**Effects of Green Tea Compounds on Irinotecan Metabolism**

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**ABSTRACT:**

The effects of green tea compounds on the metabolism of irinotecan have never been investigated. We aimed to study whether catechins ([(-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (EG), (-)-epigallocatechin (EGC), (-)-epicatechin] affect the inactivation metabolism of irinotecan into 7-ethyl-10-[4-N-(1-piperidino)-1-aminocarbonyloxycamptothecin (NPC) (by CYP3A4) and 7-ethyl-10-hydroxycamptothecin (SN-38) into 7-ethyl-10-hydroxycamptothecin glucuronide (SN-38G) (by UGT1A1). Human liver microsomes, hepatocytes and Hep G2 cells were incubated with catechins and treated with irinotecan and/or SN-38. NPC and SN-38 formation was measured by high-performance liquid chromatography. UGT1A1 mRNA levels were measured by real-time polymerase chain reaction. In human liver microsomes, a concentration-dependent decrease in the formation of NPC and SN-38G was observed. In human hepatocytes, a significant increase in SN-38G production was observed in 33% (EGCG), 44% (ECG), and 44% (EGC) of the hepatocyte preparations. Phenobarbital increased the formation of SN-38G in 100% of the same hepatocyte preparations. In Hep G2 cells, no increase in SN-38G formation was observed. With the exception of EGCG in one liver, catechins did not increase UGT1A1 mRNA levels. NPC production was also significantly increased in 40% of the hepatocyte preparations for each catechin. However, the production of 6β-hydroxytestosterone remained unaffected in other hepatocyte preparations. At pharmacologically relevant concentrations, catechins are unlikely to inhibit the formation of irinotecan inactive metabo- lites when administered concomitantly. The induction effect of catechins on UGT1A1 seems to be modest and highly variable. Catechins do not induce CYP3A4 activity. The effect of acute and prolonged use of green tea on the pharmacokinetics of irinotecan in patients remains to be evaluated.

Dietary and herbal supplements are becoming increasingly popular as preventative measures against cancer or as supportive care treatments. It is estimated that as many as 85% of cancer patients use complementary and alternative medicines. It is estimated that as many as 85% of cancer patients use complementary and alternative medicines. Dietary and herbal supplements are becoming increasingly popular as preventative measures against cancer or as supportive care treatments. It is estimated that as many as 85% of cancer patients use complementary and alternative medicines. Dietary and herbal supplements are becoming increasingly popular as preventative measures against cancer or as supportive care treatments. It is estimated that as many as 85% of cancer patients use complementary and alternative medicines.

**Abbreviations:** EGCG, (-)-epigallocatechin gallate; ECG, (-)-epicatechin gallate; EC, (-)-epicatechin; EGC, (-)-epigallocatechin; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, SN-38-glucuronide; UGT, UDP-glucuronosyltransferase; P450, cytochrome P450; NPC, 7-ethyl-10-[4-N-(1-piperidino)-1-aminocarbonyloxycamptothecin; CPT, camptothecin; HMM, hepatocyte maintenance medium; HMM”, serum-free HMM; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide.
also directly metabolized by CYP3A4 and 3A5 into APC and NPC. NPC is the major oxidation product formed by CYP3A4 in vitro liver preparations.

It has been demonstrated that modulation of irinotecan metabolism might change the pharmacokinetics and the required doses of irinotecan. By inhibiting CYP3A4 with ketoconazole (also a UGT1A1 inhibitor) or cyclosporin A, the exposure of patients to SN-38 is significantly increased, leading to increased irinotecan toxicity (Kehrer et al., 2002; Innocenti et al., 2004). By inducing UGT1A1 with enzyme-inducing anticonvulsants in brain tumor patients, the exposure of patients to SN-38 is significantly reduced, and these patients required higher irinotecan doses (Crews et al., 2002). Significant alterations of the CYP3A4 and UGT1A1 systems by herbal compounds might have clinically important consequences.

Combined use of herbs and chemotherapy may result in clinically important interactions (Sparreboom et al., 2004; Komoroski et al., 2005). Recent studies have been focused on ascertaining the effect of herbal compounds on inducing or inhibiting irinotecan metabolism. The herbal components of St. John’s wort can alter irinotecan disposition, resulting in differences among patients in the severity of myelosuppression (Mathijsen et al., 2002). This interaction is likely to be mediated via the inducing effects of St. John’s wort on CYP3A4-mediated oxidation of irinotecan. However, milk thistle, a CYP3A4 and UGT1A1 inhibitor, had no effect on the pharmacokinetics of irinotecan and its metabolites in patients (van Erp et al., 2005).

The effect of catechins on UGT and P450 enzymes has been tested mainly in rodents. UGT microsomal activity has been enhanced by chronic treatment with green tea beverages or extracts to a variable degree in animal studies (Sohn et al., 1994; Bu-Abbas et al., 1998; Zhu et al., 1998; Embola et al., 2002). Concerning the P450 enzymes, the data are discordant. In rats, midazolam metabolism was increased by green tea extracts, suggesting in vivo induction of CYP3A4. Inhibition of P450 activity in human liver microsomes was found by coadministering green tea extracts with P450 substrates (Muto et al., 2001; Nishikawa et al., 2004). However, in healthy volunteers, treatment with green tea extracts did not alter CYP3A4 or CYP2D6 activity (Donovan et al., 2004).

The effect of catechins on the inactivation of irinotecan has never been elucidated. Hence, we aimed to investigate the effects of catechins on the metabolism of irinotecan, focusing on SN-38 glucuronidation (mainly through UGT1A1) and NPC formation (mainly through CYP3A4). Establishing an interaction between catechins and irinotecan is important to guide the design of studies investigating the clinical impact of green tea in patients treated with irinotecan.

Materials and Methods

Chemicals and Reagents. Irinotecan and SN-38 were kindly provided by Dr. Kigoshi Terada (Yakult Honsha Co., Ltd., Tokyo, Japan). Green tea components, phenobarbital, camptothecin (CPT), bovine serum albumin, UDP-glucuronic acid, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). NPC was kindly provided by Dr. Laurent P. Rivory (University of Queensland, Queensland, Australia). Hepatocyte maintenance medium (HMM), insulin, and dexamethasone were supplied from Cambrex Bio Science Walkersville Inc. (Walkersville, MD). William’s E medium, t-glutamine, penicillin, streptomycin, and amphotericin B were obtained from Invitrogen (Carlsbad, CA). iScript cDNA Synthesis Kit and IQ SYBR Green Supermix were obtained from Bio-Rad (Hercules, CA). All other chemicals and reagents were of the highest grade and commercially available.

Preparation of Human Liver Microsomes. Human liver microsomes were prepared by differential centrifugation methods from normal human livers (n = 15) obtained through the Liver Tissue Procurement and Distribution System funded through National Institutes of Health Contract N01-DK-9-2310 with human subjects approval. Total protein content in microsomes was determined by the Bradford (1976) method using bovine serum albumin as the standard. Microsomes from 15 livers were pooled and stored at −80°C.

Incubations of Human Liver Microsomes with Catechins and SN-38. Incubations were performed as described previously (Innocenti et al., 2002) by using SN-38 (5 μM) and different concentrations of EGCG, ECG, EGC, and EC (1, 10, and 100 μM). Samples were analyzed by high-performance liquid chromatography (HPLC) as described below. Because the interday and intra-day variation in SN-38G formation was less than 10% (data not shown), experiments were performed in duplicate. The production of SN-38G was shown to be linear after 1 h of incubation (data not shown), as described previously (Iyer et al., 1998).

Incubations of Human Liver Microsomes with Catechins and Irinotecan. The incubation mixture contained 2 mg/ml microsomal protein, 0.5 nM NADPH, 5 mM MgCl₂, 80 μM irinotecan, different concentrations of EGCG, ECG, EGC, and EC (1, 10, and 100 μM), and 0.1 M sodium phosphate buffer (pH 7.4) in a final volume of 200 μl. After a 5-min preincubation, the reaction was initiated by adding NADPH. Samples were incubated in a 37°C water bath for 45 min. The reaction was terminated by adding 10 μl of ice-cold 12 M HCl. Samples were centrifuged for 20 min at 20,000×g and 4°C. After addition of internal standard (CPT, 1 μg/ml in 0.01 M HCl), samples were mixed and injected into the HPLC. The linearity in the production of NPC was not tested.

Isolation of Primary Human Hepatocytes. Primary human hepatocytes were obtained with the approval of the institutional review boards of the institutions involved through the Liver Tissue Procurement and Distribution System (Pittsburgh, PA), which were funded by National Institutes of Health Contract N01-DK-9-2310. Human hepatocytes were isolated by a three-step collagenase perfusion technique as described previously (Strom et al., 1996). Viability of cells was determined by the trypan blue exclusion method and was at least 80%. Hepatocytes were plated on Falcon six-well culture plates (1.5 × 10⁶ cells), previously coated with rat-tail collagen in HMM supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B, and 10% bovine calf serum. After cells attached for 4 h, medium was changed with serum-free medium (HMM) containing all of the supplements described above. Cells were maintained in culture at 37°C in atmosphere containing 5% CO₂ and 95% air. After 24 h in culture, unattached cells were removed by gentle agitation and the medium was changed every 24 h.

Treatments of Hepatocytes. Forty-eight hours after plating, medium was aspirated and replaced with HMM containing catechins (EGCG, ECG, EGC), phenobarbital, or rifampicin. EC was not used because of the limited availability of isolated cells. Catechins were used at 2 μM, a concentration that is likely to 1) have minimal inhibitory effects on SN-38G and NPC formation (Table 1) and 2) be comparable to that found in human plasma after chronic exposure to green tea (Donovan et al., 2004; Kroon et al., 2004). Phenobarbital (2 mM) was used as positive control for induction of UGT1A1 and CYP3A4 in irinotecan treatments. Rifampicin (10 μM) was used as positive control for CYP3A4 in incubations with testosterone (Komoroski et al., 2004). Control treatments (no treatment) consisted of DMSO (0.1% final concentration). Data from six hepatocyte preparations of phenobarbital incubation and SN-38 treatment were previously published (Ramirez et al., 2006). Treatments were performed in triplicate (i.e., three wells of a plate). Medium containing the compounds above was changed every 24 h for 6 days. At the end of the exposure, hepatocytes were washed for 1 h with plain medium (HMM) and exposed to either 100 μM irinotecan (n = 5 cell preparations) or 5 μM SN-38.
(n = 9 cell preparations) for 1 h, or testosterone (250 μM, n = 4 cell preparations) for 30 min. Testosterone hydroxylation is a marker of CYP3A4 activity (Yuan et al., 2002; Parkinson et al., 2004). Medium and cells were collected and stored at −80°C until analysis.

**Hep G2 Cell Culture.** Hep G2 cells were obtained from The American Type Culture Collection (Manassas, VA). The cells were plated in six-well plates and maintained in Williams’ Medium E with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin solution (10,000 units/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate) at 37°C in an atmosphere containing 5% CO2 and 95% air. When approximately 80% of the cells were confluent, they were treated with catechins (EGCG, ECG, EGC; 2 μM) or chrysos (25 μM) for 3 days. Chrysos was used as a positive control for induction of UGT1A1 in Hep G2 cells. Control treatments (no treatment) consisted of DMSO (0.1% final concentration). The medium was changed every 24 h. At the end of the exposure, cells were washed for 1 h with plain medium (without additives) and exposed to SN-38 (5 μM) for 1 h. Medium and cells were collected and stored at −80°C until analysis.

**HPLC Measurement of the Formation of NPC, SN-38G, and 6β-Hydroxytestosterone.** To quantify NPC in irinotecan-treated microsomes and hepatocytes, we used high-performance liquid chromatography (Hitachi High Technologies America, San Jose, CA) with fluorescence detection (λex = 355, λem = 515), and a Symmetry RPS column (3.0 × 150 mm, 5 μm) and precolumn (3.9 × 20 mm, 5 μm; Waters Corp., Milford, MA). The mobile phase consisted of 13:87 acetonitrile:0.1 M ammonium acetate (pH 4). The flow rate was 1 ml/min. NPC peak identification was done by injection of the pure compound. SN-38G formation was quantified in microsomes and hepatocytes by HPLC as described previously (Innocenti et al., 2002). To quantify 6β-hydroxytestosterone in hepatocytes, we followed a previously established method (Komoroski et al., 2004). Metabolite formation was expressed as the ratio of the chromatographic peak heights of metabolites over internal standard. In the hepatocytes and Hep G2 experiments, SN-38G formation was quantified by using a SN-38 standard curve. Due to shortage of analytical grade NPC, a standard curve produced from an authentic standard was not used for NPC quantification, and NPC formation was expressed as the ratio of the chromatographic peak heights of NPC over internal standard.

**RNA Isolation from Human Hepatocytes.** Forty-eight hours after plating, medium was aspirated and replaced with HMM containing catechins (EGCG, ECG, EGC; 2 μM), or phenobarbital (positive control for induction, 2 mM) (n = 6 cell preparations). Controls (no treatment) consisted of DMSO (0.1% final concentration). Treatments were performed in triplicate (i.e., three wells of a plate). Medium containing the compounds above was changed every 24 h for 6 days. At the end of the exposure, medium was removed, and 1 ml of TRIzol reagent was added to each well and incubated for 10 min. Cells were lysed by passing the cell lysate several times through a pipette and scraping with a cell scraper. After addition of chloroform (0.2 ml), homogenates were centrifuged at 15,300 g for 15 min at 4°C. Aqueous phases of samples precipitated with isopropanol alcohol (0.5 ml), incubated at room temperature for 10 min, and centrifuged at 15,300 g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol (1 ml) and centrifuged at 6000 g for 5 min at 4°C. At the end, RNA pellet was dried and resuspended in RNase-free water by passing the solution a few times through a pipette tip. RNA concentrations were measured on a Cary spectrophotometer (Varian, Inc., Palo Alto, CA). All samples had an A260/A280 ratio >1.65.

**Real-Time PCR.** UGT1A1 mRNA levels in total RNA isolated from human hepatocytes (n = 6) were measured by real-time PCR on an Mx3000P system (Stratagene/Biocrest, Cedar Creek, TX). First, mRNA was adjusted to give an equal total mRNA concentration in all samples. cDNA was synthesized in a single experiment in a 96-well plate, diluted, and stored at −80°C until analysis. cDNA was amplified in 25 μl of reaction buffer containing IQ SYBR Green Supermix by following the manufacturer’s instructions. In brief, cDNA was amplified in 25 μl of reaction buffer containing IQ SYBR Green Supermix, specific primers (0.5 μM), and nuclease-free water. β-Actin cDNA was used as the control gene. The oligonucleotide sequences of the primers were 5′-AACAAAGGGCTCATGCGCCCT-3′ (forward) and 5′-CCACATTC-CATGTTCTCCAG-3′ (reverse) for UGT1A1. They were 5′-ACGTGGA-CATCGAAGACGCTCATGCGCCCT-3′ (forward) and 5′-CCACATTC-CATGTTCTCCAG-3′ (reverse) for UGT1A1. They were 5′-ACGTGGA-

**Table 2** Percentage reduction in SN-38G formation by catechins in pooled human liver microsomes incubated with different catechin concentrations

<table>
<thead>
<tr>
<th>Percentage Decrease in SN-38G</th>
<th>1 μM</th>
<th>10 μM</th>
<th>100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>5%</td>
<td>14%</td>
<td>84%</td>
</tr>
<tr>
<td>ECG</td>
<td>6%</td>
<td>37%</td>
<td>87%</td>
</tr>
<tr>
<td>EC</td>
<td>8%</td>
<td>13%</td>
<td>19%</td>
</tr>
<tr>
<td>EGC</td>
<td>1%</td>
<td>9%</td>
<td>25%</td>
</tr>
</tbody>
</table>

Significance refers to the comparison between control (no treatment) and samples treated with catechins (*p < 0.05).
exception being ECG in one hepatocyte preparation (Fig. 3). In contrast, significant increases in 6β-hydroxytestosterone production by rifampicin were observed in all preparations ($p < 0.05$).

**SN-38G Production in Hep G2.** UGT1A1 expression in Hep G2 is inducible by chrysin (Walle and Walle, 2002). Hence, we measured the formation of SN-38G in Hep G2 cells after incubating them with catechins. In the cells incubated with catechins, no formation of SN-38G was detected, similar to the control treatments. SN-38G formation was measured only in the cells incubated with chrysin (14.6 ± 3.0 ng/ml, $n = 3$).

**UGT1A1 mRNA Expression in Human Hepatocytes Incubated with Catechins.** After pretreatment of human hepatocytes ($n = 6$) with catechins, UGT1A1 mRNA levels were not significantly increased ($p < 0.05$), with the exception of one hepatocyte preparation treated with ECG (56% increase, Table 3). In the same hepatocyte preparations, pretreatment with phenobarbital significantly increased the UGT1A1 mRNA levels in 83.3% of the livers tested ($p < 0.05$). Increases ranged from 105% to 578%.

**Discussion**

The present study aimed to investigate the effect of green tea compounds (catechins) on irinotecan metabolism focusing on UGT1A1 and CYP3A4, the inactivating enzymes of irinotecan. Although green tea beverage and dietary supplements are widely used, there are limited data on the potential involvement of catechins in herb-drug interactions.

The results of our study showed that catechins inhibited UGT1A1-dependent glucuronidation of SN-38 and CYP3A4-dependent oxidation of irinotecan in human liver microsomes. The observed decrease in SN-38G and NPC levels was concentration-dependent, usually observed at catechin concentrations of 10 μM or higher. Our results are in agreement with a previous study in which the IC$_{50}$ of EGCG for estrone and estradiol glucuronidation was 25 μM in rat liver microsomes (Zhu et al., 1998). UGT1A1 is the main glucuronidating enzyme of estradiol (Court, 2005), and because EGCG is mainly glucuronidated by UGT1A1 in the liver (Lu et al., 2003), it is likely that reduced SN-38G formation in the presence of catechins is due to a mechanism of competitive inhibition. Concerning the effect on CYP3A4-mediated NPC formation, other studies have shown an inhibitory effect of catechins on CYP3A4 activity similar to that observed on NPC formation, using either testosterone or midazolam as CYP3A4 probes (Muto et al., 2001; Nishikawa et al., 2004). Such an inhibitory mechanism seems to be related to the inhibition of NADPH-P450 reductase (Muto et al., 2001).

Herbal compounds may have a dual effect depending upon their duration of exposure and concentration used. For example, in human hepatocytes acutely exposed to hyperforin (the active component of St. John’s wort), inhibition of CYP3A4 activity was observed after acute exposure of hepatocytes at high concentrations, whereas chronic exposure at lower concentrations had an inductive effect (Komoroski et al., 2004). The inhibition of SN-38G and NPC formation observed in our study occurred at catechin concentrations (≥10 μM) that are unlikely to be found in the blood of individuals consuming green tea. Despite different modes of consumption, plasma concentrations of EGCG, as well as that of total catechins, do not exceed 2 μM on average (Nakagawa et al., 1997; Yang et al., 1998; Chow et al., 2003;
green tea was unlikely to alter the disposition of drugs primarily metabolized by CYP2D6 and CYP3A4. In healthy volunteers, the mean plasma concentration of the unconjugated EGCG was usually less than 1 μM 2 h after administration of decaffeinated green tea extract (Donovan et al., 2004).

Our study has some limitations. First of all, the hepatocyte model has high interdonor variability, hampering the detection of mild inducing effects (Soars et al., 2004). Even for phenobarbital, the magnitude of induction is highly variable, although the UGT1A1 induction effect is consistent across all livers tested. Second, a concentration-effect study of catechins in the induction model has not been performed, because we intended to use catechin concentrations similar to those reached during average consumption of green tea products. However, when we treated two hepatocyte preparations with 2 and 20 μM EGCG, the formation of SN-38G at 20 μM was not significantly increased compared with that obtained at 2 μM (data not shown), suggesting that a higher catechin concentration is unlikely to increase the inducing potential of catechins. We also believe that the use of suprapharmacologic concentrations of herbal compounds in hepatocyte models does not provide clinically useful information. Third, we used catechins individually, and not together, ruling out possible effects resulting from the biochemical interaction of different catechins with the enzymes of interest in this study. Due to the existence of different brands of green tea products, each containing different proportions of catechins, it is quite difficult to exactly reproduce the catechin exposure in individuals taking various forms of green tea products. In addition, EGCG is the main catechin in all different green tea supplements and beverages (Fujiki, 2005; Schmidt et al., 2005), and several studies on green tea catechins have been performed using EGCG only.

Based upon the results of our study, green tea catechins, at pharmacologically relevant concentrations, are unlikely to alter the inactivating metabolism of irinotecan. However, because the pharmacokinetic variability in SN-38 and SN-38G has clinical consequences in irinotecan patients (Gupta et al., 1994; Innocenti et al., 2004), further clinical studies are necessary to elucidate the effect of acute and chronic exposure to green tea products on irinotecan pharmacokinetics and toxicity. It is conceivable to hypothesize that, in some inducible patients, the induction of UGT1A1 by catechins in the liver might
affect the systemic exposure of patients to SN-38. In addition, an induction of UGT1A1 in intestinal cells might protect the intestinal mucosa from the injury of SN-38, reducing the gastrointestinal toxicity of irinotecan. Such studies addressing these questions are warranted, because (1) only 15% of physicians or other health professionals ask about the herbal supplement intake of patients undergoing chemotherapy (Hyodo et al., 2005), (2) on average, approximately 50% of cancer patients do not disclose this information to their doctors (Werneke et al., 2004), and (3) up to 11% of cancer patients use herbal compounds at doses higher than those recommended (Werneke et al., 2004).

References