentrations of catechins are considered to be higher than blood concentrations after the oral administration of GTE. Therefore, the results suggested that a single administration of GTE mainly inhibited simvastatin metabolism in the intestine, and might not affect the elimination of simvastatin.

Previous studies suggested that both simvastatin and simvastatin acid are substrates of several drug transporters such as P-glycoprotein (P-gp) and organic anion transporting polypeptide (OATP) 1B1.[22] There is also a report showing an increased distribution of simvastatin to the brain and liver in multidrug-resistance gene (*mdr1a/b*) knockout mice as compared with wild-type mice.[23] Jodoin *et al.* demonstrated that EGCG can modulate the bioavailability of a P-gp substrate.[24] Thus, to elucidate whether GTE influences the activities of drug transporters *in vivo*, it is necessary to examine its effects on the tissue distribution of simvastatin by measuring the concentration of simvastatin in the tissue. In addition, it may also be necessary to investigate whether chronic treatment with GTE or increasing doses of GTE further alter the pharmacokinetics of simvastatin as well as the measurement of plasma concentration profiles of green tea catechins. Those studies will reveal more detailed mechanisms underlying green tea–simvastatin interaction. There is known to be a significant interspecies difference between humans and rats in the metabolism of simvastatin.[15,25] Furthermore, our group recently reported that GTE and EGCG also inhibited CYP3A-mediated midazolam 1′-hydroxylation in human liver and intestinal microsomes.[26] Therefore, further clinical studies are warranted to investigate the pharmacokinetic interaction between simvastatin and green tea.

The present study showed that GTE weakly to moderately inhibited CYP3A activity in a noncompetitive manner as evaluated by midazolam 1′-hydroxylation in rat hepatic microsomes. A single oral dose of GTE 30 min before simvastatin administration resulted in a significant elevation in plasma concentrations of simvastatin. In conclusion, GTE may affect the pharmacokinetics of simvastatin and simvastatin acid in rats, possibly by inhibiting CYP3A in the intestine.

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CONFLICT OF INTEREST
The author declared no conflict of interest.

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Regulatory Challenges in the Review of Data from Global Clinical Trials: The PMDA Perspective

K Asano[1], A Tanaka[1], T Sato[1] and Y Uyama[1]

Regulatory agencies face challenges in reviewing data from global clinical trials (GCTs) in the era of globalization of drug development. One major challenge is consideration of ethnic factors in evaluating GCT data so as to extrapolate foreign population data to one's own national population. Here, we present the Pharmaceuticals and Medical Devices Agency (PMDA) perspective in reviewing GCT data in new drug applications (NDAs) and discuss future challenges for new drug approval.

Japanese guideline: "Basic principles on global clinical trials"

Evaluating the similarities and differences in pharmacokinetics (PK) and dose–clinical response relationships between Japanese and other populations has been a key step in reviewing data from bridging studies based on the International Conference on Harmonisation (ICH) E5 guideline.[1,2] Consideration of ethnic factors has recently become more important in the era of globalization of drug development because a GCT is conducted simultaneously in multiple countries where the effects of ethnicity may differ.[2,3] The aim of a 2007 Japanese guideline focusing on GCTs[4] was to clarify points to consider in planning and conducting GCTs and to encourage Japanese participation in such trials (**Table 1**). Many important points for sponsor consideration were described, including compliance with good clinical practice, evaluation of ethnic factors, and study design. It also recommended early participation by Japan in global drug development, explained the importance of Japanese subject dose–response data, and emphasized the need, during data evaluation of GCTs, for understanding similarities and differences—both in the target population and in clinical trial methods—between countries and regions. Methods of sample-size calculation in GCTs are provided to promote appropriate evaluation of consistency in results between overall and Japanese populations. Some points had already been made in the ICH E5 guideline,[1] but more specific and practical recommendations pertinent to GCTs are described in this guideline. The present guideline enables a sponsor to plan and conduct GCTs more effectively, which may result in increased acceptance by the PMDA of GCT data for NDAs in Japan.

Increase of NDA approval based on data from GCTs

After publication of the 2007 guideline, the percentage of GCTs in Japan identified through clinical trial notification increased significantly.[5] As a result, approved new drugs whose pivotal data were collected through GCTs (i.e., GCT-based approvals) have increased in recent years (**Figure 1**). In the period from FY2007 to FY2012, 42 new drugs were GCT-based approvals. Since FY2009, the percentage of GCT-based approvals has been larger than that of drug approvals based on bridging studies following ICH E5 guidelines, probably as a result of changing drug development strategy from sequential bridging studies to simultaneous GCTs (**Figure 1**). In FY2012, 13.4% of drugs approved in Japan were GCT-based approvals. Recently, cooperation between Japan and other countries has also diversified. For example, GCTs for fesoterodine fumarate for overactive bladder and for atomoxetine hydrochloride for attention-deficit hyperactivity disorder in adults were conducted

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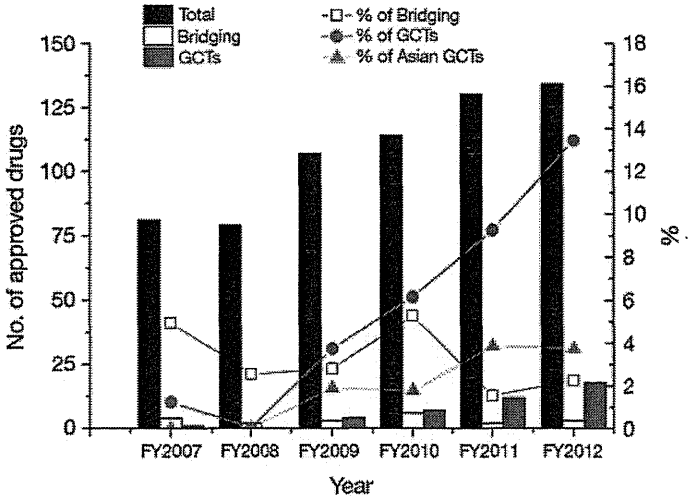

Figure 1 Trends of new drug application approvals in Japan and their pivotal data. In the period from FY2007 to FY2012, the number of approved drugs whose pivotal data were collected as global clinical trials (GCT-based approvals) increased. Black bars, total number of approved drugs; blue bars and blue circles, number and percentage, respectively, of approved drugs whose pivotal data were collected in GCTs; red triangles, percentage of approved drugs whose pivotal data were collected in Asian GCTs; open bars and open squares, number and percentage, respectively, of approved drugs whose pivotal data were collected in bridging studies based on the ICH E5 guideline.[1]

only in Asia, and such percentages have increased over the past few years (**Figure 1**). Similarly, 13 of 42 GCT-based approvals included large-scale trials of thousands of subjects, with eight of them (including denosumab for the treatment of bone lesions due to multiple myeloma and bone metastasis of solid tumor, and apixaban for prevention of ischemic stroke and systemic embolism in patients with nonvalvular atrial fibrillation) among the NDAs approved in the past two years. As described above, the 2007 guideline has provided more choice of drug development strategies for regulatory approval in Japan.

Use of GCT data in NDAs for regulatory approval is expected to increase in Japan in the year ahead because clinical trial notification of GCTs has steadily increased in recent years.[5] To respond to this recent situation, in 2012 a document to supplement the 2007 guideline was published.[6] This new document was based on recent experience and new scientific data. Its purpose is to further promote an understanding of the 2007 guideline and ensure Japan's successful participation in global drug development activities from an early stage as well as effective and appropriate conduct of GCTs in East Asia, where an increase in such trials is expected. The document contains a special section focusing on East Asian GCTs (**Table 2**). In this section, sponsors are encouraged to conduct East Asian GCTs to accumulate additional scientific data to be included in Japanese NDAs. A classification of development strategies based on PK profile was also included. A later section, covering general points, examines more practical issues, such as global communication in daily practice, evaluation of GCT data and long-term safety, inclusion of Japanese subjects living outside of Japan, handling differences in active control and concomitant medication, and special considerations for large-scale GCTs. The guideline also includes points to consider in comparing PK data between different ethnicities. The decision to address this aspect was based on the outcome of the research under China, Korea, and Japan tripartite cooperation.[7]

Regulatory-science research on ethnic factors to aid in drug approval decisions

To conduct GCTs that are acceptable to a regulatory agency, it is very

Table 1 Summary of topics addressed in the Japanese Ministry of Health, Labour, and Welfare's 2007 guideline on GCTs

Basic requirements for conducting a GCT (e.g., GCP compliance)
The appropriate time for Japan to participate in global drug development
Consideration of a phase I trial or PK information in the Japanese population before conducting a GCT with patients
Consideration of a dose-finding study in the Japanese population before participating in a global confirmatory phase III trial
Designing a GCT (e.g., evaluation of ethnic factors, analytical method, end point, standardized safety monitoring)
Methods for determining sample size and proportion of Japanese subjects when conducting an exploratory trial (e.g., a dose-finding study or a confirmatory trial) as a GCT
Acceptability of a global trial using an evaluation index that has been established overseas but not established in Japan
Evaluating data from a smaller clinical study separately performed in Japan but with a protocol that is identical to that of a GCT performed outside Japan
Use of control groups in a phase III confirmatory global trial
Use of concomitant medications or therapies in a GCT
Recommended therapeutic areas for a global trial
A reference flow chart for determining whether a GCT should be performed

GCP, good clinical practice; GCT, global clinical trial; PK, pharmacokinetics.

Adapted from ref. 4.

Table 2 Summary of topics addressed in the Japanese Ministry of Health, Labour, and Welfare's 2012 supplementary document on GCTs

Special points for East Asian GCTs
Importance of East Asian GCTs and evaluation of ethnic factors in East Asia
Recommended therapeutic areas for GCTs in East Asia
General global drug development strategy based on data on interethnic comparison of pharmacokinetic profiles relating to East Asian GCTs
Evaluation of data from a bridging study not as a Japanese clinical trial but as a GCT in East Asia for extrapolating the data from US–European studies to the Japanese population
General points
Planning Japanese clinical development strategies and a protocol for a Japanese study on the trend of globalization of drug development
Evaluating the results of a GCT
Evaluating the data for Japanese subjects living outside of Japan, e.g., those enrolled in foreign studies
Comparing pharmacokinetic data between ethnicities
Conducting a phase I (first-in-human) trial as a GCT
Conducting an exploratory GCT including Japanese subjects that investigates a combination therapy without safety data on the combination in Japanese persons
Conducting an exploratory dose–response trial as a GCT including Japanese subjects if the blood concentration of an investigational drug is different between Japanese and non-Japanese subjects
Use of an active control that has not been approved in Japan but is used in an exploratory GCT
Dealing with a case in which the active ingredient of an active control drug has been approved in Japan and foreign countries but the dosage regimen or formulation differs
Taking into account indications or dosage regimens that vary among countries for a drug used in combination with the investigational drug
Addressing failure to achieve the target sample size of Japanese subjects for a GCT using a competitive registration system
Participating in a large-scale GCT using a true end point such as survival time
Determining an appropriate number of Japanese patients in evaluating the long-term safety of a drug in global development that is intended for long-term treatment of a nonfatal disease

GCT, global clinical trial.
Adapted from ref. 6.

important to clarify—as much as possible—the regulatory perspective on considerations in planning and conducting the trials, based on current scientific knowledge. In this regard, the 2007 guideline along with the 2012 supplementary document contribute to our understanding of the regulatory perspective on GCTs. The European Medicines Agency published a reflection paper on the extrapolation of results from clinical studies conducted outside the European Union to the EU population.[8] It recommends prospective analysis of potential extrinsic and intrinsic factors when conducting a GCT to extrapolate its data to an EU population. In addition, an article by Khin *et al.* in this issue discusses the US Food and Drug Administration perspective on the use of GCT data in NDAs.[9] Not surprisingly, the points described by both agencies are similar to those addressed in the Japanese guideline, although the scope and level of detail differ on some points. The similarity indicates common matters considered by regulatory agencies about evaluation of data from GCTs.

The impact of ethnic factors on drug responses (efficacy and safety) and whether GCT data can be extrapolated to a particular national population should be carefully considered in evaluating the benefit vs. risk of a drug for regulatory approval. Ethnic factors affecting drug responses are described in the ICH E5 guideline, and effects on drug responses of some factors—e.g., a genetic polymorphism in metabolic enzymes and molecular targets of anticancer drugs—have been reported.[10,11] However, scientific data about the impact of ethnic factors on drug responses are still limited. It is not sufficiently clear how the effect of a given ethnic factor differs among various populations and how much difference in the effect is clinically meaningful. Drug development will be more globalized in the future, and populations participating in GCTs could be more diversified. In such situations, more scientific research in the field of regulatory science will be necessary to characterize the relationship between ethnic factors and drug responses and to correctly understand similarities and differences of ethnic factors among populations.

International collaboration among sponsors, investigators, academia, and regulators is important to promote this research. Research groups focusing on ethnic factors have been established in Japan (e.g., the Tohkin and Kawai study groups supported by the Japanese Ministry of Health, Labour, and Welfare). The results of their research have been shared internationally at the China–Korea–Japan Director-General Meeting/Working Group[7] as well as at other international scientific meetings, including the Asia–Pacific Economic Cooperation multiregional clinical trials workshop.[12] New research findings will facilitate better understanding concerning the impact of ethnic factors on drug response and will also enable appropriate regulatory decisions regarding drug approvals based on GCT data.

Finally, harmonization of regulatory standards regarding GCT data is still a major challenge. It is necessary for GCTs to lead to efficient and feasible drug development while being acceptable to multiple regulatory agencies as well as to avoid duplication of work in clinical development. Accumulating more scientific data on ethnic factors may clear the way for international harmonization of these efforts.

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CONFLICT OF INTEREST
The authors declared no conflict of interest.

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Genetics of Cisplatin Ototoxicity: Confirming the Unexplained?

AV Boddy[1]

Replication of findings in clinical pharmacogenetic studies is essential but often neglected. The observation that *TPMT* and *COMT* genotypes, in combination with *ABCC3* genotype, are predictive of ototoxicity following cisplatin treatment has been confirmed. However, translating this observation into a useful preventive strategy requires more mechanistic insight.

On the face of it, the paper by Pussegoda *et al.*[1] in this issue presents an empirical confirmation of a previous, surprising report[2] that germline single-nucleotide polymorphisms in *TPMT* (and *COMT*) are linked to a greater risk of cisplatin-induced ototoxicity. The importance of this chronic and debilitating side effect should not be underestimated in patients receiving potentially curative therapy for childhood cancers. This problem was recently underlined in a follow-up study in retinoblastoma patients treated with the related, less ototoxic drug carboplatin.[3] Although a therapeutic dose-monitoring approach to carboplatin dosing may obviate some of the toxicity of carboplatin,[4] it would be useful to be able to identify patients at risk of platinum-induced ototoxicity. According to their genotype, these patients could be offered less toxic alternative therapy or supportive intervention to mitigate the toxic effects of cisplatin. However, in another article in this issue, Yang *et al.*[5] present contradictory clinical and, importantly, mechanistic data refuting the association of TPMT polymorphisms with ototoxicity.

In the original article, published in *Nature Genetics* in 2009, the authors identified a link between TPMT, ABCC3, and COMT variants and risk of ototoxicity.[2] A broad panel of common genotypic variants were investigated, including several DNA repair enzymes and transporters that might be considered more likely to influence the pharmacology of cisplatin. Indeed, it is possible to imagine a scenario in which *TPMT* and *COMT* genotypes were included in the analysis as negative controls. No studies have provided a mechanistic rationale for the involvement of the methyl transferase enzymes in cisplatin activation, inactivation, or modulation of response. TPMT is itself something of an enigma, in that the endogenous substrate for this cytosolic enzyme has not been identified.

Even when there is a strong mechanistic argument linking genotype and phenotype, it is desirable—although seldom carried through systematically—that an initial observation be repeated in an independent population in order to validate the observation. The demand for independent validation is probably more pressing when the observation is discordant with preconceived expectations. Therefore, the follow-up study reported in this issue of *CPT* is welcome. However, three years later, we are no closer to understanding the mechanisms underlying this observation, augmented slightly by the additional influence of *ABCC3* genetic variants. The authors can do no more than postulate a possible role for methylation in DNA repair, with no supporting data.

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REVIEW

Characteristics of pharmacogenomics/biomarker-guided clinical trials for regulatory approval of anti-cancer drugs in Japan

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Pharmacogenomics (PGx) or biomarker (BM) has the potential to facilitate the development of safer and more effective drugs in terms of their benefit/risk profiles by stratifying population into categories such as responders/non-responders and high-/low-risks to drug-induced serious adverse reactions. In the past decade, practical use of PGx or BM has advanced the field of anti-cancer drug development. To identify the characteristics of the PGx/BM-guided clinical trials for regulatory approval of anti-cancer drugs in Japan, we collected information on design features of 'key trials' in the review reports of anti-cancer drugs that were approved after the implementation of the 'Revised Guideline for the Clinical Evaluation of Anti-cancer drugs' in April 2006. On the basis of the information available on the regulatory review data for the newly approved anti-cancer drugs in Japan, this article aims to explain the limitations and points to consider in the study design of PGx/BM-guided clinical trials.
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Keywords: biomarker; drug development; international harmonization; pharmacogenomics; PMDA; regulatory science; study design

INTRODUCTION

Pharmacogenomics or biomarker (PGx/BM), is increasingly being utilized in the drug development processes for identifying the appropriate target population of a drug in order to achieve a better benefit/risk balance, and as a consequence it has contributed to effective drug development.[1] Accordingly, in recent years, PGx/BM has been recognized worldwide as an important tool for drug development and it has also been implemented in regulatory reviews.[2]

In oncology, the use of PGx/BM has not only helped in elucidating the underlying molecular mechanism of tumor formation[3] but also contributed to major advances in personalized medicine as novel anti-cancer drugs targeting relevant molecules were successfully developed.[4] As a result, over the last decade more drugs carry PGx/BM information on their labels (prescribing information in Japan).[5] Development of anti-cancer drugs, however, still remains a challenge due to high failure rates in the later stages of clinical development.[6] Several studies indicated that the lack of efficacy is a reason for the attrition mostly seen in the later stages of development.[7–9] In addition, a poor knowledge about the methodology, including the study design in PGx/BM research, is another underlying reason that hampered the translation of PGx/BM technology from the bench to the bedside.[10,11] In this study, in order to identify the characteristics of PGx/BM-guided clinical trials in oncology for regulatory approval of drugs in Japan, we scrutinized the information found under 'key clinical trial' in the new drug applications (NDAs) of anti-cancer drugs that were approved in Japan after April 2006.

MATERIALS AND METHODS

We searched the Pharmaceuticals and Medical Devices Agency (PMDA) website, which is publically available at http://www.info.pmda.go.jp/approvalSrch/PharmacySrchInit, to identify the NDAs of anti-cancer drugs approved by the Ministry of Health, Labor and Welfare under the category of new molecular entities or new indication after April 1, 2006 on which the guidelines entitled the 'Revised Guideline for the Clinical Evaluation of Anti-cancer drugs'[12] was implemented. In this study, we excluded the NDAs approved for new route of administration, for new dosage and for combination therapy with marketed drug, as well as any NDA that was approved without clinical trial data as a special category based on the notification 'NDAs based on public knowledge'.[13] Accordingly, using 31 December 2012, as the cutoff date, we identified 52 approved NDAs, including 37 new molecular entities and 15 new indications.

To examine the characteristics of PGx/BM-guided clinical trials in oncology for the drug approval in Japan, we focused on the 'key trial', defined as an important clinical trial described in the Pharmaceuticals and Medical Devices Agency review report for each NDA as the key clinical evidence used in evaluating the efficacy and safety of the drug for approval. On the basis of the review reports of 52 NDAs, 108 clinical trials were identified as the key trials. We then classified the key trials whether or not any PGx/BM was used for the drug development. For the analysis, the key trials involving PGx/BM were further classified into three categories as outlined in Figure 1.[14,15] Briefly, the

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