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ORIGINAL ARTICLE

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Optimal antidiarrhea treatment for antitumor agent irinotecan hydrochloride (CPT-11)-induced delayed diarrhea

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Abstract *Purpose*: An antitumor camptothecin derivative CPT-11 has proven a broad spectrum of solid tumor malignancy, but its severe diarrhea has often limited its more widespread use. We have demonstrated from a rat model that intestinal β-glucuronidase may play a key role in the development of CPT-11-induced delayed diarrhea by the deconjugation of the luminal SN-38 glucuronide, and the elimination of the intestinal microflora by antibiotics or dosing of TJ-14, a Kampo medicine that contains β-glucuronidase inhibitor baicalin, exerted a protective effect. In the present study, we assessed the efficacy of several potential treatments in our rat model to clarify which is the most promising treatment for CPT-11-induced delayed diarrhea. *Methods and results*: Oral dosing (twice daily from days −1 to 4) of streptomycin 20 mg/kg and penicillin 10 mg/kg (Str/Pen), neomycin 20 mg/kg and bacitracin 10 mg/kg (Neo/Bac), both of which inhibited almost completely the fecal β-glucuronidase activity, or TJ-14 1,000 mg/kg improved the decrease in body weight and the delayed diarrhea symptoms induced by CPT-11 (60 mg/kg i.v. from days 1 to 4) to a similar extent. The efficacy was less but significant in activated charcoal (1,000 mg/kg p.o. twice daily from days −1 to 4). In a separate experiment using rats bearing breast cancer (Walker 256-TC), TJ-14, Neo/Bac, and charcoal at the same dose regimen improved CPT-11-induced intestinal toxicity without reducing CPT-11's antitumor activity. In contrast, oral dosing (twice a day) of cyclosporin A (50 mg/kg), a P-glycoprotein and cMOAT/MRP2 inhibitor or valproic acid (200 mg/kg), a UDP-glucuronosyltranferase inhibitor, exacerbated the intestinal toxicity without modifying CPT-11's antitumor activity. *Conclusions*: The result clearly demonstrated the ability of Neo/Bac, Str/Pen, and TJ-14, less but significant ability of activated charcoal, to ameliorate CPT-11-induced delayed-onset diarrhea, suggesting the treatments decreasing the exposure of the intestines to the luminal SN-38 are valuable for improvement of CPT-11-induced intestinal toxicity. In contrast, the treatments affecting the biliary excretion of CPT-11 and its metabolites might have undesirable results.

Keywords CPT-11 · Irinotecan · Diarrhea · β-Glucuronidase

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Introduction

Irinotecan hydrochloride (CPT-11), a water-soluble semisynthetic derivative of camptothecin, is an inhibitor of DNA topoisomerase I enzyme by its main active metabolite SN-38 [1, 2], and a promising antitumor agent, approved worldwide for use in patients with advanced colorectal cancer [3, 4], lung cancer [5, 6], and malignant lymphoma [7]. One of the major dose-limiting toxicities of CPT-11 therapy is unpredictable and severe diarrhea, especially delayed-onset severe diarrhea [inci-

dence of National Cancer Institute (NCI) grade 3 or 4 diarrhea is 20–40%] [8, 9]. It has limited the further evaluation of more aggressive antitumor regimens using CPT-11 [4, 5, 7, 10]. The great interpatient variations in the severity of diarrhea, the pharmacokinetics [11–16], and the efficacy of conventional antidiarrhea agents [5, 17, 18] make it difficult to understand the mechanisms of CPT-11-induced diarrhea, although preclinical and clinical studies have yielded some critical insight into the mechanisms and advances in treatment of the CPT-11-induced side effects [9].

CPT-11 is hydrolyzed by carboxylesterase to form the active metabolite SN-38 [19]. SN-38 is further conjugated to an inactive glucuronic acid conjugate (SN-38 glucuronide) by UDP-glucuronosyltransferase UGT1A1, the same isoenzyme responsible for glucuronidation of bilirubin, and excreted into the bile with other major component CPT-11 and SN-38 by P-glycoprotein (P-gp) and canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2 (cMOAT/MRP2) [20–24]. SN-38 glucuronide may be deconjugated by β-glucuronidase produced by the intestinal microflora, releasing SN-38. The SN-38 deconjugated may largely be responsible for the accumulation of SN-38 in the intestine [25–27].

We have first demonstrated from a rat model that β-glucuronidase produced by microflora in the large intestine may play a key role in the development of CPT-11-induced delayed-onset diarrhea by the deconjugation of the SN-38 glucuronide, and administration of antibiotics exerted a protective effect against the diarrhea by completely inhibiting the β-glucuronidase activity, thereby decreasing the exposure of the large intestine to the luminal SN-38 [28, 29]. Furthermore, we reported that TJ-14 or TJ-114, a Chinese herbal medicine that contains β-glucuronidase inhibitor baicalin, also exerted a protective effect on the delayed-onset diarrhea in the same model [30].

Based on our findings, several nonclinical and clinical studies to alleviate CPT-11-induced diarrhea have been performed especially focusing on the attenuating antiproliferating activity of SN-38 excreted into the intestinal lumen via the bile acid. Up to date, various attractive, and promising treatments for attenuating CPT-11-induced diarrhea, including (1) inhibition of intestinal β-glucuronidase using Kampo medicine TJ-14 [31] or other antibiotics neomycin or bacitracin [32, 33], (2) prevention of intestinal transport (re-absorption) of SN-38 and/or CPT-11 by oral alkalization [34, 35] or by adsorbing of these compounds using activated charcoal [36, 37], or various nonspecific treatments for cancer chemotherapy-induced diarrhea [9], have been clinically demonstrated. However, since these studies were performed under different experimental conditions with each other, no one can expect which is the most promising and effective treatment for CPT-11-induced delayed diarrhea.

To address the question, we compared the antidiarrhea activity of the several potential treatments on CPT-11-induced diarrhea in our rat model.

Methods

Reagents

CPT-11 (Topotecin® Injection, Yakult Honsha, Tokyo, Japan); penicillin G, streptomycin, and valproic acid (Sigma, St Louis, MO, USA); neomycin, bacitracin, and cyclosporin A (Wako Pure Chemicals, Tokyo, Japan); activated charcoal (Iwaki Seiyaku, Tokyo, Japan); and TJ-14 (Hange-Shasin-To, Tsumura, Tokyo, Japan) were commercially purchased. All the potential antidiarrhea agents were dissolved and/or suspended in distilled water for oral administration (Fuso Pharmaceutical Industries, Osaka, Japan) as a volume of 10 or 20 ml/kg.

RPMI1640 medium (Invitrogen Corp.; Carlsbad, CA, USA) and fetal bovine serum (FBS; Hyclone Laboratories Inc.; Logan, UT, USA) were also commercially purchased.

Animals

The experiment was conducted using male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing about 150–180 g ($n=4$–5). The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 15% with a 12-h light–dark cycle. A commercial animal chow (F-2, Funabashi Farms, Funabashi, Japan) and tap water were freely available throughout the acclimatization and experimental periods.

Experimental schedule

Animals were intravenously administered CPT-11 (60 mg/kg) from the tail vein once a day (a.m.) for four consecutive days (from days 1 to 4). In the following three antibiotic groups, 2 mg streptomycin and 1 mg penicillin, 2 mg neomycin, or 2 mg neomycin per ml of drinking water was administered from 5 days before the start of CPT-11 administration and throughout the experiment (days −5 to 4), respectively, to aspire complete individual antibiotic efficacy. Antibiotics (Str/Pen, streptomycin 20 mg/kg and penicillin 10 mg/kg; Neo, neomycin 20 mg/kg; Neo/Bac, neomycin 20 mg/kg and bacitracin 10 mg/kg), TJ-14 (1,000 mg/kg), or activated charcoal (1,000 mg/kg) were orally administered twice a day (a.m. and p.m.) from the day before (day −1) to 4 days after the start of CPT-11 injection. Under the CPT-11's regimen adopted [60 mg/kg i.v. once daily for consecutive 4 days (days 1–4)], the diarrhea monitored throughout days 5–8 was similar to human diarrhea in terms of being resistant to conventional antidiarrhea agents [38]. Diarrhea, the onset which was on or after day 5, was defined delayed diarrhea. The severity of delayed diarrhea and the daily body weight were monitored, and the results were used as an index of intestinal toxicity. The severity of delayed diarrhea was scored as follows:

- Normal (0, normal stool)
- Slight (1, slightly wet stool without staining of the coat)
- Moderate (2, wet and unformed stool with moderate perianal staining of the coat)
- Severe (3, watery stool with severe staining of the coat around the anus)

The total diarrhea score area under the score-day curve during days 5–9, and the mean score at each day were calculated. Watery diarrhea which appeared within about 2 h after the administration of CPT-11 was defined acute diarrhea. Before the start of CPT-11 administration on day 1, the fecal β-glucuronidase activity was determined by a modification of the procedure of Akao et al. [39], and the fecal pH using pH meter (HM-50G, Toa Electrics, Tokyo, Japan) after homogenization of the samples in 0.9% physiological saline.

In a separate experiment, the effects of several potential antidiarrhea treatments on CPT-11-induced antitumor activity and diarrhea were evaluated using rats bearing breast cancer. The rat breast cancer cell line Walker 256-TC cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan) and were cultured in vitro in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum. The cultures were grown at 37°C in a 5% CO_2–95% air atmosphere, and the passages were performed twice a week. The Walker 256-TC cells (1×10^5 cells/0.1 ml) were inoculated subcutaneously into the right flank of rats. When the mean estimated tumor volume reached about 300 mm^3 on day 7 after tumor inoculation, the rats were randomly divided into experimental groups (five rats per group) to have the similar mean estimated tumor volume, and were given CPT-11 at the same regimen mentioned above with the following potential antidiarrhea compounds. (1) TJ-14 1,000 mg/kg, (2) activated charcoal 1,000 mg/kg, (3) Neo/Bac, neomycin 20 mg/kg and bacitracin 10 mg/kg—these three treatments showed an obvious antidiarrhea activity in the normal rats in the present study, (4) cyclosporin A 50 mg/kg, (5) valproic acid 200 mg/kg. Both cyclosporine A and valproic acid had been expected to enhance CPT-11's antitumor activity with reduced intestinal toxicity because both increase the area under plasma concentration–time curve of SN-38 by lowering biliary excretion of SN-38 or by inhibiting SN-38 conjugation. All these compounds were orally administered twice daily (a.m. and p.m.) for days −1 to 4, except that Neo/Bac group received 2 mg neomycin per ml of drinking water from 5 days before the start of CPT-11 administration. The estimated tumor volume was measured 4, 7, and 10 days after the start of CPT-11 administration, and the severity of delayed diarrhea and the daily body weight were also monitored.

The estimated tumor volume was calculated using the formula:

$$\text{The estimated tumor volume } (mm^3) = \frac{L \times W^2}{2},$$

where L and W represent the length and the width of the tumor mass, respectively.

All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co., Ltd.

Results

Fecal pH and β-glucuronidase activity in normal rats

On day 1 (6 days after the start of antibiotics administration), the fecal pH and β-glucuronidase activity in the control group were 6.42 and about 190 nmol/min/mg protein, respectively. The fecal pH in the treatment groups was similar to that in the control group, except for Str/Pen group in which the fecal pH was 7.20. The fecal β-glucuronidase activities in Str/Pen and Neo/Bac treated groups were reduced to less than approximately 10% of the control group. The fecal β-glucuronidase activity of Neo group was also reduced but it remained in about 20% of the control group. The fecal β-glucuronidase activities in TJ-14 and activated charcoal groups had somewhat higher values as compared with that of the control group but it was not a statistically significant change (Fig. 1).

Effects on CPT-11-induced body weight loss and diarrhea symptoms in normal rats

Following the i.v. administration of CPT-11 (60 mg/kg once daily for the consecutive 4 days: days 1–4), body weight decreased from day 2 and reached a nadir on day 8, being about 23% decrease as compared with the initial value (day 1). No diarrhea was present during the first 2 days, but acute watery diarrhea occurred on days 3 and 4 within 1–2 h after CPT-11 injection. Thereafter, diarrhea was chronically present during days 5–8 (delayed diarrhea).

Each treatment had little or no effect on CPT-11-induced decrease in body weight during days 2–3. On or after day 4, either treatment inhibited the decrease in body weight, and improved the delayed diarrhea symptoms. Str/Pen, Neo, Neo/Bac, and activated charcoal, but not TJ-14, also inhibited the acute watery diarrhea that appeared on days 3 and 4. There was an obvious difference of the effectiveness among the treatments. In consideration of the changes of body weight and diarrhea score, the rank order for beneficial effect on CPT-11-induced intestinal toxicity was Str/Pen = TJ-14 = Neo/Bac > activated charcoal > Neo (Figs. 2, 3).

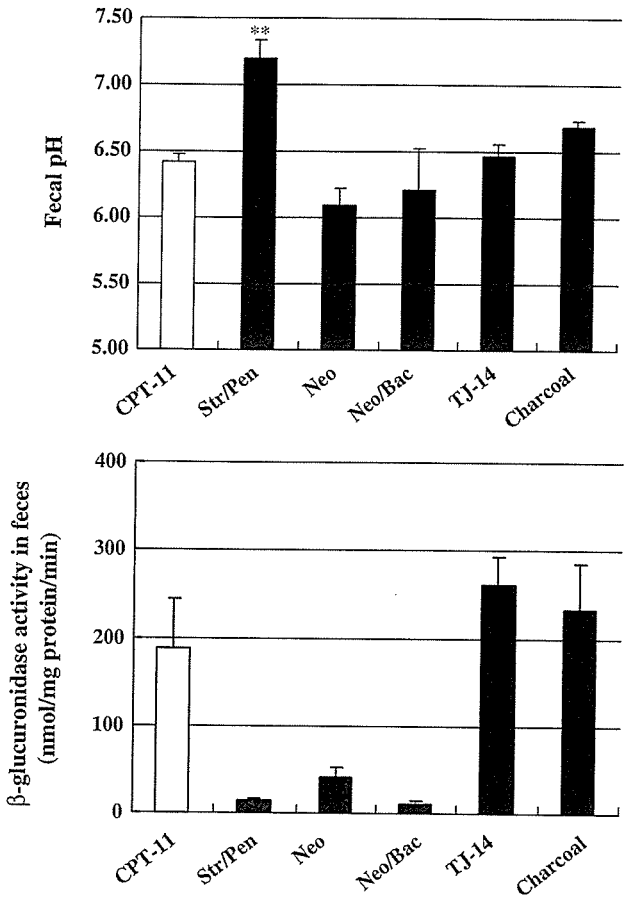

Fig. 1 The pH and β-glucuronidase activity of the feces on the day (day 1) of the start of CPT-11 (60 mg/kg, once daily for 4 days) injection in normal rats. Antibiotics, except for bacitracin, were administered in drinking water from 5 days (day −5) before the start of CPT-11 injection. Each data represents the mean of 4–5 animals. $^{**}P<0.01$ versus CPT-11 group (Dunnett test). *Str/Pen* streptomycin (2 mg/ml) and penicillin (1 mg/ml) in drinking water + streptomycin 20 mg/kg and penicillin 10 mg/kg p.o.; *Neo* neomycin (2 mg/ml) in drinking water + neomycin 20 mg/kg p.o.; *Neo/Bac* neomycin (2 mg/ml) in drinking water + neomycin 20 mg/kg and bacitracin 10 mg/kg p.o.; *TJ-14* TJ-14 1,000 mg/kg p.o. *Charcoal* activated charcoal 1,000 mg/kg p.o

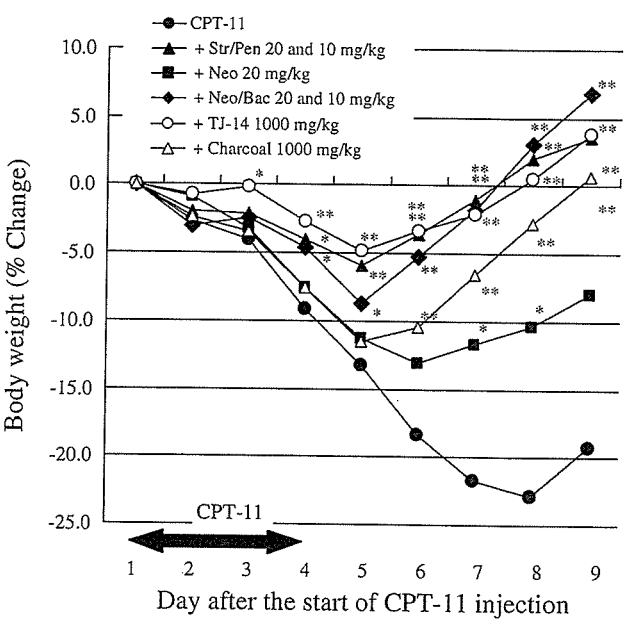

Fig. 2 Effects of several agents on CPT-11-induced body weight loss in rats. CPT-11 was given intravenously at a dose of 60 mg/kg once daily for four consecutive days (days 1–4). The agents were orally administered twice daily from the day before to 4 days after the start of CPT-11 injection. In addition, antibiotics, except for bacitracin, were administered in drinking water from 5 days before to 4 days after the start of CPT-11 injection. The change in body weight was calculated on the basis of that on day 1. Each point represents the mean of 4–5 animals. The abbreviations are referred in Fig. 1. $^{*}P<0.05$, $^{**}P<0.01$ versus CPT-11 group (Dunnett test)

Effects on CPT-11-induced antitumor activity and intestinal toxicity (body weight loss and diarrhea symptoms) in rats bearing breast cancer

The mean estimated tumor volume in the vehicle control group increased linearly and reached about 10,000 mm^3, which was approximately 30-fold its initial mean estimated tumor volume of 300 mm^3, on day 10. CPT-11 (60 mg/kg once daily for the consecutive 4 days: days 1–4) showed moderate but significant reduction of the mean estimated tumor volume on days 4 and 7, but its antitumor effect was no longer apparent (not significant) on day 10. The body weight decreased from day 2 and reached a nadir on day 6, being about 15% decrease as compared with the initial value (day 1). No diarrhea was present during the first 2 days, but acute watery diarrhea

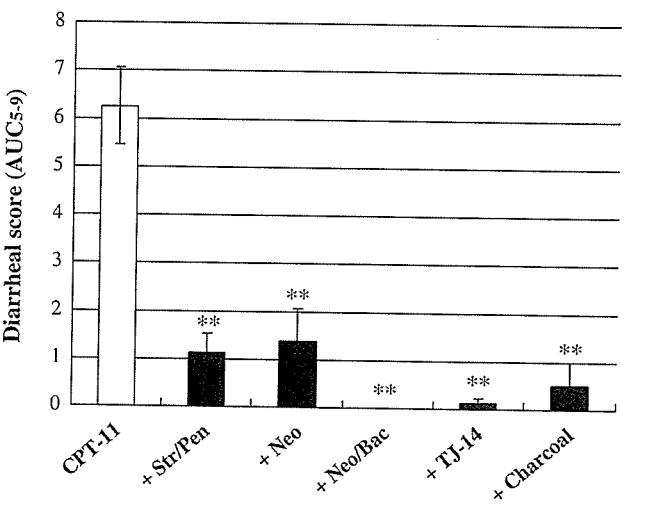

Fig. 3 Effects of several agents on CPT-11 (60 mg/kg i.v., once daily for 4 days)-induced delayed diarrhea symptoms in rats. The agents were orally administered twice daily from the day before to 4 days after the start of CPT-11 injection. In addition, antibiotics, except for bacitracin, were administered in drinking water from 5 days before to 4 days after the start of CPT-11 injection. Each data represents the mean of 4–5 animals. The abbreviations are referred in Fig. 1. $^{**}P<0.01$ versus CPT-11 (Wilcoxon rank sum test)

occurred on days 3 and 4 within 1–2 h after CPT-11 injection. Thereafter, diarrhea was chronically present during days 6–7 (delayed diarrhea).

TJ-14, Neo/Bac, and activated charcoal inhibited the decrease in body weight, and improved the delayed diarrhea, but had no effect on the antitumor effect of CPT-11. Neo/Bac, but not TJ-14 or activated charcoal, also inhibited the acute watery diarrhea that appeared on days 3 and 4. In contrast, cyclosporin A and valproic acid augmented the loss of body weight gain and delayed diarrhea symptom score while those had no effects on CPT-11's antitumor activity (Figs. 4, 5). In addition, the acute diarrhea appeared not only on days 3 and 4 but also on day 1 in cyclosporin A and valproic acid groups.

Discussion

The clinical use of CPT-11 has been associated with early onset diarrhea that is observed immediately after CPT-11 injection (acute diarrhea) and delayed-onset diarrhea that occurs more than 24 h after CPT-11 injection and usually continues for several days (delayed diarrhea) [9, 10]. The former was usually accompanied with cholinergic symptoms such as salivation, cramps, and diaphoresis, and could be controlled with cholinergic receptor blocker atropine [40]. Therefore, its anticholinesterase activity [41, 42] is at least involved in the cholinergic symptoms including acute diarrhea. Indeed, we have confirmed that CPT-11 has not only anti-acetylcholinesterase activity but also anti-butyrylcholinesterase activity which plays a major role in the intestinal tract (unpublished data).

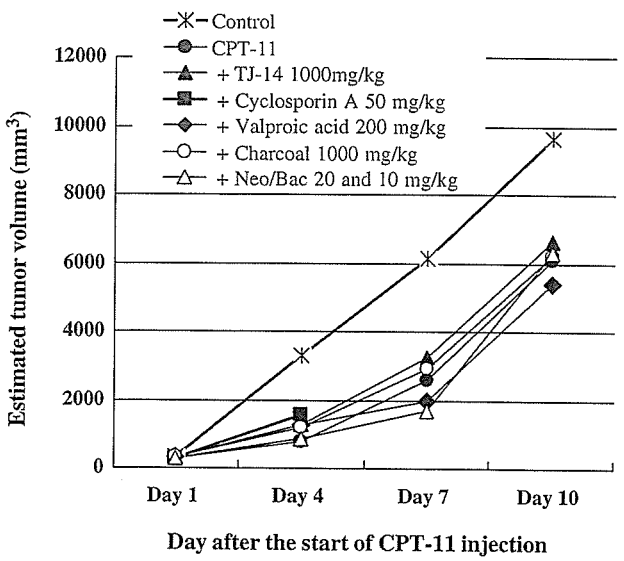

Fig. 4 Effects of several agents on antitumor activity of CPT-11 (60 mg/kg iv for 4 days) in rats bearing breast cancer (Walker 256-TC). The agents were orally administered twice daily from the day prior to the start of CPT-11 injection for total of 5 days. In addition, neomycin was administered in drinking water from 5 days prior to the start of CPT-11 injection for total of 9 days. All rats died until day 7 in cyclosporin A-treated group. One rat died on day 8 in valproic acid-treated group. *TJ-14* TJ-14 1,000 mg/kg, p.o.; *cyclosporin A* cyclosporin A 50 mg/kg, p.o.; *valproic acid* valproic acid 200 mg/kg, p.o.; *charcoal* activated charcoal 1,000 mg/kg, p.o.; *Neo/Bac* neomycin (2 mg/ml) in drinking water + neomycin 20 mg/kg and bacitracin 10 mg/kg, p.o. Each value represents the mean of 2–5 animals. There are no significant differences of the estimated tumor volumes between the CPT-11 alone and CPT-11 with agents on days 4, 7, or 10 (Student's *t* test)

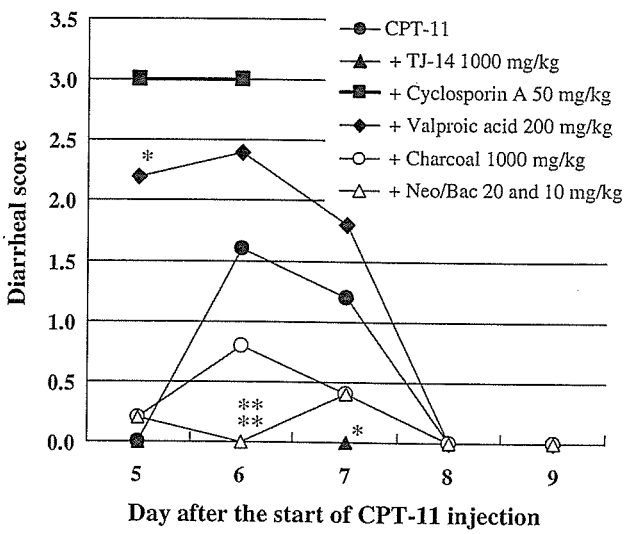

Fig. 5 Effects of several agents on delayed diarrhea symptoms caused by CPT-11 (60 mg/kg iv for 4 days) in rats bearing breast cancer (Walker 256-TC). The agents were orally administered twice daily from the day prior to the start of CPT-11 injection for total of 5 days. In addition, neomycin was administered in drinking water from 5 days prior to the start of CPT-11 injection for total of 9 days. All rats died until day 7 in cyclosporin A-treated group. One rat died on day 8 in valproic acid-treated group. The abbreviations are referred in Fig. 4. Each value represents the mean of 2–5 animals. $*P<0.05$, $**P<0.01$: Significantly different from the group treated with CPT-11 alone (Wilcoxon rank sum test)

In contrast, the latter is unexpected diarrhea, the severe [National Cancer Institute-Common Toxicity Criteria (NCI-CTC) grade 3 or 4] diarrhea might be a potentially life-threatening disorder, especially when concomitant with severe neutropenia. Although many pharmacokinetic analysis in humans have been made to predict the incidence or the mechanisms of delayed diarrhea, there are somewhat conflicting results [11–16]. Namely, there are no generally accepted relationship between the severity of diarrhea and any of the studied pharmacokinetic parameters.

Intensive loperamide regimens have been considered as the standard antidiarrhea treatment for CPT-11-induced diarrhea in Europe and the United States. It is one of the nonspecific treatments for cancer chemotherapy-induced diarrhea and probably reduces diarrhea by delaying intestinal transit allowing increased time for fluid absorption or reducing the fluid secretion [43], but the clinical studies could not necessarily confirm its satisfied efficacy [44]. Other potential approaches are (1) altering the metabolism [(a) inhibition of deconjugation

of SN-38 glucuronide excreted into the intestinal lumen [28, 30, 45], (b) inhibition of glucuronidation of SN-38 in the liver [20], (c) inhibition of biliary excretion of CPT-11 and its metabolites [21–24], (d) selective inhibition of intestinal SN-38 production [46], (2) prevention of intestinal re-absorption of SN-38 and/or CPT-11 by alkalization of the intestinal lumen [34, 35] or by adsorbing of CPT-11 and its metabolites using activated charcoal [36, 37], or (3) blockade of CPT-11-induced fluid secretion [43]: these are all specific measures for CPT-11-induced diarrhea (Fig. 6). In addition, other potential nonspecific treatments have also been reported (Table 1). Some of those have already been shown to improve CPT-11-induced diarrhea, but no one can expect which is the most promising and effective treatment for CPT-11-induced delayed-onset severe diarrhea.

In the present study, we assessed the efficacy of several potential antidiarrhea treatments that have been shown to be effective clinically or currently under clinical trial, in comparison with those of Str/Pen or TJ-14 treatment that has been confirmed effective in our rat model [28, 30]. The present result was well in agreement with our previous reports, namely both Str/Pen and

Table 1 Summary of nonspecific approaches and other potential approaches to prevent CPT-11-induced diarrhea

Nonspecific measures
Enkephalinase inhibitor (Tiorphan) [51]
COX_2 inhibitor (Celecoxib) [52]
IL-15 [53]
Sandostatin [44, 54, 55]
Lipopeptide JBT-3002 [56, 57]
RDP58 [58]
Radical scavenger (amifostine) [59]
Sucralfate and nifuroxazide [60]
Thalidomide [61, 62]
Glutamate [63]
Steroid (budesonide) [64]
Fish oil [65]
Modified schedule of CPT-11 dosing [66–68]
Pharmacogenetic analysis of UDP-glucuronosyltransferase [69]

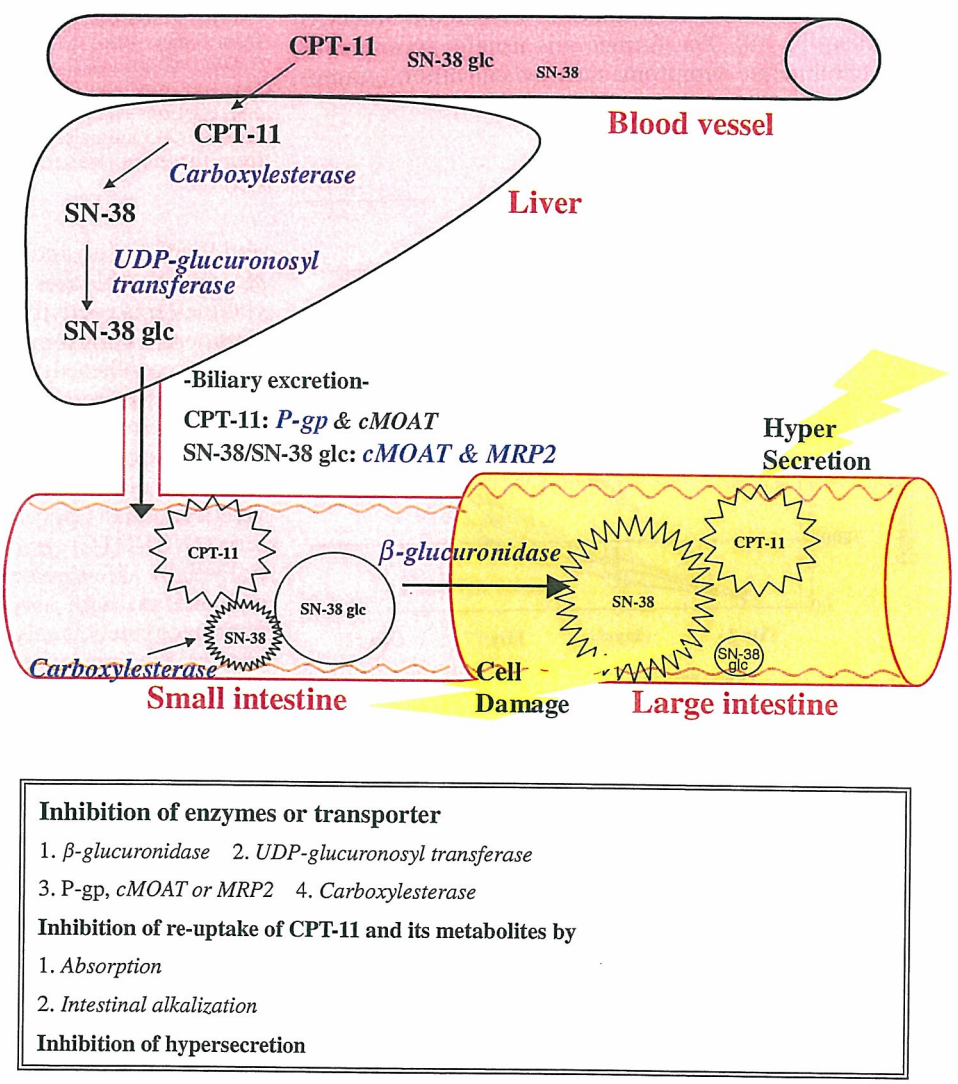

Fig. 6 Schematic representation of CPT-11 metabolism, expecting CPT-11-specific approaches to prevent intestinal toxicity of CPT-11

TJ-14 showed good antidiarrhea activity against CPT-11-induced delayed diarrhea, and first revealed that their efficacy was almost equivalent. The other poorly absorbed aminoglycoside antibiotics Neo/Bac also showed good antidiarrhea activity similar to that of Str/Pen or TJ-14. In contrast, the efficacy of single co-administration of neomycin was relatively low as compared with the above three treatment regimens despite inhibition (about 80%) of intestinal β-glucuronidase activity. The possible reason why neomycin could not ameliorate the CPT-11-induced intestinal toxicity as other two antibiotic regimens might be due to an incomplete inhibition of β-glucuronidase activity (<80% in Neo vs. >90% in Str/Pen or Neo/Bac). Another possible reason might be due to the change of fecal pH. Takeda et al. [34] and Ikegami et al. [35] have recently reported that intestinal alkalization by sodium bicarbonate supplementation ameliorated CPT-11-induced diarrhea with reduction of the histopathological damage to the mucosa of the intestine by influencing the conversion of SN-38/CPT-11 from lactone to carboxylate. In the present study, the fecal pH in Str/Pen group changed to be about pH 7.2 from the pH 6.4 (control group). The respective rates of intestinal uptake for CPT-11 and SN-38 were shown to be pH sensitive, with uptake decreasing by more than 65% at pH levels greater than 6.8 [47], suggesting that intestinal alkalization by Str/Pen might, at least in part, be involved in the ameliorating mechanism while the reason of the change in pH is not known. Since, however, there is no change in the fecal pH of Neo/Bac or TJ-14 group which exerted efficacy comparable to Str/Pen, alkalization in the intestinal lumen might not play a key role in the ameliorating efficacy of these treatments used in the present study.

An alternative measure for the inhibition of β-glucuronidase in the intestinal lumen is pharmacological inhibition using specific inhibitors including natural glucuronides [48]. Indeed, we have reported that TJ-14 or TJ-114 (0.5 and 1 g/kg twice daily), a Chinese herbal medicine that contains β-glucuronidase inhibitor baicalin, or baicalin itself (25 mg/kg) exerted a protective effect on the delayed-onset diarrhea in rats [30]. D-Glucaric acid-1,4-lactone monohydrate, a specific β-glucuronidase inhibitor, has recently been shown to reduce CPT-11-induced mucosal damage in the small intestine in rats [45]. Our preliminary study, however, did not confirm its antidiarrhea activity. The dose of glucaro-1,4-lactone used in the preliminary study (25 mg/kg orally twice daily) might be enough to inhibit β-glucuronidase because it has β-glucuronidase inhibitory activity comparable to baicalin [48]. The reason why glucaro-1,4-lactone had no efficacy in our rat model remains to be determined. We reported that CPT-11-induced delayed-onset diarrhea would be attributable to the damage to the cecum, which has the highest β-glucuronidase activity in the luminal contents, and the inhibition of β-glucuronidase by antibiotics resulted in mainly the reduction of the cecal damage, not of the small intestine [28]. Since Fittkau et al. [45] reported that glucaro-1,4-lactone reduced CPT-11-induced mucosal damage in the small intestine which almost lacks β-glucuronidase activity in the luminal contents [28], other mechanisms apart from β-glucuronidase inhibition in the intestinal lumen might be involved. Alternatively, the therapeutic effect of Kampo medicine TJ-14 on CPT-11-induced delayed diarrhea might be solely based on the inhibition of SN-38 glucuronide deconjugation but also on other mechanisms including a suppression prostaglandin E_2 production in the colon [49].

Chowbay et al. [50] reported that activated charcoal was not effective in the prevention of CPT-11-induced diarrhea as compared with inhibition of β-glucuronidase in the intestinal microflora by ceftriaxone, a third generation cephalosporin. In the present study, activated charcoal showed clearly improved CPT-11-induced intestinal toxicity though its activity was slightly weak as compared with Str/Pen, Neo/Bac, or TJ-14. Therefore, the adsorption of CPT-11 and its metabolites using activated charcoal could offer some help in reducing CPT-11-induced diarrhea as reported by Michael et al. [36] and Maeda et al. [37].

Although the pharmacokinetic or histopathologic examinations were not conducted in the present study, we have shown good correlation between the severity of indices of intestinal toxicity adopted in this study and histopathological changes in the intestine [28, 30]. Moreover, the inhibition of β-glucuronidase in the intestinal microflora by antibiotics [29, 50] or TJ-14 (unpublished data) did not affect SN-38 or CPT-11 plasma pharmacokinetics. It is suggested that the antibiotics or TJ-14 could prevent CPT-11-induced intestinal toxicity without reducing antitumor activity. Indeed, antibiotics (Neo/Bac), TJ-14, or activated charcoal ameliorated CPT-11-induced intestinal toxicity with maintenance of CPT-11's antitumor activity in rats bearing breast cancer in the present study. Since the biliary excretion of CPT-11, its active metabolite SN-38 and SN-38 glucuronide are mediated by the P-gp and cMOAT/MRP2 in the bile canalicular membrane [20–24], inhibition of the transporters, or UDP-glucuronosyl transferase has been proposed to reduce the intestinal toxicity of CPT-11 by decreasing the biliary excretion of particularly SN-38 and SN-38 glucuronide or potentially increase the CPT-11 therapeutic index by decreasing the intestinal toxicity associated with more aggressive antitumor regimens. In the present study, contrary to ones expectations, cMOAT/MRP2 inhibitor cyclosporin A or UDP-glucuronosyl transferase inhibitor valproic acid exacerbated the intestinal toxicity, and did not modify CPT-11's antitumor activity. As we do not confirm whether or not CPT-11 can show dose-dependent antitumor activity in the present rat model bearing breast cancer, we cannot conclude that co-administration of cyclosporin A or valproic acid does not enhance CPT-11's antitumor activity from the present study. However, the fact that cyclosporin A and valproic acid caused a worsening intestinal toxicity, and may produce adverse systemic reaction, probably keeping SN-38 serum and

tissue levels too high [20, 21, 23], is suggesting that it is at risk of potentiating the systemic and/or intestinal side effect of CPT-11 to control biliary excretion of CPT-11 and its metabolites by drugs for preventing CPT-11-induced intestinal toxicity in consideration of individual difference of biliary pharmacokinetics.

The present results conclude that the optimal combined use of antibiotics which completely reduces the intestinal, bacterial β-glucuronidase activity prevents CPT-11-induced intestinal toxicity to a similar extent of Kampo medicine TJ-14, and activated charcoal, a more inexpensive agent, may also be useful when antibiotics or TJ-14 could induce severe secondary complications. Moreover, the treatments affecting the biliary excretion of CPT-11 and its metabolites might have undesirable results.

To date, a lot of CPT-11 specific and nonspecific (Fig. 6, Table 1) antidiarrhea treatment designs have been proposed in animal and human studies. However, to our knowledge only Kampo medicine and antibiotics could improve CPT-11-induced delayed-onset diarrhea in both animal and human studies although our rat model might be different from human in terms of types of intestinal microflora and anatomical distribution. Therefore, we currently think that Kampo medicine, antibiotics, or both treatments if possible, are the optimal antidiarrhea treatments against CPT-11-induced diarrhea.

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Comparative *Cytochrome P450 -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* Expressions in Human Larynx Tissue Analysed at mRNA Level

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ABSTRACT: The metabolic activation of numerous exogenous and endogenous chemicals is catalysed by cytochrome P450 enzymes (CYPs). The aim of this study was to analyse the expression of the individual forms of CYP at the mRNA level in human larynx and quantitatively to compare their expressions in human liver, the main organ of CYP expression. Individual forms of CYP mRNAs were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers for the *CYPs -1A1, -1A2, -2A6, -2B6, -2C, -2D6, -2E1, -3A3/4, -3A5, -3A7* and *-4B1*. An RNA competitor of known copy number, covering the primer sequences necessary to amplify the entire object *CYPs* within a single molecule, was used as reference. This study reports a consistent detection of mRNAs for the *CYPs -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* in the human larynx tissue. The data indicate that the human larynx highly resembles the lung tissue in CYP content, as a comparable subset of CYP mRNAs was detected in the larynx previously reported for human lung with the exception of *CYP1A2*. The results are discussed in quantitative ratios of the detected CYP mRNAs in relation to the hepatic CYP expression. Copyright © 2006 John Wiley & Sons, Ltd.

Key words: cytochrome P450; laryngeal tissue; drug metabolizing enzymes

Introduction

Cytochrome P450 (CYP) (E.C. 1.14.14.1) is a heme-containing enzyme responsible for the metabolism of numerous endogenous and exogenous compounds [1]. Approximately 20 individual CYPs have been identified in man [1,2]. The regulation of *CYP* expression is, in part, tissue-specific leading to a tissue-selective response for a given xenobiotic compound. The majority of *CYP* genes are expressed most abundantly in the liver, yet many *CYPs* were also reported in extrahepatic tissues [3]. Relatively little is known about the individual CYP forms present in the human larynx. The larynx forms a connection from the pharynx to the trachea and it has histological resemblances to the nasal mucosa, trachea and lung, which are formed by a set of hyaline and elastic cartilages in a complex muscular architecture [4]. Its function is to maintain patency of the passage to prevent swallowed food or liquid from entering the trachea in a valve-like manner. Being a tissue with a high exposure to both inhaled and blood-borne xenobiotic compounds, the larynx is an important target for cancer, which makes it a

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valuable tool in the search for metabolic bioactivators of carcinogens [5]. In order to understand the metabolism of xenobiotics and endogenous compounds, information on the expression of individual members of the CYP superfamily in larynx tissue is required as the individual CYPs may protect the body against orally ingested xenobiotics as well as influence the bioavailability of therapeutic compounds. The aim of this study was to detect the presence of mRNAs for *CYPs -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* as an indication of the potential expression in human larynx tissue and to compare them with hepatic CYP expression using the reverse transcriptase-polymerase chain reaction (RT-PCR) method.

Materials and Methods

Subjects

A total of ten larynx tissue samples (two malignant, four premalignant and four normal) of about 10–100 mg from individuals between the ages of 33 and 84 were excised with direct laryngoscopy under general anesthesia. A total of six liver biopsy specimens about 10–100 mg were obtained and pooled from the hepatectomic material of patients undergoing liver transplantation at Ege University Hospital, Izmir, Turkey. Samples tissues were frozen immediately in liquid nitrogen. A written informed consent was obtained from each patient and the study was approved by the Ethics Committee of Ege University, Izmir, Turkey.

RT-PCR

Tissues were homogenized in a microtube using a homogenizer (Ika-Werke, Staufen, Germany). Total RNA was isolated using a Highpure RNA tissue kit (Roche, Penzberg, Germany) and cDNA was synthesized using a Takara RNA PCR kit (AMV.Ver.2) (Takara, Otsu-Shiga, JA) according to the manufacturer's specifications. The primers for detecting *CYPs -1A1, -1A2, -2B6, -2C, -2D6, -2E1, -3A3/4, -3A5, -3A7, -4B1* and *GAPDH* were purchased as a human cytochrome P450 competitive RT-PCR kit (Takara, Otsu-Shiga, JA). The human cytochrome P450 RNA competitor with a 3′-poly (A) tail (1.0×10^8 copies/μl) was purchased from Takara Bio Inc (Takara, Otsu-Shiga, JA). It covered the primer sequences necessary to amplify all the object templates in a single molecule with +25% to −25% size differences with target mRNAs in order to have similar amplification efficiency for each fragment. The *CYP2A6* primers (2A6-KK) were designed using the Genbank database (accession number; NM 00762 and NG 000008); 5′-CCCTCA TGAAGATCAGTGAGC-3′ (forward) and 5′-GC GCTCCCCGTTGCTGAATA-3′ (reverse), giving a band of 200 bop upon PCR amplification. Another set of *CYP2A6* primers (2A6-DS) were synthesized according to Vondracek and his coworkers [6]. The PCR conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and polymerization at 72 °C for 1 min.Both cDNA synthesis and PCR amplifications included negative control reactions, which were set up by excluding RNA and DNA templates, respectively. The products were analysed on 2% agarose gels in TBE buffer and gels were photographed under UV light after staining with ethidium bromide (EtdBr) (0.5 μg/ml). All reactions were carried out in nuclease-free microcentrifuge tubes.

Data evaluation

Band intensities were quantified from gel photographs using BioRad Multianalyst (Ver.1.1). The lane with the same band intensity between the products derived from the target mRNA and from the RNA competitor was determined. The expression level in copies per ng total RNA for a given CYP was determined using the following formula [7].

Expression level (copies/ng total RNA)=RNA competitor concentration (copies/μl) × volume of competitor (μl)/the amount of total RNA (ng).

Quantification for *CYP2A6* was carried out by comparing with the *GAPDH* amplification upon densitometric analyses. To control the reproducibility, all amplifications were repeated at least once.

Results

In this study a competitive RT-PCR method was employed to analyse the expression of *CYP* genes

in larynx tissues by including a competitor RNA with a 3′-poly (A) tail for reverse transcription. Primers were used covering *CYPs -1A1, -1A2, -2A6, -2B6, -2C, -2D6, -2E1, -3A3/4, -3A5, -3A7* and *-4B1* (Table 1). A representative agarose gel photograph of the amplified *CYPs* using a RNA competitor in larynx tissue is given in Figure 1A. All the amplified products were detected at their expected molecular weights (Table 1). In Figure 1A, the PCR products of *CYPs -2C, -2E1, -3A5, -2D6, -4B1, -1A1* and *-2B6* are shown as two bands (Figure 1A, lanes 2–8). The lower bands in the lanes for *CYPs -2D6, -4B1, -1A1* and *-2B6* (Figure 1A, lanes 5, 6, 7 and 8, respectively) originated from their corresponding mRNAs while the competitor gave rise to the upper DNA bands in this group of *CYPs* (Table 1). The mRNAs for *CYPs -2C, -2E1* and *-3A5* (Figure 1A, lanes 2–4, respectively) gave products with slower mobility due to the higher molecular weight relative to the competitor (Table 1). There were no detectable reaction products for the *CYPs -1A2, -3A3/4* and *-3A7* either in tumor or healthy larynx tissues (data not shown). On the other hand, the amplification for *CYP2A6* was carried out using two sets of primers, 2A6-KK and 2A6-DS, with expected PCR amplification products of 200 bp and 423 bp, respectively (Figure 1A, lanes 9 and 10, respectively). The two other bands detected above 200 bp were due to non-specific amplification products in the course of polymerization (Figure 1A, lane 9). The competitor was not included in this particular reaction as its sequence did not cover *CYP2A6*-specific primers. The products of *GAPDH* primers, used as a positive control, are shown in Figure 1A, lane 11. No amplification was detected in the negative control reaction which was set up without including the template (Figure 1A, lane 12).

The procedure was repeated using a pooled liver tissue homogenate (Figure 1B). Being the major organ of CYP synthesis, the liver tissue gave rise to the additional *CYP* bands. The products for the *CYPs -2C, -2E1, -3A5, -2D6, -3A3/4, -1A1, -2B6, -1A2* and *-3A7* were amplified in liver in the presence of RNA competitor (Figure 1B, lanes 3–7, 9–12, respectively). Unlike larynx samples, *CYP1A2, CYP3A3/4* and *CYP3A7* mRNAs were detected (see Figure 1B, lanes 11, 7,

Table 1.

Primer	Amplified product (bp)	
	Competitor	mRNA
CYP1A1	507	433
CYP1A2	370	309
CYP2B6	445	376
CYP2C	287	333
CYP2D6	387	339
CYP2E1	300	366
CYP3A3/4	284	324
CYP3A5	384	471
CYP3A7	545	475
CYP4B1	442	398
CYP2A6-KK	NA	200
CYP2A6-DS	NA	423
GAPDH	486	546

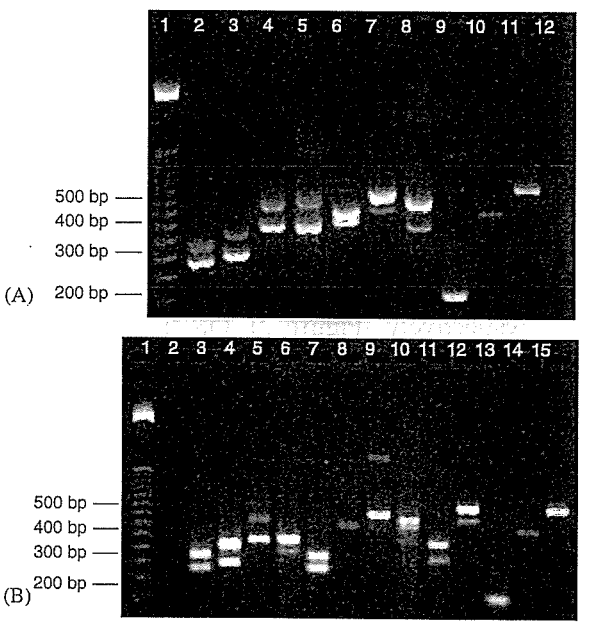

Figure 1. A representative agarose gel photograph of the amplified products in larynx (A) and liver tissues (B). (A) The PCR products (20 µl) were applied on to 2% agarose gel, run in TBE (5 V/cm) and stained with EtdBr (0.5 µg/ml). Lanes (1) 50 bp. DNA standards, PCR products with (2) CYP2C-; (3) CYP2E1-; (4) CYP3A5-; (5) CYP2D6; (6) CYP4B1-; (7) CYP1A1-; (8) CYP2B6-; (9) CYP2A6-KK; (10) CYP2A6-DS; (11) GAPDH-specific primers; (12) negative control reaction. (B) Lanes (1) 50 bp. DNA standards; (2) negative control reaction, PCR products with (3) CYP2C-; (4) CYP2E1-; (5) CYP3A5-; (6) CYP2D6-; (7) CYP3A3/4-; (8) CYP4B1-; (9) CYP1A1-; (10) CYP2B6-; (11) CYP1A2-; (12) CYP3A7-; (13) CYP2A6-KK; (14) CYP2A6-DS; (15) GAPDH-specific primers

12, respectively) while no *CYP4B1* mRNA was found in the liver (Figure 1B, lane 8). The *CYP2A6* amplification using both 2A6-KK and 2A6-DS primer sets gave bands at the expected molecular weights (Figure 1B, lanes 13 and 14, respectively, and Table 1). No product was detected in the negative control reaction (Figure 1B, lane 2) and *GAPDH* amplification was used as a positive control for *CYP2A6* (Figure 1B, lane 15).

The next step was to compare target RNA-based amplifications with competitive RNA-based amplifications by using a competitor of known copy number. The results for the individual *CYPs* for larynx and liver tissues in averaged copy numbers per μl obtained from ten larynx tissue samples are given in Figure 2. As seen in Figure 2, the liver had an average of approximately 2.3 fold mRNA copy number for *CYP2C* (9.0×10^6 copy/μl vs 3.9×10^6 copy/μl, for liver and larynx, respectively) and 3.4 fold mRNA copy number for *CYP2E1* (2.5×10^6 copy/μl vs 8.4×10^6 copy/μl, in larynx and liver, respectively) (Figure 2). The *CYP2D6* and *CYP1A1* mRNA copy numbers were comparable in both tissues. Larynx and liver tissues gave *CYP2D6* mRNAs of 3.4×10^6 copy/μl and 3.6×10^6 copy/μl, respectively, and *CYP1A1* mRNAs of 2.7×10^6 copy/μl and 2.4×10^6 copy/μl, respectively (Figure 2). On the other hand, *CYP3A5* and *CYP2B6* mRNAs were found to be slightly higher in larynx (1.5 and 1.6 fold, respectively) compared with that of the liver (Figure 2). Comparison for *CYP2A6* was carried out from the average band intensities of the larynx samples relative to *GAPDH*. There was a lower average detection of *CYP2A6* mRNA in larynx based on the comparison of the amplified products to *GAPDH* control (5.9 vs 9.8 pmol/μl, in larynx and liver, respectively) (data not shown).

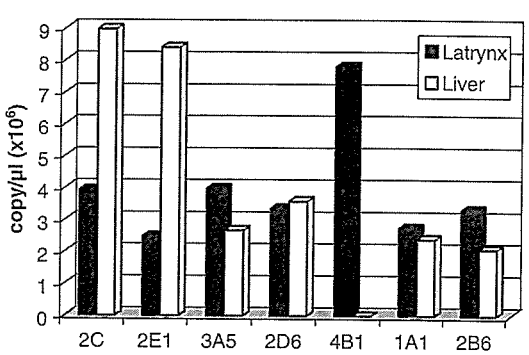

Figure 2. Comparative CYP expression levels in larynx and liver tissues. Band intensities were quantified from gel photographs using BioRad Multianalyst (Ver.1.1) (see Materials and Methods for the details of the experiments)

Discussion

The expression of CYPs can be determined by various methods including immunoblotting, immunohistochemistry and RT-PCR [8]. Given that interpretation of antibody-requiring methods is influenced by the non-specific binding of antibodies, RT-PCR is generally considered as a highly sensitive method for the detection of small amounts of RNA molecules. However, accurate quantification with normal cycling is difficult as the amount of amplified product does not reflect the amount of template because of the plateau phase of PCR, which indicates that a similar amount of amplified products would be obtained, regardless of the initial template levels by performing sufficient cycling of PCR. Competitive RT-PCR, on the other hand, is a sensitive and specific method developed to overcome this difficulty in quantification [7].

The human liver samples used were not obtained from the same individuals that the larynx samples came from. It should be taken into account that the liver samples were used as positive control for the amplifications, as it is the major organ of CYP expression, i.e. reporting a minus result for a particular CYP in larynx would be valuable if the same primer set was capable of amplifying the same form of CYP already known to be expressed in liver. Therefore, it was not intended to compare individual's CYP profile between laryngeal and liver tissues. Liver tissue also served as a reference in the quantification of CYP mRNA levels in larynx. A particular CYP isoform, unless shown to be expressed in the larynx, was not subjected to quantification.

Because of its histological resemblance to nasal mucosa, trachea and lung, the CYP content of larynx is potentially comparable to this group of

extrahepatic tissues. Many microsomal *CYPs* were reported in human lung, including *CYPs -1A1, -1A2, -2B6, -2A6, -2E1, -3A4, -4B1, -2C* and *-2D6*, while the expression of *CYPs -2A6, -2A13* and *-2B6* were detected in trachea at mRNA level [3,9,10]. The expression of *CYPs -2A6, -2A13, -2B6, -2C, -2J2* and *-3A* were reported in human nasal mucosa [11,12]. Reports on CYP expressions in these reports were only qualitative.

The results demonstrated a consistent estimation of mRNAs for the *CYPs -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* in larynx. Although the range of individual CYP forms, covered in our study, do not exactly match those searched by the others, detecting a comparable subset of CYP mRNAs in larynx that is reported to be preferentially expressed in the respiratory tract strengthens its physiological resemblances to lung, trachea and nasal mucosa [12]. This study shows that human larynx is most comparable to the lung tissue in terms of CYP transcriptional levels. The *CYPs -1A2, -3A3/4* and *-3A7* were not detected in larynx. The absence of mRNA for *CYP1A2* in human lung was reported by a number of other studies [13–15]. Moreover, the absence of *CYP3A7* in larynx is supported by the CYP subset of respiratory tract organs, reported so far [16,17].

The mRNA copy numbers for *CYP2C* and *CYP2E1* in larynx were below that of their hepatic counterparts because these individual *CYP* forms, like several other individual forms, are predominantly expressed in liver [18]. However, a slightly higher copy number of mRNAs was detected for *CYP3A5* and *CYP2B6* in larynx compared with liver (1.5 fold and 1.6 fold, respectively). On the other hand, CYP3A5 is the main extrahepatic form of the CYP3A subfamily enzymes and not induced in human liver while inducible in other tissues such as human lung [17,19]. A number of other studies reported low to undetectable levels of hepaticCYP3A5 in 80%–90% of Caucasian populations [20,21], confirming our finding of high *CYP3A5* transcription in the larynx. This particular CYP was also reported to be polymorphically expressed in human liver [17]. Therefore the polymorphic nature of CYP3A5 should also be considered when interpreting its expression in the larynx.

Among the individual CYP mRNAs reported here, the human CYP2B6 is known as a minor drug-metabolizing enzyme accounting for only 0.2% to 2% of total hepatic CYP [22,23] while CYP1A1 is known for its role in the oxidative activation of polycyclic aromatic hydrocarbons and nitrosamines [1,24]. Together with CYP3A4, CYP1A1 forms ketoaldehydes. Both CYP1A1 and CYP4B1 forms have been detected mainly in extrahepatic tissues [2,25]. There is no published result on the expression of CYP4B1 protein, but its mRNA was reported in the human lung [19,26].

The CYP2Cs are an important subfamily of P450 enzymes that metabolize approximately 20% of clinically used drugs and it comprises four members in humans (CYP2C8, CYP2C9, CYP2C18, CYP2C19), however, virtually no role in bioactivation of toxic and carcinogenic chemicals is attributed to CYP2C [27]. Both CYP2D6 and CYP2E1 expressions were reported in several other tissues [21,28,29].

The CYP2A6 represents a relatively minor component (~4%) of the human CYPs and but it is a highly important CYP member because CYP2A6 catalyses the metabolic activation of a number of clinically used drugs and several procarcinogens, such as nitrosamines [30]. Besides the liver, CYP2A6 expression was also reported in human nasal and bronchial mucosa, trachea and lung [9–12]. In agreement with its profound localization in the liver, the larynx *CYP2A6* mRNA level was found to be half that of liver *CYP2A6* mRNA. However, the CYP2A6 is also a polymorphic enzyme [30], therefore its expression needs further analyses in extended research.

Studies correlating the quantitative levels of CYP expression with the amount of cigarette smoking were reported. Among the CYPs, the -1A1, -2E1, -3A5 and -2D6 were inducible by cigarette smoking in human lung [12]. Some other studies reported comparative CYP expression in tumor and non-tumor tissues [12]. The mRNA copies for individual CYP forms investigated in our study did not vary significantly due to either smoking habits or health conditions among individuals (data not shown). However, it should be noted that a total of ten tissue samples was not adequate to make a

comparison for the subjects with different health conditions and habitual characteristics.

In conclusion, this is the first report on the expression profile of *CYPs* in larynx tissue. Our detection method was limited to monitoring *CYP* mRNAs. The current data show that *CYPs -1A1, -2A6, -2B6, -2C, -2D6, -2E1, -3A5* and *-4B1* are transcribed in human larynx tissue. Although it is difficult to assess *in vitro* detection of mRNAs to *in vivo* protein expression, the results establish a base for further studies on the role of CYPs in larynx tissue.

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PHARMACOGENETICS

Impact of *CYP2D6*10* on H1-antihistamine-induced hypersomnia

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Abstract
Objective This study investigated the relevance of the cytochrome P450 (CYP) 2D6 genotype to the adverse drug reactions (ADRs) of H1-antihistamines and the level of sedation.
Methods Japanese participants in a health screening program were asked to describe any past history of ADRs. Any subjects reporting ADRs induced by H1-antihistamines were then individually interviewed and defined as cases. Excessive daytime sleepiness, which had occurred in the cases as an H1-antihistamine-induced ADR, was assessed by the Epworth sleepiness scale (ESS), and an ESS score ≥12 was considered hypersomnia. *CYP2D6*4*, **5*, **14*, and **10* were genotyped by a panel of polymerase chain reaction techniques.
Results Out of 2,074 participants, 100 cases (M:F=37:63, mean age 51.9±9.2 years) were eligible for analysis. The most common etiological drug was chlorpheniramine, which is the most frequently used H1-antihistamine in Japan. *CYP2D6*10* allele and genotypes were more frequently found in the cases than in the healthy Japanese population in a large study ($P<0.005$ and $P=0.039$, respectively), but no difference was observed in the null alleles and genotypes. The ESS scores in 75 cases (M:F=25:50) who had experienced excessive daytime sleepiness were 9.5±5.5 in men and 12.9±6.1 in women ($P<0.001$, cases vs. 34 subjects without symptoms; $P=0.001$ men vs. women). The occurrence of hypersomnia increased as the number of *CYP2D6* mutant alleles increased ($P=0.045$).
Conclusion The results suggest that the presence of the *CYP2D6*10* allele is a risk factor for development of H1-antihistamine-induced ADRs in Japanese.

Keywords Cytochrome P450 2D6 · Gene polymorphism · H1-antihistamine · Adverse drug reaction · Epworth sleepiness scale

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Introduction

Cytochrome P450 (CYP) 2D6 is involved in the metabolism of more than 50 clinically important drugs, including antidepressants, antipsychotics, β-blockers, and H1-antihistamines [1–3]. It is polymorphically expressed, thus causing marked interindividual and interethnic variations [1, 4]. Many case reports as well as retrospective and prospective studies have demonstrated that individuals exhibiting extremes in CYP2D6 activity tend to have significantly more problems when they receive medications metabolized by CYP2D6 [5–9].

The frequency of a poor metabolizer (PM) phenotype has been estimated to be 5–10% in Caucasians and less than 1% in Japanese [1, 2, 4]. In Caucasians, *CYP2D6*3*, **4*, **5*, and **6* alleles account for over 95% of PMs [4]. In Japanese PMs, the allele frequencies of *CYP2D6*3*, **4*, **5*, **6*, and **14* are 0.000, 0.002, 0.051, 0.000, and 0.014, respectively; these variants are too rare to be epidemiologically relevant in the Japanese population [4]. Approximately 10–15% of Caucasians and up to 50% of East Asians appeared to exhibit impaired CYP2D6 activity [i.e., intermediate metabolizer (IM)] [1, 2, 4]. Several common IM alleles (e.g., *CYP2D6*9*, **10*, **17*, and **41*) have been identified [1, 2, 4]. The most common allele in Asians (allele frequency in Chinese of >50%) and thus perhaps the most common *CYP2D6* allele in the world is *CYP2D6*10*, with a frequency of about 40% in Japanese [2, 4]. *CYP2D6*10* has been shown to influence the pharmacokinetics of propranolol, codeine, haloperidol, and tramadol [10–13]. Although previous studies have reported that Japanese IMs were prone to antipsychotic-induced tardive dyskinesia [14, 15], the clinical relevance of the IM phenotype and/or the genotype to the CYP2D6 substrate-induced adverse drug reactions (ADRs) remains to be elucidated.

H1-antihistamines are frequently used as over-the-counter (OTC) or prescribed drugs, and many of them are metabolized either mainly (e.g., promethazine, mequitazine) or partially (e.g., chlorpheniramine) by CYP2D6 [3, 16]. The most common ADR of the first-generation H1-antihistamine is sedation, which is clearly governed by dose-response relationships [3, 16]. Therefore, individuals with a PM and/or IM phenotype of CYP2D6 might be identified from a large population by their past history of H1-antihistamine-induced excessive daytime sleepiness, without the loading of a probe drug.

In the present study, we investigated the impact of *CYP2D6* polymorphisms on the risk of ADRs induced by H1-antihistamines in a Japanese population, while paying special attention to the level of the sedating effect of H1-antihistamines.

Methods

Subjects

Approximately 3,300 participants in a health screening program at the Japanese Red Cross Kumamoto Health Care Center (Kumamoto, Japan) were asked by mail in 2003 about their past history of ADRs. Completed replies were available from 2,074 subjects (1,239 men and 835 women; mean age 52.0±10.2 years) for a response rate of 63.1%. The subjects who reported a past history of ADRs related to H1-antihistamines were then individually interviewed by pharmacists. A semistructured questionnaire was used to record pertinent data, the name of the etiological drugs, the doses, the indications, concomitant diseases, comedications, adverse symptoms, and detailed relationships between the symptoms and the H1-antihistamine use. The records were reevaluated by investigators, consisting of two pharmacists and a physician. The decision regarding inclusion and exclusion was made before genotyping *CYP2D6*. The following criteria were used to define cases that were included: an association of adverse reactions with H1-antihistamine treatment at standard doses, the absence of potent inhibitors of CYP2D6, and the absence of renal and hepatic failure. The subjects were excluded if sedation was a possible reason for receiving promethazine or other antihistamines. The study protocol was approved by the ethical review boards of the Graduate School of Medical and Pharmaceutical Sciences of Kumamoto University, the Japanese Red Cross Kumamoto Health Care Center, and Hokkaido University. In addition, all subjects gave their written informed consent to participate in the study.

Assessment of daytime sleepiness caused by H1-antihistamines

Excessive daytime sleepiness that had occurred in the cases as a symptom of the H1-antihistamine-induced ADR was assessed using the Epworth sleepiness scale (ESS) [17]. The ESS is a self-reported scale to measure sleep propensities in eight different real-life situations (range of an item score, 0–3). The ESS score is the sum of the eight item scores (range, 0–24); higher scores indicate being more sleepy. The ESS has a high sensitivity and high specificity, with a cut-off score ≥11 or 12 for daytime sleepiness [18]. In the present study, those who showed an ESS score ≥12 were diagnosed as having impairment in their daily activities due to their extraordinary sleepiness (hypersomnia). To determine the baseline ESS score, 34 age-matched controls without ADRs completed the ESS.

Determination of *CYP2D6* genotype

Genomic DNA was isolated from peripheral blood samples obtained from the cases with the use of an extraction kit (Wako Pure Chemical Industries, Osaka, Japan). The null allele **5*, in which the *CYP2D6* gene is deleted, was identified by long-range allele-specific polymerase chain reaction (PCR) using the method of Steen et al. [19]. The genotyping of null alleles **4* and **14*, as well as the reduced function allele **10*, was performed by a combined approach of PCR-restriction fragment length polymorphisms (RFLP) and allele-specific PCR. Briefly, the point mutation 188C>T that is associated with these alleles (i.e.,

*4, *10, and *14) was detected by the PCR-RFLP method as described by Wang et al. [20]. Next, these three alleles were discriminated by identifying mutations 1934G>A and 1846G>A using the PCR-RFLP method of Wang et al. [20] and the allele-specific PCR method of Kubota et al. [21], respectively. When these variant alleles were absent, the allele was identified as wild type allele with a normal function (e.g., *1 or *2).

A large study on the genotype and allele frequencies of general Japanese populations was used to calculate the expected genotype frequencies [4].

Data analysis

All data are given as the mean ± standard deviation. The chi-square test was used to compare allele and genotype frequencies, along with other categorical tests. Two-way analyses of variance, the Mann-Whitney U–test, and Fisher's exact test were used to analyze any differences in the ESS scores. These statistical analyses were done using the SPSS software package (version 12.0, SPSS, Chicago, USA). A score of $P<0.05$ was defined as statistically significant.

Results

One hundred cases (37 men and 63 women; mean age 51.9± 9.2 years) were thus eligible for analysis. All cases were judged to be healthy based on physical examination and blood chemistry and urinalysis data. The etiological drugs were chlorpheniramine ($n=20$), promethazine ($n=16$), clemastine ($n=5$), and mequitazine ($n=2$). Five cases had had repeated ADRs caused by the different H1-antihistamines, and 62 cases could not specify the drug name(s) of the etiological H1-antihistamine(s). Indications for the H1-antihistamines were upper respiratory tract infection (common cold) in 56 cases, allergic rhinitis in 36 cases, and urticaria in eight cases. These cases had had no concomitant diseases or comedications that might have interacted with the H1-antihistamines. They had had one or more adverse symptoms. The most prevalent was excessive daytime sleepiness (75/100), followed by fatigue (18/100), mouth dryness (15/100), gastrointestinal symptoms (12/100), and others (18/100).

The frequencies of alleles and genotypes in the present study and in a referential study of healthy Japanese populations [4] are shown in Table 1. The observed genotype frequency distribution was consistent with Hardy-Weinberg equilibrium. The genotype and allele frequencies in the cases significantly differed from those in the healthy Japanese populations. The *CYP2D6*10* allele and its genotypes were observed significantly more frequently in these cases than in the healthy Japanese, but those of the null alleles (*0: i.e., *4, *5, and *14) or genotypes containing null alleles did not differ between the groups.

Table 1 Genotype and allele frequencies in the cases and references

	Cases[a] (n=100) [n (%)]	References[b] [%]	Cases vs. references[c]
CYP2D6 genotype			
*Wild/*wild*	22 (22.0)	29.7	P=0.039
*Wild/*10*	41 (41.0)	42.3	χ^2=10.101
*Wild/*0*	6 (6.0)	7.3	
*10/*10*	26 (26.0)	15.1	
*10/*0*	5 (5.0)	5.2	
*0/*0*	0	0.4	
CYP2D6 allele[d]			
Wild	91 (45.5)	54.5	P=0.013
10	98 (49.0)	38.8	χ^2=8.765
0	11 (5.5)	6.7	
*CYP2D6*10* allele			
Noncarriers	28 (28.0)	37.5	P=0.005
Heterozygous carriers	46 (46.0)	47.5	χ^2=10.399
Homozygous carriers	26 (26.0)	15.1	

[a] Cases: subjects with a past history of H1-antihistamine-induced adverse drug reactions
[b] References: healthy Japanese populations from a large study [4]
[c] The chi-square goodness of fit test and test of homogeneity were used to assess the genotype and allele frequencies of healthy Japanese. [4]
[d] Total allele number =200

The distribution of the ESS scores in the 75 cases (25 men and 50 women; mean age 51.3±9.2 years) who had experienced excessive daytime sleepiness and the baseline scores in 34 subjects (21 men and 13 women; mean age 49.4±6.9 years) without ADRs are shown in Fig. 1. The mean ESS scores of the female and male cases were 12.9± 6.1 and 9.5±5.5, respectively, and the female and male baseline scores were 6.5±4.1 and 4.0±2.3, respectively. The mean ESS score of the cases was significantly higher than the baseline and was higher in women than in men. We assessed the effect of the *CYP2D6* genotypes among three groups of the homozygotes for wild type alleles (*wild/ *wild*), heterozygotes for a wild type allele and a variant allele (*wild/*0* or *10), and heterozygotes or homozygotes for variant alleles (*0 or *10/*0 or *10), because the frequencies of the null alleles were low. The ratio of cases with hypersomnia, as diagnosed by an ESS score ≥12, increased as the number of variant alleles increased (P=0.045; Fig. 2). The mutant allele frequency in cases with hypersomnia (0.65) was also higher than that of controls (0.44; P=0.015). The mean ESS scores of the *wild/*wild, the *wild/*0 or *10, and the *0 or *10/*0 or *10 were 8.9±4.6, 11.9±6.2, and 13.5±6.3, respectively (P=0.085, Kruskal-Wallis test), and that of mutant allele(s) carriers was 12.5±6.2 (P=0.045 vs.