A Mechanistic Study on Reduced Toxicity of Irinotecan by Coadministered Thalidomide, a Tumor Necrosis Factor-α Inhibitor

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ABSTRACT

Dose-limiting diarrhea and myelosuppression compromise the success of irinotecan (7-ethyl-10-[4-[1-piperidino]-1-piperidino]carbonyloxycamptothecin) (CPT-11)-based chemotherapy. A recent pilot study indicates that thalidomide attenuates the toxicity of CPT-11 in cancer patients. This study aimed to investigate whether coadministered thalidomide modulated the toxicities of CPT-11 and the underlying mechanisms using several in vivo and in vitro models. Diarrhea, intestinal lesions, cytokine expression, and intestinal epithelial apoptosis were monitored. Coadministered thalidomide (100 mg/kg i.p. for 8 days) significantly attenuated body weight loss, myelosuppression, diarrhea, and intestinal histological lesions caused by CPT-11 (60 mg/kg i.v. for 4 days). This was accompanied by inhibition of tumor necrosis factor-α, interleukins 1 and 6 and interferon-γ, and intestinal epithelial apoptosis. Coadministered thalidomide also significantly increased the systemic exposure of CPT-11 but decreased that of SN-38 (7-ethyl-10-hydroxy-camptothecin). It significantly reduced the biliary excretion and cecal exposure of CPT-11, SN-38, and SN-38 glucuronide. Thalidomide hydrolytic products inhibited hydrolysis of CPT-11 in rat liver microsomes but not in primary rat hepatocytes. In addition, thalidomide and its major hydrolytic products, such as phthaloyl glutamic acid (PGA), increased the intracellular accumulation of CPT-11 and SN-38 in primary rat hepatocytes. They also significantly decreased the transport of CPT-11 and SN-38 in Caco-2 and parental MDCKII cells. Thalidomide and PGA also significantly inhibited P-glycoprotein (PgP/MDR1), multidrug resistance-associated protein (MRP1)- and MRP2-mediated CPT-11 and SN-38 transport in MDCKII cells. These results provide insights into the pharmacodynamic and pharmacokinetic mechanisms for the protective effects of thalidomide against CPT-11-induced intestinal toxicity.

Irinotecan (CPT-11, 7-ethyl-10-[4-[1-piperidino]-1-piperidino]carbonyloxycamptothecin), a potent DNA topoisomerase I inhibitor, has been widely used for the treatment of advanced colorectal cancer as a first-line therapy in combination with 5-fluorouracil and other malignancies (Pizzolato and Saltz, 2003). As a prodrug, CPT-11 is activated by carboxylesterases (CEs) to SN-38 (7-ethyl-10-hydroxy-camptothecin). It significantly reduces the biliary excretion and cecal exposure of CPT-11, SN-38, and SN-38 glucuronide. Thalidomide hydrolytic products inhibited hydrolysis of CPT-11 in rat liver microsomes but not in primary rat hepatocytes. In addition, thalidomide and its major hydrolytic products, such as phthaloyl glutamic acid (PGA), increased the intracellular accumulation of CPT-11 and SN-38 in primary rat hepatocytes. They also significantly decreased the transport of CPT-11 and SN-38 in Caco-2 and parental MDCKII cells. Thalidomide and PGA also significantly inhibited P-glycoprotein (PgP/MDR1), multidrug resistance-associated protein (MRP1)- and MRP2-mediated CPT-11 and SN-38 transport in MDCKII cells. These results provide insights into the pharmacodynamic and pharmacokinetic mechanisms for the protective effects of thalidomide against CPT-11-induced intestinal toxicity.

ABBRVIATIONS: CPT-11, irinotecan, 7-ethyl-10-[4-[1-piperidino]-1-piperidino]carbonyloxycamptothecin; CE, carboxylesterase; SN-38, 7-ethyl-10-hydroxy-camptothecin; SN-38G, SN-38 glucuronide; AUC, area under the concentration time curve; Mdr, multidrug resistance; MRP, multidrug resistance-associated protein; TNF-α, tumor necrosis factor-α; RT-PCR, reverse transcription polymerase chain reaction; IL, interleukin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; TEER, transepithelial electric resistance; PGA, phthaloyl glutamic acid; IFN-γ, interferon-γ; PgP, P-glycoprotein; MK-571 (or L-660,711), 3-[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio]methylthiopropionic acid; BNPP, bis(2-nitrophenyl) phosphate sodium salt; UGT, uridine diphosphate glucuronosyltransferase; i.p., intraperitoneal; i.v., intravenous; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; MDCK, Madin-Darby canine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide kidney; CL, clearance; AP, apical; BL, basolateral; HBSS, Hanks’ balanced salt solution; ANOVA, analysis of variance; RT, reverse transcription; PCR, polymerase chain reaction; Brij 58, polyoxyethylene 20 cetyl ether; Brij 38, polyoxyethylene 23 cetyl ether.
ecin), which is 100- to 1000-fold more cytotoxic than CPT-11. SN-38 is further converted to its glucuronide (SN-38G) by uridine diphosphate glucuronosyltransferase (UGT) 1A isoforms (Fig. 1) (Gupta et al., 1994). SN-38G can be converted back to SN-38 by intestinal microbial β-glucuronidase and undergo enterohepatic recycling (Mathijssen et al., 2001). A second less important metabolic pathway of CPT-11 is cytochrome P450 (CYP3A)-catalyzed bipiperidine side chain oxidation, giving rise to 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin and 7-ethyl-10-[4-(1-piperidino)-1-amino]carbonyloxycamptothecin (Gupta et al., 1994). Biliary excretion is the major elimination route for CPT-11 and its major metabolites, with the urinary excretion being a less important pathway. In vitro and animal studies have indicated that CPT-11, SN-38, and SN-38G are actively transported by P-glycoprotein (PgP/MDR1), multidrug resistance-associated proteins (MRP1, MRP2, and MRP4), and the breast cancer resistance protein (Chu et al., 1998; Tian et al., 2005).

However, dosing-intensified regimens of CPT-11-based chemotherapy are often limited by severe myelosuppression and intestinal mucositis characterized by severe diarrhea. In

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**Fig. 1.** The metabolic scheme of CPT-11. APC, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin; NPC, 7-ethyl-10-[4-(1-piperidino)-1-amino]carbonyloxycamptothecin; CYP3A, cytochrome P450 3A.
particular, the incidence of grade 3 or 4 diarrhea was up to 40% of patients treated with CPT-11, which may cause dehydration, renal failure, and thromboembolic events (Rothenberg et al., 1996). High-dose loperamide is recommended to manage CPT-11-induced diarrhea (Benson et al., 2004). However, a number of patients do not respond to this agent. Therefore, new and effective agents are needed to alleviate CPT-11-induced intestinal mucositis. The increasing understanding of the molecular events that lead to chemotherapy-induced intestinal mucosal injury may allow us to identify new approaches to manage chemotherapy-induced intestinal damage. The mucosal lesions seem to be associated with intestinal exposure to these cytotoxic drugs, which induce epithelial apoptosis, decreased crypt cell renewal, complicated inflammatory responses, and destruction of the mucosal architecture (Thompson, 1995). Cytotoxic drugs, including CPT-11, induce severe intestinal inflammatory responses with hypersecretion of proinflammatory cytokines, including tumor necrosis factor (TNF-α) and interleukins (ILs) (Sonis, 2004). TNF-α, mainly produced by activated macrophages, is a key cytokine in initiation of mucosal immunoinflammatory responses and lesions. It is a critical cytokine that could orchestrate inflammatory responses by activating a wide range of cells, including neutrophils, macrophages, and natural killer cells. Activation of these cells in turn induces the production of proinflammatory cytokines, such as IL-1β and IL-6, and up-regulation of adhesion molecules on cell surface. There exists a close interaction between intestinal epithelial cellular apoptosis and intestinal proinflammatory cytokine expression. TNF-α has been proven to play an essential role in regulating intestinal epithelial cell apoptosis and/or survival during chronic inflammation (Marini et al., 2003). Therefore, TNF-α plays a critical role in initiation of chemotherapy-induced primary mucosal damage responses, including early lesion to connective tissue and endothelium, reduction of epithelial oxygenation, and ultimately, epithelial basal-cell death and injury (Sonis, 2004).

Thalidomide [2-(2,6-dioxo-3-piperidinyl)]-1H-isoindole-1,3-(2H)-dione], a derivative of glutamic acid, has been widely used in the treatment of various inflammatory and autoimmune disorders, as well as a variety of tumors because of its immunomodulating and antiangiogenic effects (Franks et al., 2004). It has shown inhibitory effects on TNF-α production by enhancing the degradation of TNF-α mRNA (Moreira et al., 1993). Thalidomide is often used in combination with other cytotoxic agents aimed at generating additive/synergistic activity, alleviating toxicity, and overcoming tumor resistance (Catley et al., 2005). A recent pilot study demonstrated that concomitant thalidomide almost eliminated CPT-11-induced gastrointestinal toxicities, including nausea and diarrhea (Govindarajan et al., 2000). However, the mechanisms underlying this striking protective effect are not clear. Spontaneous hydrolysis is the major elimination pathway of thalidomide, whereas CYP2 mediated metabolism plays only a minor role in its elimination (Ando et al., 2002).

In the present study, we investigated whether combination of thalidomide modulated the toxicities of CPT-11 using a rat model and explored the underlying pharmacokinetic and pharmacodynamic mechanisms using a variety of in vivo and in vitro models. The rats were chosen in this study because of the similarity in the metabolic and disposition pathways of CPT-11 and SN-38 between rats and humans (Yang et al., 2005a) and because the rat is relatively sensitive to CPT-11 and has been widely used in the pharmacological and toxicological studies of CPT-11. In an established rat toxicity model induced by CPT-11, the effects of coadministered thalidomide on the intestinal and blood toxicities, expression of TNF-α and other proinflammatory cytokines, including IL-1β, IL-2, IL-6, and interferon (IFN)-γ, and intestinal epithelial apoptosis were monitored. The effects of coadministered thalidomide on the plasma and intestinal (cecal) pharmacokinetics and biliary excretion of CPT-11 and its major metabolites were also investigated. Rat liver microsomes were used to study the possible metabolic interactions between CPT-11 and thalidomide and its hydrolytic products. Herein we also included freshly isolated primary rat hepatocytes to study the effects of thalidomide, its total hydrolytic products, and one of its major hydrolytic products, phthaloyl glutamic acid (PGA), on the metabolism and intracellular accumulation of CPT-11 and SN-38. In addition, the effects of thalidomide, its total hydrolytic products, and PGA on the transport in Caco-2 cells and MDCKII cells overexpressing MDR1, MR1, or MR2 were investigated. Moreover, the effects of thalidomide, its total hydrolytic products, and PGA on rat plasma protein binding of CPT-11 and SN-38 were examined to identify possible displacement of the substrates from the binding sites of plasma proteins. As such, the roles of both pharmacodynamic and pharmacokinetic components in the protective effect of thalidomide against CPT-11-induced toxicity were comprehensively explored in the present study.

Materials and Methods

Chemicals and Reagents. Camptothecin (CPT) analogs, including irinotecan (CPT-11), CPT, and SN-38 in lactone form [all compounds with a purity >99.0% as determined by high-performance liquid chromatography (HPLC)], were purchased from SinoChem Ningbo Import and Export Co. (Ningbo, China). An injectable formulation of CPT-11 was prepared as described previously (Kurita et al., 2003). Sodium 1-heptane-sulfonate, lyophilized type IX-A glucuronidase (from Escherichia coli, activity 1,724,400 U/g solid form), probenecid, nifedipine, Brij 58, Brij 38, verapamil, probenecid, bilirubin, bis[p-nitrophenyl] phosphate sodium salt (BNPP), uridine diphosphate glucuronic acid (UDPGA), Dulbecco’s modified Eagle’s medium, β-sorbitol, and β-lactacid were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Fetal bovine serum was obtained from Hyclone Lab Inc. (Logan, UT). Thalidomide (purity >99.0%, determined by HPLC) was provided by Celgene Co. (Summit, NJ). The leukotriene D4 receptor antagonist 3-[[[3-(2-thienyl)ethenyl]-(E)-ethyl]phenyl][(3-dimethylamino)-3-oxo-propyl][thiomethyl]thioliopropionic acid (MK-571 or L-660,711) was a gift from Dr. Ford Hutchinson (Merck Frosst Canada Ltd., Kirkland, QC, Canada). PGA, a hydrolysis product of thalidomide, with a purity >99.0% as determined by HPLC was a gift provided by Dr. Tom Pui-Kai Li (School of Pharmacy, Ohio State University, Cincinnati, OH). The control (wild-type) MDCKII cell line with empty vector and its human MDR1-, MR1-, or MR2-recombinantly transfected derivatives, MDR1-MDCKII, MR1-MDCKII, and MR2-MDCKII cells, were obtained as a kind gift from Professor Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Mouse monoclonal antibody to MDR1 (C219), mouse monoclonal antibody against MR1 (m6), and mouse monoclonal [M2IIII-5] antibody to MR2 were purchased from Abeam Co. (Cambridge, UK). The stable overexpression of MDR1, MR1, and MR2 in transfected cells were monitored and confirmed every two to four passages by Western blotting analysis. The water was purified by a Milli Q water purification System (Millipore, Bedford, MA). All other chemicals
were of analytical grade or HPLC grade obtained from commercial sources.

**Animals.** Healthy male Sprague-Dawley rats (200–250 g) were purchased from the Laboratory Animals Center, the National University of Singapore (Singapore, Singapore). They were used after 2 to 4 days of acclimatization with free access to water and regular diet. Rats were kept in a room under controlled temperature (22 ± 1°C) and automatic day-night rhythm (12-h cycle) and housed on wire-bottom cages with paper underneath. All animal procedures were approved by the Ethical Committee of the National University of Singapore in accordance with the guidelines of the National Institutes of Health (Bethesda, MD).

**Cell Culture.** Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). Caco-2, control MDCKII, and MDCKII cells overexpressing MDR1, MRPI, or MRP2 were cultured in complete Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. The cells were grown in an atmosphere of 5% CO₂ and 95% air at 37°C and given fresh medium every 3 to 5 days. Experiments were performed on cells within 10 passages. Viable cells were counted using the trypan blue exclusion method.

**Preparation of Primary Cultured Rat Hepatocytes.** Hepatocytes were isolated from the livers of healthy male Sprague-Dawley rats (200–250 g) by using collagenase perfusion as described previously (Fang et al., 2005). After isolation, 3 x 10⁵ viable hepatocytes were plated onto 12-well plates for metabolism and transport experiments in Williams’ Medium E. Medium was changed 4 h after plating and every 24 h thereafter.

**MMT Assay.** The cytotoxicity of CPT-11 and SN-38 in primary rat hepatocytes, Caco-2, MDRI-MDCKII, MRPI-MDCKII, and MRP2-MDCKII cells were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were exposed to drugs for 2 to 48 h, 100 μl of MTT reagent (0.5 mg/ml) was added to each well after removal of medium, and cells were incubated for an additional 4 h at 37°C. The absorbance of formazan, a metabolite of MTT, in the resulting solution was photometrically measured at a wavelength of 595 nm using a microplate reader (Tecan Instruments Inc., Research Triangle Park, NC).

**Preparation of Rat Hepatic Microsomes.** Livers were collected from healthy male Sprague-Dawley rats and stored at −80°C. Hepatic microsomes were prepared by differential centrifugation. The rat liver homogenates were centrifuged at 9000g for 20 min at 4°C. The supernatant was then centrifuged at 105,000 g for 20 min at 4°C. The resultant supernatant was collected for cytokine (TNF-α, IFN-γ, IL-1β, IL-2, and IL-6) concentration determination using enzyme-linked immunosorbent assay set from Biomed Diagnostics (BD Biosciences). The cytokine levels in each tissue sample (in picograms/milliliter) were then normalized to the protein concentration (milligrams/milliliter) of each tissue sample to give a final cytokine result expressed as picograms/milligram of protein. The cytokine levels in serum were expressed as picograms/milliliter.

**Reverse Transcription-Polymerase Chain Reaction.** Total RNA was extracted from intestine samples using TRIZol (Invitrogen, Carlsbad, CA), and the TNF-α mRNA expression levels were determined by reverse transcription-polymerase chain reaction (RT-PCR) amplification using Access RT-PCR System (Promega, Madison, WI) according to the manufacturer’s instructions. The housekeeping gene β-actin was used in the same reaction as a reference for standardization of the procedure. The primer sequences for TNF-α were as follows: 5’-TAC TGA TCT CCG GGG TGA TTG GTC G-3’ and 3’-CAC CCT TGT CCC TTG AAG AGA ACC-5’. The resultant PCR products were separated by 1.0% agarose gel electrophoresis and visualized by means of ethidium bromide staining using Gel-Doc (Bio-Rad, Hercules, CA). The levels of TNF-α mRNA were normalized to that of β-actin present in the same sample.

**Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay.** The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed in formalin-fixed and paraffin-embedded rat intestine tissue slides by using an apoptosis detection kit (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI) according to the manufacturer’s instructions. An apoptosis index was obtained by counting the TUNEL-positive cells per crypt. Results are means from at least five animals per experiment (at least three crypts per rat).

**Pharmacokinetic Study.** For the 1-day single-dose study, rats were randomized to two groups with one group receiving CPT-11 at 60 mg/kg i.v. and control vehicle (1% DMSO, w/v) at 1 ml/kg i.p. and...
another group of rats treated with CPT-11 at 60 mg/kg i.v. in combination with thalidomide (100 mg/kg i.p. given 30 min prior to CPT-11 injection). Blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h after CPT-11 administration. Plasma was obtained by immediate centrifugation at 1500g for 10 min at 4°C. Similar blood sampling procedure was used for the 5-day multidose kinetic study, but thalidomide at 100 mg/kg by i.p. injection or the control vehicle (1% DMSO at 1 ml/kg/day i.p.) was given for 5 consecutive days before CPT-11 injection. CPT-11 was administered by i.v. 30 min after thalidomide i.p. injection on the 5th day.

In independent experiments, 10 groups (n = 6 per group) of rats were CPT-11 at 60 mg/kg i.v. and control vehicle (1% DMSO, v/v) at 1 ml/kg i.p. and another group of rats were treated with CPT-11 at 60 mg/kg i.v. in combination with thalidomide (100 mg/kg i.p. given 30 min before CPT-11 injection). For the 5-day multidose kinetic study, 10 groups of rats were treated with thalidomide at 100 mg/kg by i.p. injection or the control vehicle (1% DMSO at 1 ml/kg/day i.p.) for 5 consecutive days before CPT-11 injection. CPT-11 was administered by i.v. 30 min after thalidomide i.p. injection on the 5th day. The animals were killed by exsanguination at 1, 2, 6, 8, and 24 h after the start of infusion, and their cecal tissues were extirpated, homogenized, and stored at −20°C until analysis.

**Bile-Duct Cannulation of Rats.** The effects of coadministered thalidomide on the biliary excretion of CPT-11 and its metabolites were determined in bile duct-cannulated rats after i.v. administration of CPT-11 at 60 mg/kg with or without thalidomide (100 mg/kg i.p.) pretreatment for 1 or 5 consecutive days. The bile duct of rats (n = 6 per group) was cannulated as described previously (Li et al., 2005). The bile duct was cannulated with a PE-10 tubing (Clay Adams; BD Biosciences). CPT-11 was administered as a single i.v. injection at 60 mg/kg. The bile was collected at an interval of 1 to 4 h over 12 h, and thereafter, bile was collected over a 12-h interval.

**Plasma Protein Binding Assay.** The effects of thalidomide (25 and 250 μM) and its hydrolysis products (total concentration of 10 μM) on the protein binding of CPT-11 and SN-38 were investigated in drug-free fresh plasma from healthy male Sprague-Dawley rats (200–220 g) using an ultrafiltration method followed by HPLC drug determination as described previously (Zhou et al., 2001a). The concentration of thalidomide used is an important factor affecting the outcomes in the in vitro studies. A concentration of 25 μM thalidomide is physiologically relevant. The common dose used in cancer patients is 200 to 1000 mg/day, i.e., approximately 3 to 15 mg/kg assuming a body weight of 65 kg, and the maximal plasma concentration (C_{max}) is approximately 5 to 25 μM (Franks et al., 2004). The inclusion of a higher concentration of thalidomide (250 μM) used in our study is based on our in vivo studies, where a C_{max} of 100 to 300 μM was achieved when 100 mg/kg thalidomide was administered to the rat (Yang et al., 2005b). Thus, in the following metabolism and transport studies, both 25 and 250 μM thalidomide were also included. The hydrolysis products of thalidomide were prepared by incubation of thalidomide with 0.1 M phosphate buffer at pH 7.4 overnight at room temperature (22°C). The substrate and the potential inhibitor were incubated at 37°C for 30 min in a water bath with gentle shaking. Control incubations with 1% DMSO were also undertaken, which showed no significant effects on CPT-11 or SN-38 plasma protein binding. After incubation, an aliquot (400 μl) of the plasma sample was transferred to the Centrisart ultrafiltration device (20,000 molecular weight cut-off; Sartorius AG, Goettingen, Germany) and centrifuged at 2000g for 30 min at 37°C. The fraction of unbound (f_u) CPT-11 or SN-38 was calculated by the ratio of the drug concentration in the ultrafiltrate to that in the rat plasma before ultrafiltration.

**Metabolic Inhibition Study in Rat Liver Microsomes.** The effects of thalidomide (25 and 250 μM), its total hydrolysis products (10 μM), and PGA at 10 μM on CPT-11 hydrolysis and SN-38 glucuronidation in rat hepatic microsomes were carried out using optimized incubation conditions. In brief, CPT-11 or SN-38 was incubated with hepatic microsomes (2.0 mg/ml for CPT-11 and 1.0 mg/ml for SN-38) in 200-μl incubation buffers containing either thalidomide or its hydrolysis products for 30 min. The incubation buffer was 0.1 M sodium phosphate buffer, pH 7.4, for CPT-11 and 0.1 M sodium phosphate buffer, pH 6.8, containing 4 mM UDPGA, 10 mM MgCl_2, and 1 mM β-saccharic acid 1,4-lactone (used as a β-glucuronidase inhibitor) for SN-38. Both low and high concentrations of CPT-11 (0.5 or 78 μM) or SN-38 (5 or 18.24 μM) that corresponded to the high-affinity K_{mi} and low-affinity K_{ma} obtained from the best fit two-binding site model were used. Because of the high lipophilicity of CPT-11 (logP = 4.37) and moderate hydrophobicity of SN-38 (logP = 0.89), we had to use DMSO to dissolve these two compounds. Control incubations with 1% DMSO (v/v) were also undertaken, which showed no significant effect on the liver microsomal metabolism of CPT-11 to form SN-38 and SN-38 glucuronidation (<8.5%). When we decreased the DMSO concentrations to 0.25% (v/v), CPT-11 and SN-38 precipitated out from the solutions. Control incubations with 1% DMSO (v/v) were also undertaken, which showed no significant effect on the liver microsomal metabolism of CPT-11 and SN-38 (<8.5%). SN-38 and SN-38G formation was measured as picomoles/minute/milligram of protein. In addition, the known CE inhibitor nifedipine (100 μM) and BNPP (100 μM) (Charasso et al., 2002) were used as positive controls for CPT-11 hydrolysis, whereas nifedipine (100 μM) and bilirubin (100 μM) were used as positive controls for SN-38 glucuronidation (Hauz et al., 1998). For SN-38G determination, it was converted to SN-38 by incubation with β-glucuronidase.

**Metabolism and Intracellular Accumulation Studies in Primary Rat Hepatocytes.** Primary rat cultured hepatocyte monolayers were preincubated with thalidomide or its hydrolysis products for 2 h in 56-cm² plastic culture dishes, and the cells were washed twice with PBS. Cells were then incubated with CPT-11 (0.5–50 μM) or SN-38 (0.05–5.0 μM) over 120 min for metabolism and transport study. CPT-11 (10 μM) incubated for 30 min and SN-38 (1 μM) incubated for 5 min were used for intracellular accumulation inhibition study. In addition, control incubations containing an equal amount of 0.5% DMSO or 0.5% of 0.1 M sodium phosphate buffer, pH 7.4 (used for thalidomide hydrolysis products), were conducted. Furthermore, the positive control inhibitors used for rat microsomal metabolic inhibition studies as described above and PgP and/or MRP inhibitors, including MK-571 (100 μM), verapamil (100 μM), probencid (200 μM), and nifedipine (100 μM), were also included in the metabolism and accumulation studies. All inhibitors used at the reported concentrations did not show significant cytotoxicity (<10%) when incubated with rat primary hepatocytes for 2 to 4 h. The substrates in the medium and cellular lysates were extracted using the solution (0.01 M HCl/methanol, 1:1, v/v) containing the internal standard (CPT), and the concentrations of CPT-11, SN-38, and SN-38G were determined using validated HPLC methods.

**Transport of CPT-11 and SN-38 in Caco-2 Cells and MDRckII Cells Overexpressing PgP, MRP1, or MRP2.** The effects of thalidomide, its total hydrolytic products, and PGA on the transport of CPT-11 and SN-38 in Caco-2 and MDR1-MDCKII, MRP1-MDCKII, and MRP2-MDCKII monolayers were investigated on an orbital shaker as described previously (Zhang et al., 2006). In brief, the cells were seeded onto polycarbonate membrane Transwell inserts (Corning Co., Corning, NY) in 12-well plates. The effective transepithelial electric resistance (TEER) of the monolayers (∼100-1000 Ω·cm²) was measured routinely before and after the experiment using a MilliCell-ERS apparatus (purchased from Millipore Corporation). Caco-2 cells were used for transport experiments 21 days after seeding when the effective TEER values exceeded 300 Ω·cm². Caco-2 cells were conducted on cells between passages 30 and 36. MDCCKII, MDR1-MDCKII, MRP1-MDCKII, and MRP2-MDCKII cells were used in experiments at passages 4 to 9 after receipt from the Netherlands Cancer Institute. Cells were used in transport experiments at days 4 to 7 postseeding where the effective TEER values were typically 50 to 80 Ω·cm².
Thalidomide Reduced the Toxicity of CPT-11

Thalidomide attenuates the body weight loss, myelosuppression, and diarrhea induced by CPT-11. Rats treated with CPT-11 alone at 60 mg/kg/day i.v. for 4 consecutive days experienced rapid decrease in body weight, reached a nadir by day 8 with a decrease of 10% compared with the baseline (day 1, \( P < 0.05 \)), and recovered to 106% of the baseline by day 11 (Fig. 2). In addition, treatment of rats with CPT-11 induced severe early- (days 1–4) and late-onset (days 5–8) diarrhea, with mean severity scores of 0.32, 0.28, 0.76, 1.63, 1.86, 2.21, 1.63, and 0.67 by days 1 through 8. However, rats receiving the vehicle alone did not show any significant signs of toxicity, with gradual body weight increase over 11 days, and the body weight loss was less significant than the control rats receiving CPT-11 and the control vehicle (Fig. 2) (\( P < 0.05 \) for days 6, 7, 8, 10, and 11 after CPT-11 injection by ANOVA). Similar results were observed in rats treated with CPT-11 and control vehicle [DMSO (1%, v/v)]. These rats experienced rapid decrease in body weight, reached a nadir by day 8 with a decrease of 11% compared with the baseline (day 1), and recovered to 104% of the baseline by day 11.

The counts of blood cells were also monitored throughout studies, the cell monolayers were washed twice with warm HBSS containing 25 mM HEPES, pH 6.5, prior to the transport experiments. A pH of 6.5 was chosen as it stabilized CPT-11 and SN-38, and this pH resulted in maximal apical (AP) to basolateral (BL) and BL to AP transport. For the measurement of AP to BL transport, 0.5 ml of HBSS containing CPT-11 (2, 10, and 50 \( \mu \)M) or SN-38 (0.2, 1, and 5.0 \( \mu \)M) was added to the AP side, and 1.5 ml of HBSS without the drug was added on the BL side. After drug loading at AP side and incubation at 37°C, an aliquot (0.1 ml) was collected from the BL side at predetermined times (5, 10, 15, 20, and 30 min) over 30 min.

In experiments to investigate the effects of thalidomide, its total hydrolytic products and PGA on the transport of CPT-11 and SN-38 from AP to BL or BL to AP across the Caco-2 and various MDCKII monolayers, the inhibitor was added to the incubation medium on both the apical and the basolateral sides of the cell monolayers and preincubated with the cells for 30 min before the addition of CPT-11 or SN-38. Various PgP or MRP1/2 inhibitors, including verapamil (100 \( \mu \)M), MK-571 (100 \( \mu \)M), and probenecid (200 \( \mu \)M), were used as positive controls and added to both apical and the basolateral sides with a final DMSO concentration of 0.5% (v/v). All inhibitors used at the reported concentrations did not show significant cytotoxicity (<10%) when incubated with Caco-2 or various MDCKII cells overexpressing PgP, MRP1, or MRP2 for 2 to 4 h. Control vehicle was used for the control inserts. The apparent permeability coefficient \( (P_{app}) \) is expressed in centimeters/second and calculated as

\[
P_{app} = \frac{\Delta Q / \Delta t}{A} \times \frac{1}{C_0} \times \frac{1}{60}
\]

where \( \Delta Q / \Delta t \) is the permeability rate (nanomoles/minute); \( A \) is the surface area of the membrane (centimeter\(^2\)); and \( C_0 \) is the initial concentration in the donor chamber (nanomoles/milliliter). Samples from the 30-min point were used for \( P_{app} \) calculations.

High-Performance Liquid Chromatography and Liquid Chromatography-Mass Spectrometry Analysis. A Shimadzu HPLC system was used to quantify the CPT-11, SN-38, and SN-38G concentrations in different biological matrices as described previously (Hu et al., 2005). An analytical column (200 × 4.6 mm i.d.) packed with 5-μm Hyperclon ODS (C18) material (Phenomenex, Torrance, CA) preceded by a Phenomenex C18 guard cartridge was used for separation of test compounds. The mobile phase for the chromatography (LC)-mass spectrometry (MS) analysis was conducted to confirm the formation and structures of major metabolites of CPT-11 or SN-38 as the substrate in rat liver microsomes, Caco-2, MDCKII cells, and primary rat hepatocytes. The LC-MS system was equipped with an Agilent 1100 LC (Agilent Technologies, Palo Alto, CA) connected to a Q-Trap mass spectrometer (Applied Biosystems, Foster City, CA) through an electrospray ionization source.

Pharmacokinetic Calculations. Pharmacokinetic parameters were calculated by standard model-independent pharmacokinetic formulae using WinNonlin program (Pharsight Co., Mountain View, CA). The \( t_{1/2p} \) value was calculated as 0.693/\( k_p \), where \( k_p \) is the elimination rate constant calculated from the terminal linear portion of the log plasma concentration-time curve. The total areas under plasma or cecal concentration-time curve from time 0 to the last quantifiable time point \( (\text{AUC}_{0-\infty}) \) and from time 0 to infinity \( (\text{AUC}_{0-\infty}) \) were calculated using the log trapezoidal rule. The clearance \( (CL) \) was estimated by dividing the total administered dose by the \( \text{AUC}_{0-\infty} \). The \( C_{\text{max}} \) for the metabolites of CPT-11 (SN-38 and SN-38G) was obtained by visual inspection of the plasma concentration-time curve, whereas the initial drug concentration \( (C_0) \), the extrapolated concentration at zero time) of CPT-11 was calculated by back extrapolation of the plasma concentration-time curve to y-axis. The volume of distribution \( (V_d) \) was calculated by dividing CL by \( \beta \).

Statistical Analysis. Data are expressed as mean ± S.D. To describe the metabolism and transport kinetics of CPT-11 and SN-38, several models (e.g., the single and two binding site model, substrate inhibition model, and the sigmoid model) were fitted and compared using the Prism 3.0 program (GraphPad Software, Inc., San Diego, CA) described previously. These models included choice of model that was confirmed by F-test and comparison of Akaike's information criterion values (Yamaoka et al., 1978). Diarrhea scores were analyzed using Wilcoxon rank sum test. Statistical comparisons for multiple groups were performed using a one-way analysis of variance (ANOVA) followed by Student's Newman-Keuls test. Differences between groups for continuous variables on more than one occasion were evaluated with ANOVA followed by a Dunnett's multiple comparison test at \( P < 0.05 \). Differences between two groups were analyzed using unpaired Student's t test. Statistical significance was set as \( P < 0.05 \).
the toxicity study. Numbers of neutrophils and lymphocytes were significantly decreased and reached a minimum by day 7 in rats treated with CPT-11 and the control vehicle (1% DMSO, v/v) (Fig. 3). The combination of CPT-11 and thalidomide resulted in lesser decrease in the numbers of these types of blood cells. After day 7, rats receiving either CPT-11 with the control vehicle (1% DMSO, v/v) or combination therapy (CPT-11 plus thalidomide) had significantly increased neutrophils and lymphocytes. This may be due to a systemic response to severe inflammation of the intestines and other organs caused by CPT-11 administration. However, there was no significant difference in the erythrocyte and platelet levels of rats treated with CPT-11 and the control vehicle (1% DMSO, v/v) compared with rats treated with CPT-11 in combination with thalidomide.

Severe early- (days 1–4) and late-onset (days 5–8) diarrhea, with mean severity scores of 0.5, 0.42, 1.08, 1.83, 2.0, 2.33, 2.0, and 0.58 by days 1 through 8, respectively, were observed in these rats treated with CPT-11 and control vehicle. However, coadministration of thalidomide with CPT-11 resulted in lesser body weight loss compared with rats receiving CPT-11 and control vehicle (1% DMSO, v/v), with a decrease of 2% by day 8 and recovery to 114% of the baseline by day 11 (P < 0.01). Furthermore, the severity scores for both early- and late-onset diarrhea were significantly (P < 0.05, by Wilcoxon rank sum test) brought down in rats treated with CPT-11 and control vehicle (1% DMSO, v/v) in combination with thalidomide.

![Changes of counts of lymphocytes (A) and neutrophils (B) in rats treated with CPT-11 (60 mg/kg by i.v. bolus injection) and control vehicle (1% DMSO, v/v) or CPT-11 in combination with thalidomide (60 mg/kg i.p.).](image)

**TABLE 1**

Incidence of acute and delayed-onset diarrhea in rats treated with CPT-11 (60 mg/kg by i.v. bolus injection) and control vehicle (1% DMSO, v/v) or CPT-11 in combination with thalidomide (100 mg/kg i.p.).

The values are the number of animals (n) with scores of severity of diarrhea.

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<th>n</th>
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</table>

*P < 0.05 vs CPT-11 + 1% DMSO (v/v) group. Mean = Sum of scores (1–3)/rat number.

Similar severe histological damages were induced in the rats treated with CPT-11 and control vehicle (1% DMSO, v/v). However, these lesions were significantly alleviated in rats treated with CPT-11 in combination with thalidomide (Fig. 4). Our studies showed that combination of thalidomide effectively reduced the intestinal damages induced by CPT-11.

The histological lesion, including increased intestinal permeability and leukocyte infiltration accompanied with anticancer agent such as CPT-11, leads to an inflammatory response through the overproduction of proinflammatory cytokines, such as TNF-α by epithelial cells and infiltrating leukocytes within the intestinal mucosa. Moreover, the overall pathology initiated by CPT-11 is exacerbated with increased TNF-α expression by TNF-α-mediated apoptosis, tissue matrix degradation, and vascular leakage (Sonis, 2004).

**Thalidomide Inhibits the Intestinal Production of Proinflammatory Cytokines.** Treatment of CPT-11 increased the levels of TNF-α, IFN-γ, IL-1β, and IL-6 5- to 10-fold compared with day 0 (rats without any drug therapy), which achieved peak levels by day 5, 7, or 9 and declined thereafter, whereas the level of IL-2 decreased 2- to 5-fold compared with day 0, reaching a minimal level by day 5 or 7 in the above tissues examined (data not shown). These cytokines were undetectable or at very low levels in rats treated with the vehicle.

The levels of TNF-α in the ileum, cecum, colon, spleen, and serum were brought down by the combination of thalidomide (Fig. 5). Significant inhibition of TNF-α was seen in ileum...
from rats treated with combination therapy on day 5 by 65.0% ($P < 0.001$) and on day 7 by 59.0% ($P < 0.01$); in colon on day 5 by 46.5% ($P < 0.05$); and in cecum on day 5 by 44.9% and on day 7 by 46.6% ($P < 0.05$). Splenic TNF-$\alpha$ was also diminished on day 5 by 53.3% ($P < 0.01$) and in serum on day 7 by 35.4% ($P < 0.05$).

Coadministered thalidomide caused differential effects on the levels of IFN-$\gamma$ in different tissues (Fig. 6). Coadministered thalidomide caused differential effects on the levels of IFN-$\gamma$ in different tissues (Fig. 6).
tration of thalidomide significantly inhibited the production of IFN-γ in colon on day 5 and cecum on day 7 with 40.5% \((P < 0.05)\) and 57.6% \((P < 0.01)\) decreases, respectively. However, the combination of thalidomide resulted in increases in the levels of IFN-γ in the liver on day 5 by 44.6% \((P < 0.05)\) and in spleen on days 5 and 7 by 233.3 \((P < 0.001)\) and 103.7% \((P < 0.01)\), respectively, compared with the control rats that received CPT-11 and 1% DMSO (v/v).

Coadministration of thalidomide significantly increased the IL-1β expression in ileum on days 5, 7, and 11 by 127.6, 108.7 \((P < 0.01)\), and 64.3% \((P < 0.05)\), respectively (data not shown). Likewise, IL-1β protein levels in serum were also increased on days 5 and 7 by 196.9 and 161.5% \((P < 0.01)\), respectively. However, combination of thalidomide caused inhibitory effects on IL-1β levels in ileum on day 9 by 37.5% \((P < 0.05)\), and in spleen on days 5 and 7 by 38.0–57.1% \((P < 0.05)\) compared with the control rats that received CPT-11 and 1% DMSO (v/v).

Combination with thalidomide significantly increased IL-2 protein levels in ileum on day 5 by 514.0% and in serum on days 5 and 9 by 282.2 and 222.7% \((P < 0.001)\), respectively (data not shown). However, combination of thalidomide inhibited IL-2 protein levels in colon on day 5 by 47.1%, in spleen on day 7 by 41.0%, and in serum on days 7 and 11 by 74.0 \((P < 0.001)\) and 60.5% \((P < 0.01)\), respectively, compared with the control rats that received CPT-11 and 1% DMSO (v/v). In addition, combination with thalidomide significantly reduced the levels of IL-6 on day 5 in ileum by 67.3% \((P < 0.001)\), in colon by 41.3%, and in cecum by 35.4% \((P < 0.05)\) (data not shown). In addition, significant inhibitory effects on the production of IL-6 could be seen in spleen on days 5 and 7 with decreases of 62.1 and 48.5% \((P < 0.01)\), respectively, and in serum on days 9 and 11 with decreases of 60.4 and 57.8% \((P < 0.01)\), respectively, compared with the control rats that received CPT-11 and 1% DMSO (v/v).

**Thalidomide Inhibits Intestinal TNF-α mRNA Expression.** TNF-α can activate invading T cells and natural killer cells to produce IFN-γ, which directly leads to increased mucosal damage. Because of the critical role of TNF-α in CPT-11-induced intestinal lesions, we examined the expression pattern and kinetics of the transcripts for TNF-α gene over 11 days after administration of CPT-11 in intestinal tissues, including ileum, cecum, and colon. The transcripts for TNF-α in ileum and colon were detectable in healthy rats. However, the band intensity increased after CPT-11 injection and reached a peak level on day 7 in ileum and colon and then started to drop until day 11. Although the transcripts for TNF-α in cecum were almost undetectable from healthy rats, they were readily detectable in rats treated with CPT-11 and thalidomide (100 mg/kg i.p.). +, \(P < 0.05\); ++, \(P < 0.01\); and +++, \(P < 0.001\). Data are the mean ± S.D. of six rats per group of treatment.

Furthermore, expression pattern and kinetics of the transcripts for TNF-α gene over 11 days were also observed in rats treated with CPT-11 with or without thalidomide in intestinal tissues, including ileum, cecum, and colon (Fig. 8).
The band intensity in rat ileum increased after CPT-11 injection and reached a peak level on day 9 (2.8-fold increase compared with that of day 0) and then started to drop until day 11. However, combination with thalidomide resulted in less increase in the expression levels of TNF-α mRNA, as indicated by decreasing fold increase in band intensity compared with that of day 0 on days 5, 7, and 9 by 27.4, 29.2, and 29.2%, respectively ($P < 0.05$).

Transcripts for TNF-α in rat cecum were readily detectable on days 5 to 11, with gradual decrease in the expression level after CPT-11 administration. Decreased TNF-α transcript was observed in the group treated with combined thalidomide over 11 days. The fold increase of band intensity was brought down by 19.1% ($P < 0.05$) on day 5, 61.5% on day 7, 95.7% on day 9, and 95.6% ($P < 0.001$) on day 11. The transcripts for TNF-α were almost undetectable on days 9 and 11 in the rat receiving combination therapy.

Transcripts for TNF-α in rat colon were increased after CPT-11 injection and achieved the maximal level on day 7 in the control group treated with CPT-11 and 1% DMSO (v/v) and then decreased. Similar trends could be seen in the combination group with decreased expression levels of TNF-α over 11 days. Significantly smaller fold-increase of band intensity was observed on day 7 by 23.6% ($P < 0.05$) in the combination group.

Study in mice treated with CPT-11 showed overexpression of TNF-α after CPT-11 administration and its role involved in CPT-11-induced histological injuries (Zhao et al., 2004). Consistent with our data in the rat model, administration of CPT-11 for 4 consecutive days in rats in our study showed significant increase in TNF-α production in rat intestinal tissues both at protein and mRNA levels, associated with severe diarrhea and histological damages. These findings further support the hypothesis that the intestinal lesions induced by CPT-11 results from increased overproduction of proinflammatory cytokines like TNF-α.

**Thalidomide Reduces Intestinal Epithelial Apoptosis Induced by CPT-11.** Studies have shown that chemo-
therapy-induced intestinal mucositis is associated with intestinal exposure to these drugs, which induce epithelial apoptosis characterized by the generation of DNA fragments, decreased crypt cell renewal, and destruction of the mucosal architecture (Thompson, 1995). In normal rats without any drug treatment, the number of epithelial apoptotic cells per crypt in ileum, cecum, and colon were $2.0 \pm 0.7$, $5.0 \pm 1.6$, and $3.2 \pm 1.3$, respectively. Administration of CPT-11 caused a maximal epithelial apoptosis ($68.9 \pm 12.6$ per crypt) on day 5 in the ileum and then decreased thereafter. Likewise, maximal epithelial apoptosis was detected in cecum and colon on day 5 with gradual decreases until day 11 (Fig. 9).

Intestinal epithelial apoptosis was also evaluated in rats treated with CPT-11 alone (with control vehicle, 1% DMSO, v/v) or in combination with thalidomide. CPT-11 caused a maximal epithelial apoptosis ($62.6 \pm 11.5$ per crypt) on day 5 in the ileum and then decreased thereafter ($12.2-40.2$ per crypt over 7–11 days). Combination of thalidomide significantly reduced ileal epithelial apoptosis on day 5 by $47.9\% (P < 0.001)$, on day 7 by $39.3\% (P < 0.05)$, and on day 11 by $52.5\% (P < 0.05)$ compared with rats receiving CPT-11 and 1% DMSO (v/v). Likewise, maximal epithelial apoptosis was detected in the cecum and colon on day 5. Thalidomide pretreatment caused less epithelial apoptosis by $52.4\%$ on day 5 ($P < 0.001$) and by $45.7\%$ on day 7 ($P < 0.001$) in the cecum. In addition, coadministered thalidomide reduced the apoptosis by $47.0\%$ on day 5 ($P < 0.001$), by $34.1\%$ on day 7 ($P < 0.05$), and by $45.3\%$ on day 11 ($P < 0.05$) in the colon.

Studies also showed venous congestion-induced mucosal apoptosis via TNF-$\alpha$-mediated cell death in the rat small intestine with a variety of intermediates and protein-protein interactions involved (Wu et al., 2004). This finding is consistent with our result that congestion is observed in intestinal tissues accompanied with increased TNF-$\alpha$ level and apoptosis. This indicated that the reduced TNF-$\alpha$ level after

Fig. 9. Detection of apoptotic epithelial cells in cecum tissues (4-µm slices) on day 5 after first CPT-11 i.v. injection (60 mg/kg) using the TUNEL assay. Fragmented DNA of TUNEL-positive apoptotic cells (green spots) were incorporated with fluorescein-dUTP at free 3'-hydroxyl ends and visualized under fluorescence microscopy (magnification $\times 100$). A, healthy rats without drug therapy. B, rats received CPT-11 at 60 mg/kg by i.v. injection for 4 consecutive days. C, rats received CPT-11 and control vehicle (1% DMSO, v/v). D, rats treated with CPT-11 in combination with thalidomide (100 mg/kg i.p.). TH, thalidomide.

Fig. 10. Representative plasma concentration-time profiles for CPT-11, SN-38, and SN-38G in rats treated with CPT-11 and 1% DMSO (v/v) (control vehicle) or CPT-11 in combination with thalidomide (100 mg/kg i.p.). CPT-11 was dosed at 60 mg/kg by a bolus i.v. injection. A, B, and C, the kinetic study with a single-dose of thalidomide (100 mg/kg i.p.). D, E, and F, the kinetic study with 5-day multiple doses of thalidomide (100 mg/kg i.p.). $\bullet$, CPT-11 + thalidomide; $\circ$, CPT-11 with 1% DMSO (v/v).
Thalidomide combination also inhibited the intestinal damages caused by apoptosis arising from venous congestion. In addition, the inhibitory effects of thalidomide on IL-1β, IL-2, IL-6, and IFN-γ expression may also contribute to the alleviation in intestinal epithelial cellular apoptosis.

Thalidomide Alters the Plasma Pharmacokinetics of CPT-11 and Its Major Metabolites. For the single-dose study, coadministered thalidomide significantly (P < 0.05) increased the AUC0–10h and AUC0–∞ of CPT-11 by 32.50 and 39.02%, respectively. By contrast, these values for SN-38 were significantly (P < 0.01) decreased by 24.58 and 42.62%, respectively (Fig. 10A and Table 2). The value of t1/2B for SN-38 in rats treated with CPT-11 in combination with thalidomide was significantly lower compared with rats treated with CPT-11 and control vehicle (1% DMSO, v/v) (4.38 versus 7.18 h; P < 0.001). However, the combination of thalidomide did not significantly alter the pharmacokinetic parameters of SN-38G.

For the 5-day multiple-dose study, pretreatment of rats with thalidomide at 100 mg/kg i.p. significantly (P < 0.05) increased the C0, AUC0–10 h, and AUC0–∞ of CPT-11 by 85.56, 45.49, and 44.82%, respectively, and decreased the Vd and CL of CPT-11 by 38.98 and 43.03%, respectively (P < 0.01) (Fig. 10B and Table 2). The values of Cmax, AUC0–10 h, and AUC0–∞ for SN-38 were significantly (P < 0.05) decreased in rats treated with CPT-11 in combination with thalidomide by 26.36, 28.12, and 37.53%, respectively, compared with rats receiving CPT-11 and the control vehicle (1% DMSO, v/v). However, there was no significant change in the pharmacokinetic parameters of SN-38G, although the combination of thalidomide increased Cmax and AUC0–∞ of SN-38G by 24.56 and 13.01%, respectively, and decreased its t1/2B by 36.12%.

Thalidomide Decreases the Biliary Excretion of CPT-11 and Its Major Metabolites. The effects of coadministered thalidomide (100 mg/kg i.p.) for one single dose or multiple doses for 5 consecutive days are shown in Fig. 11. In bile duct-cannulated rats, CPT-11, SN-38, and SN-38G were rapidly excreted into the bile by 23.2 ± 2.2, 0.92 ± 0.06, and 1.43 ± 0.08% with 4 h following a 60-mg/kg bolus i.v. injection. The biliary excretion rates in rats receiving CPT-11 bolus injection alone were largely constant for several hours after the start of CPT-11 administration and then slightly declined. The cumulative excretion of CPT-11, SN-38, and SN-38 in rat bile over 12 h was 35.1 ± 2.2 (of total dose), 2.2 ± 0.17, and 3.78 ± 0.31%, respectively. These values increased to 35.9 ± 3.1 (of total dose), 2.99 ± 0.21, and 5.24 ± 0.30%, respectively, when the bile was collected over 24 h.

In bile duct-cannulated rats receiving one single dose of CPT-11 and thalidomide (100 mg/kg i.p.), the cumulative

### TABLE 2
Comparison of pharmacokinetic parameters between two groups of rats treated with CPT-11 (60 mg/kg by i.v. bolus injection) and control vehicle (1% DMSO, v/v) or CPT-11 with 1-day or 5-day thalidomide treatment (100 mg/kg i.p.)

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<td>CPT-11 + Control Vehicle</td>
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<td>C0 (µg/ml)</td>
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<tr>
<td>Cmax (µg/ml)</td>
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<td>Cmax (µg/ml)</td>
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<td>AUC0–10 h (µg · h/ml)</td>
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<td>AUC0–∞ (µg · h/ml)</td>
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Kinetic study with 5-day multiple doses of thalidomide

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<td>AUC0–∞ (µg · h/ml)</td>
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<td>Cmax (µg/ml)</td>
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<td>7.38 ± 1.67</td>
<td>5.68 ± 2.43</td>
<td>28.92</td>
</tr>
<tr>
<td>AUC0–∞ (µg · h/ml)</td>
<td>7.90 ± 1.74</td>
<td>6.99 ± 0.40</td>
<td>13.01</td>
</tr>
</tbody>
</table>

* Comparison of the two groups using the Student’s unpaired t test.
excretion of CPT-11, SN-38, and SN-38G in rat bile over 12 h was lesser than rats receiving CPT-11 alone (combination group: 32.1 ± 1.9, 1.95 ± 0.14, and 3.36 ± 0.24%, respectively; \( P < 0.05 \)) (Fig. 11). These values in rats with combination treatment were significantly \( P < 0.05 \) decreased to 32.9 ± 2.2, 2.32 ± 0.16, and 4.52 ± 0.25%, respectively, when the bile was collected over 24 h compared with the control rats treated with CPT-11 alone. Similar results for the biliary excretion of CPT-11, SN-38, and SN-38G were observed in bile-duct cannulated rats when treated with 5-day thalidomide (100 mg/kg). The decrease magnitude of biliary excretion of CPT-11 and its major metabolites was slightly higher than those in the one single-dose thalidomide study (Fig. 11).

**Thalidomide Alters the Intestinal Pharmacokinetics of CPT-11 and Its Major Metabolites.** The effects of coadministered thalidomide for 1 or 5 days are shown Table 3. Cecal CPT-11 concentrations rapidly achieved a maximum (42.13 ± 4.67 \( \mu g/g \) tissue) at the end of bolus infusion and then decreased exponentially in the control rats receiving CPT-11 alone. However, SN-38 and SN-38G concentrations in cecal tissues peaked at 8 or 12 h after the start of CPT-11 injection and then declined rapidly in this group of rats, with a \( C_{\text{max}} \) of 0.335 ± 0.062 and 0.162 ± 0.057 \( \mu g/g \) tissue, respectively. The AUC\(_{0-24h} \) values of CPT-11, SN-38, and SN-38G in control group rats were 234.5 ± 34.6, 3.42 ± 0.323, and 2.24 ± 0.178 \( \mu g \) h/g tissue, respectively. When a single dose of thalidomide at 100 mg/kg was combined with...
for 5 days also significantly decreased the
P/H11021 caused a significant (at 25 or 250 99%) in blood, we further examined the effects of thalidomide
CPT-11 and SN-38 are highly plasma protein-bound (80–
deincreased the 
Because coadministered thalidomide for 5 days significantly 
on Rat Plasma Protein Binding of CPT-11 and SN-38.
are able to modulate the hepatobiliary and intestinal trans-
are excreted into the intestine via the bile. Multiple dosing of thalidomide significantly reduced the intestinal exposure of CPT-11, SN-
and SN-38G, probably through reduction of biliary excretion of these compounds and/or inhibition of secretory efflux of these compounds mediated by PgP, MRP1, and MRP2 in enterocytes. Another possibility is reduced formation of these compounds mediated by PgP, MRP1, and MRP2 in cecal tissue, which thus reduces the reabsorption of SN-38 from the intestinal lumen into circulation. These findings also suggest that thalidomide and/or its hydrolytic products are able to modulate the hepatobiliary and intestinal transport.
Effects of Thalidomide and Its Hydrolysis Products on Rat Plasma Protein Binding of CPT-11 and SN-38.
Because coadministered thalidomide for 5 days significantly decreased the Vd of CPT-11 by 38.98% compared with the rats receiving CPT-11 alone (P < 0.001) and because both CPT-11 and SN-38 are highly plasma protein-bound (80–99%) in blood, we further examined the effects of thalidomide at 25 or 250 μM of its total hydrolytic products (10 μM) and PGA (10 μM) on rat plasma protein binding of CPT-11 and SN-38. As expected, CPT-11 and SN-38 bound extensively to the rat plasma proteins. The binding of CPT-11 to rat plasma proteins was concentration-dependent, whereas the concentration-dependent binding of SN-38 was insignificant. Thalidomide, its total hydrolysis products, and PGA did not significantly alter the rat plasma protein binding of CPT-11 and SN-38 (data not shown). However, thalidomide at 250 μM caused a significant (P < 0.05) increase in f0 of CPT-11 (6.7%), suggesting a decrease in CPT-11 plasma protein binding. Although thalidomide at 250 μM caused significant increase in the f0 of CPT-11, such high concentration was only achieved in vivo when high doses of thalidomide (e.g., >250 mg/kg) was administered. In addition, thalidomide is only moderately bound to plasma proteins (by 55–66% only). This moderate value for thalidomide would not significantly alter the plasma protein binding of combined drugs, such as CPT-11 and SN-38, with high plasma protein binding in vivo. However, modulation of the tissue binding of CPT-11 and SN-38 and its binding to target protein (i.e., topoisomerase I) by thalidomide and its hydrolytic products could not be excluded.
Thalidomide and Its Hydrolytic Products Alters the Metabolism of CPT-11 and SN-38 in Rat Liver Microsomes.
For the hydrolysis of CPT-11, linear SN-38 formation was observed with incubation time from 5 to 90 min and with rat liver microsomes from 0.125 to 4.0 mg/ml microsomal protein. An optimal protein concentration of 2.0 mg/ml and incubation time of 30 min were chosen for the inhibition studies. For the glucuronidation of SN-38, linear SN-38 formation was observed with incubation time from 15 to 60 min and with rat liver microsomes from 0.25 to 2.0 mg/ml microsomal protein. The addition of a detergent including Brij 58 or Brij 38 did not significantly affect the formation of SN-38G (data not shown). Thus, a protein concentration of 1.0 mg/ml microsomal protein and an incubation time of 30 min were chosen for the further inhibition studies without addition of detergent. The use of 1 to 2 mg/ml protein concentrations was necessary to conduct enzyme kinetic studies under optimal conditions and to surpass the assay limit of quantitation at low-substrate concentrations. Such microsomal protein concentrations and the enzyme activities observed were comparable with those reported in the literature (Yokoi et al., 1995). By comparing the models fitted, hydrolysis of CPT-11 to form SN-38 in rat liver microsomes was best fit by a two binding-site model with an estimated Kmax1 and Kmax2 of 0.50 and 78 μM, respectively, and Vmax1 and Vmax2 of 0.58 and 2.34 pmol/min/mg protein, respectively. In addition, glucuronidation of SN-38 in rat liver microsomes was best fit by a one-enzyme equation with an estimated Kmax of 18.24 μM and a Vmax of 185.6 pmol/min/mg protein. Our results from the
binding site fitting indicate that there are at least two CEs with differential affinity and catalyzing capacity involved in the hydrolysis of CPT-11 in rat liver microsomes, whereas a predominant UGT1A enzyme or multiple UGT1A enzymes with similar capacities catalyze SN-38 glucuronidation in rat liver microsomes. The observed enzyme kinetic constants for CPT-11 and SN-38 metabolism are comparable with those reported previously in rat or human liver microsomes (Haaz et al., 1998).

The effects of thalidomide and its hydrolysis products on CPT-11 hydrolysis and SN-38 glucuronidation are shown in Fig. 12. Thalidomide (25 or 250 μM) had no inhibitory effects on CPT-11 (0.5 and 78 μM) hydrolysis, whereas the hydrolysis products of thalidomide (10 μM) significantly (P < 0.05) decreased the CPT-11 (0.5 μM) hydrolysis by 16% and insignificantly (P > 0.05) decreased CPT-11 (78 μM) hydrolysis by 8%. Thalidomide hydrolysis products significantly inhibited CPT-11 (0.5 μM) hydrolysis in a concentration-dependent manner. However, both thalidomide and its hydrolysis products had no significant effects on SN-38 glucuronidation.

As expected, nifedipine (100 μM) significantly reduced CPT-11 hydrolysis (0.5 μM) by 17% (P < 0.01), whereas it had no significant effect for CPT-11 hydrolysis at a higher concentration (78 μM). By contrast, BNPP at 100 μM showed significant (P < 0.01) inhibitory effect on CPT-11 hydrolysis at 5 (a 50% decrease) or at 78 μM substrate concentration (43.5% decrease). In addition to its inhibitory effect on CPT-11 hydrolysis, nifedipine (100 μM) also had significant (P < 0.05) inhibitory effect on the glucuronidation of SN-38 at 5 (a 60% decrease) or at 18.24 μM (53% decrease). However, bilirubin at 100 μM had no significant effect on SN-38 glucuronidation (5 and 18.24 μM). These results indicated the effectiveness of our microsomal system in the metabolic inhibition studies.

**Cytotoxicity of CPT-11 and SN-38 in Caco-2, Primary Rat Hepatocytes, and MDCKII Cells Overexpressing MDR1, MRPI, and MRP2.** The IC_{50} values were 46.54 ± 14.74 and 0.11 ± 0.03 μM for CPT-11 and SN-38 in Caco-2 cells when the drug was incubated with the cells for 48 h, respectively. These values were 35.34 ± 12.12 and 0.06 ± 0.02 μM for CPT-11 and SN-38 in primary rat cultured hepatocytes when the drug was exposed for 48 h, respectively. These results indicate that SN-38 had 422- to 588-fold higher cytotoxicity than CPT-11 in Caco-2 cells and primary rat cultured hepatocytes, respectively.

The MTT assays with 48-h drug exposure time showed that the IC_{50} values for control MDCKII, MDR1-MDCKII, MRPI-MDCKII, and MRP2-MDCKII cells for CPT-11 and SN-38 were 5.43 ± 0.23, 35.12 ± 3.12, 26.78 ± 2.43, and 48.39 ± 5.36 μM and 0.051 ± 0.022, 0.527 ± 0.062, 0.468 ± 0.033, and 0.713 ± 0.061 μM, respectively. These results indicate that MDR1, MRPI, and MRP2 conferred 4.9- to 8.9-fold resistance to CPT-11 and 9.2- to 14.0-fold resistance to SN-38. However, CPT-11 at up to 100 μM and SN-38 at up to 10 μM did not show significant cytotoxicity (<10%) for all above cells when the drug exposure time was within 2 h.

**Thalidomide and Its Hydrolytic Products Affect the Metabolism of CPT-11 and SN-38 in Primary Rat Cultured Hepatocytes.** The metabolism of CPT-11 and SN-38 in primary rat cultured hepatocytes incubated with culture medium with regard to incubation time and substrate concentration is shown in Fig. 13. The generated metabolites SN-38 and SN-38G were structurally confirmed by LC-MS. Both SN-38 and SN-38G were detectable and achieved the peak levels after 5-min incubation of CPT-11 with primary rat cultured hepatocytes and peaked at 60 min; thereafter, the formation of both metabolites appeared saturaible with slightly declined formation over the rest of time in the medium.

The formation of both SN-38 and SN-38G in primary rat cultured hepatocytes increased with increasing CPT-11 concentration and followed Michaelis-Menten kinetics with the one-binding site model being the best fit. The estimated K_{m} and V_{max} for SN-38 formation in culture medium were 53.68 ± 3.56 μM and 8.49 ± 0.34 pmol/min/10^6 cells, respectively, and for SN-38G formation, the K_{m} and V_{max} were 22.32 ± 3.88 μM and 11.29 ± 0.91 pmol/min/10^6 cells, respectively (Fig. 13). In addition, when SN-38 as the substrate was incubated with primary rat cultured hepatocytes, SN-38G appeared rapidly in the medium and peaked at 90 min and then slowly declined over the rest of the time. The formation of SN-38 increased in a substrate concentration-dependent manner and followed Michaelis-Menten kinetics. One-binding site model was the best fit for the formation of...
SN-38G in primary rat cultured hepatocytes, with a $K_m$ of $2.13 \pm 0.12 \mu M$ and a $V_{\max}$ of $224.9 \pm 5.95 \text{pmol/min/10}^6 \text{cells}$.

Thalidomide and its hydrolysis products did not show any significant effects on CPT-11 hydrolysis and SN-38G formation when CPT-11 was incubated with primary rat cultured hepatocytes and SN-38 glucuronidation from SN-38 in primary rat cultured hepatocytes. Of the positive control inhibitors, BNPP at $100 \mu M$ significantly inhibited the hydrolysis of CPT-11 by $19.8\% (P < 0.05)$, and nifedipine at $100 \mu M$ significantly inhibited the glucuronidation of SN-38 by $34.9\% (P < 0.05)$. In addition, bilirubin at $100 \mu M$ significantly inhibited SN-38 glucuronidation by $33.1\%$ in primary rat cultured hepatocytes (Fig. 14).

Compared with rat liver microsomes, primary rat hepatocytes hydrolyzed CPT-11 much faster than rat liver microsomes, probably because of higher CE levels in fresh hepatocytes, but SN-38G formation when incubated with CPT-11 as the substrate was markedly slower compared with rat liver microsomes, with much lower $V_{\max}$ probably because of concentrated and higher UGT1A levels in rat liver microsomes compared with fresh hepatocytes. In contrast, the UGT1A inhibitor BNPP at $100 \mu M$ significantly inhibited CPT-11 hydrolysis by $50\%$ at $5 \mu M$ substrate concentration and $43.5\%$ at $78 \mu M$ substrate concentration in rat liver microsomes, and it similarly inhibited the hydrolysis of CPT-11 by $33.1\%$ in primary rat hepatocytes. When SN-38 was incubated with primary rat hepatocytes, the formation of SN-38G was comparable with that in rat liver microsomes. Thus, there are similarities and differences with regard to concentrations of enzymes and other compounding factors affecting the metabolism of CPT-11 and SN-38. This may be due to the presence of competition between metabolism and efflux and binding to target protein for SN-38 in primary rat hepatocytes but not in rat liver microsomes. These findings also highlight the necessity of application of a combination of in vitro models to overcome the intrinsic limitation of these in vitro systems in drug metabolism studies.

**Thalidomide and Its Hydrolytic Products Decreases the Intracellular Accumulation of CPT-11 and SN-38 in Primary Rat Cultured Hepatocytes.** The accumulation of CPT-11 and SN-38 in primary hepatocytes significantly depended on the incubation time and peaked at 30 and 15 min, respectively, and thereafter declined gradually, probably because of saturaibility or increased cytotoxicity. Their accumulation in primary rat hepatocytes also increased depending on the substrate concentration and followed Michaelis-Menten kinetics with one-binding site model being the best fit (Fig. 15). The $K_m$ and $V_{\max}$ for CPT-11 was $22.57 \pm 3.18 \mu M$ and $36.74 \pm 2.43 \text{pmol/min/10}^6 \text{cells}$ in HBSS, respectively. For SN-38, the values were $1.53 \pm 0.35 \mu M$ and $0.96 \pm 0.09 \text{pmol/min/10}^6 \text{cells}$ in HBSS, respectively. It seems that the transports for SN-38 has higher affinity than CPT-11, but the accumulation of CPT-11 was much faster than SN-38 in primary rat hepatocytes.

The effects of coincubation and 2-h preincubation of potential inhibitors, including thalidomide and its hydrolysis products on the intracellular accumulation of CPT-11 and SN-38 in primary rat-cultured hepatocytes, are shown in Fig. 16. Coincubation of thalidomide at $250 \mu M$, PGA at $10 \mu M$, and thalidomide hydrolys products at $10 \mu M$ significantly increased the intracellular accumulation of CPT-11 by $23.1 \pm 2.6$, $26.6 \pm 3.7$, and $32.5 \pm 4.0\%$, respectively, in primary rat cultured hepatocytes. Verapamil (100 \mu M), nifedipine (100 \mu M), MK-571 (100 \mu M), and probenecid (200 \mu M) significantly increased CPT-11 accumulation by $23.5 \pm 1.5$ to $65.6 \pm 10.8\% (P < 0.05)$. When thalidomide at $25$ or $250 \mu M$, PGA at $10 \mu M$, and thalidomide hydrolys products at $10 \mu M$ were preincubated for 2 h with the rat hepatocytes, CPT-11 accumulation was significantly ($P < 0.05$) increased by $26.7 \pm 3.3$, $89.8 \pm 8.8$, $30.4 \pm 2.1$, and $44.4 \pm 5.7\%$, respectively. The positive control inhibitors, including verapamil (100 \mu M), nifedipine (100 \mu M), MK-571 (100 \mu M), and
probenecid (200 μM) significantly increased CPT-11 intracellular accumulation by 83.1 ± 9.5 to 145.6 ± 20.2% (P < 0.01). These findings indicated that preincubation for 2 h with the potential inhibitor resulted in higher CPP-11 accumulation in primary rat hepatocytes. 

Coincubation of thalidomide at 25 or 250 μM, PGA at 10 μM, or thalidomide hydrolysis products at 10 μM significantly increased the intracellular accumulation of SN-38 by 78.4 ± 12.4, 193.3 ± 29.1, 145.4 ± 21.45, and 223.5 ± 31.44%, respectively (P < 0.01) in primary rat cultured hepatocytes (Fig. 16). The positive control inhibitors including verapamil (100 μM), nifedipine (100 μM), MK-571 (100 μM), and probenecid (200 μM) significantly increased SN-38 accumulation by 43.5 ± 5.5 to 62.0 ± 10.8% (P < 0.05). When the primary rat hepatocytes were preincubated with thalidomide at 25 or 250 μM, PGA at 10 μM, and thalidomide hydrolysis products at 10 μM for 2 h, the accumulation of SN-38 was significantly (P < 0.05) increased by 78.4 ± 6.5, 193.3 ± 23.4, 145.4 ± 15.1, and 44.4 ± 5.7%, respectively.

The positive control inhibitors, including verapamil (100 μM), nifedipine (100 μM), MK-571 (100 μM), and probenecid (200 μM), significantly increased SN-38 intracellular accumulation by 83.1 ± 9.5 to 145.6 ± 20.2% (P < 0.01). In addition, preincubation of MK-571 (100 μM) in combination with nifedipine (100 μM for each inhibitor) for 2 h significantly increased the intracellular accumulation of CPT-11 and SN-38 by 543.2 ± 75.4 and 753.6 ± 101.6% (P < 0.01), respectively, in primary rat cultured hepatocytes, suggesting the presence of synergistic effect of combined use of PgP (MDR1) and MRP1/2 inhibitors on CPT-11 and SN-38 intracellular accumulation in rat hepatocytes.

These results indicate that thalidomide and its hydrolytic products could inhibit transporter-mediated efflux of CPT-11 and SN-38 in primary rat hepatocytes and that PGA is the major contributor for the inhibitory effects of thalidomide hydrolytic products on transporters in rat hepatocytes. The mechanism for the accumulation of CPT-11 and SN-38 by primary rat hepatocytes is unknown, but both active and passive transport are probably involved. CPT-11 and SN-38 can be readily taken up by human intestinal Caco-2 cells through passive diffusion (Gupta et al., 2000). However, the best fit of one binding-site model for the accumulation of both CPT-11 and SN-38 in primary rat hepatocytes indicated the involvement of one predominant transporter or multiple transporters with similar affinity to the substrates, whereas passive diffusion played a minor or negligible role. PgP, MRP1–2, and MRP4 are all possibly involved in the transport mechanism for the accumulation of CPT-11 and SN-38 in primary rat hepatocytes, as indicated by the significant enhanced accumulation of both substrates in the presence of nifedipine, verapamil (both PgP inhibitors), MK-571 (an inhibitor for MRP1–4), and probenecid (a Pgp inhibitor). Both CPT-11 and SN-38 appeared to enter tumor cells at a rapid rate and are then distributed within cells and bound by subcellular organelles, drug-metabolizing enzymes located on endoplasmic reticulum, and the target protein in nucleus (topoisomerase I). The intracellular accumulation of CPT-11 in primary rat hepatocytes is different from that of SN-38 with differential Michaelis-Menten constants. This may be due mainly to the different physiochemical properties of these two compounds. SN-38 has higher lipophilicity than CPT-11, probably resulting in differential affinity to the transporters, uptake, subcellular compartmentalization, and efflux compared to CPT-11. Different uptake rate and extent of CPT-11 and SN-38 have also been observed in intestinal and lung cancer cells (Chu et al., 1999). Uptake, efflux, and metabolism are all considered important determinants for the intracellular accumulation of CPT-11 and SN-38.

**Thalidomide and Its Hydrolytic Products Alters the Transport of CPT-11 and SN-38 in Caco-2, MDCKII, MDR1-MDCKII, MRP1-MDCKII, and MRP2-MDCKII Monolayers.** No marked formation of metabolites was observed when CPT-11 or SN-38 was loaded on apical or baso-
lateral side of cellular monolayers tested at all drug concentrations over 30 min. The time course and concentration effect of CPT-11 and SN-38 flux from AP to BL or BL to AP has been examined. After apical or basolateral loading, CPT-11 or SN-38 appeared on the receiving side in 5 min in all monolayers examined. The transport rate of CPT-11 and SN-38 from AP to BL or BL to AP was largely proportional to the substrate concentrations for CPT-11 over 2 to 50 μM and SN-38 over 0.2 to 5.0 μM and linear up to 30 min of incubation time in all of the monolayers examined (data not shown).

The permeability of CPT-11 and SN-38 across Caco-2 and control MDCKII monolayers from BL to AP side was 2.4- to 5.5-fold higher than that from AP to BL (Tables 4 and 5). These results demonstrated a polarization in the permeability toward both CPT-11 and SN-38 and a predominantly secretory rather than absorptive transport of these two compounds in Caco-2 and wild-type MDCKII monolayers. The fold increases in permeability of CPT-11 and SN-38 in MDR1-MDCKII, MRPI-MDCKII, and MRP2-MDCKII monolayers were significantly higher (6.9-11-fold) than control (wild-type) MDCKII cells. These results indicate that PgP (MDR1), MRPI, and MRP2 conferred significant resistance to both CPT-11 and SN-38. The P_app values of CPT-11 over 2 to 50 μM and SN-38 over 0.2 to 5.0 μM from BL to AP in all of the monolayers tested were concentration-dependent and appeared saturable, with marked decrease in their P_app values for both AP to BL and BL to AP flux at increasing substrate concentrations. This may reflect that the P_app of CPT-11 and SN-38 is affected by a number of complicating factors associated with the drugs and the epithelial cells, in particular, for substrates such as CPT-11 and SN-38 that probably have high-intrinsic permeability and substantial tissue uptake and binding.

The effects of thalidomide and its hydrolytic products on the transport and the permeability of CPT-11 and SN-38 were investigated in Caco-2 and MDCKII monolayers (Tables 4 and 5). The addition of thalidomide at 250 μM, thalidomide hydrolytic products at 10 μM, and PGA at 10 μM significantly increased the AB to BL permeability of CPT-11 at 10 μM in Caco-2 monolayers by 29.6, 22.2, and 55.5%, respectively (P < 0.05). On the other hand, these potential inhibitors significantly decreased the BL to AP permeability of CPT-11 at 10 μM in Caco-2 monolayers by 31.8, 32.7, and 36.4%, respectively (P < 0.05). Similar results for CPT-11 were observed with control (wild-type) MDCKII monolayers. For SN-38, incubation of Caco-2 monolayers with thalidomide at 250 μM, thalidomide hydrolytic products at 10 μM, and PGA at 10 μM significantly increased the AB to BL permeability of CPT-11 at 10 μM by 78.6, 128.6, and 135.7%, respectively (P < 0.01). Consistently, thalidomide at 250 μM, thalidomide hydrolytic products at 10 μM, and PGA at 10 μM significantly decreased the BL to AP permeability of CPT-11 at 10 μM by 25.6, 25.6, and 30.2%, respectively (P < 0.05). Similar results for SN-38 were observed with control MDCKII monolayers. The inclusion of verapamil (100 μM), MK-571 (100 μM), or probenecid (200 μM) significantly decreased the BL to AP permeability more and enhanced the AP to BL flux of both CPT-11 and SN-38 in Caco-2 and MDCKII monolayers compared with thalidomide and its hydrolytic products. These results indicate that thalidomide and its hydrolytic products, including PGA, could inhibit the secretory transport of CPT-11 and SN-38 but enhance their...
Fig. 16. Effects of thalidomide (TH) at 25 or 250 μM, its total hydrolysis products at 10 μM, PGA at 10 μM, nifedipine at 100 μM, probenecid at 200 μM, MK-571 at 100 μM, and verapamil at 100 μM on the intracellular accumulation of CPT-11 at 10 μM (A and B) and SN-38 at 1.0 μM (C and D) in primary rat hepatocytes cultured in medium. A and C, coincubation of the substrate with the potential inhibitor in rat hepatocytes. B and D, 2-h preincubation of the rat hepatocytes with the potential inhibitor. *, P < 0.05; **, P < 0.01. Data are the mean ± S.D. from at least three to nine determinations.
The secretory transport of CPT-11 and SN-38 in both absorptive and non-absorptive transport. MDR1, MRP1, and MRP2 play a major role in the secretory transport of CPT-11 and SN-38 in both Caco-2 and MDCKII cells.

To further characterize the inhibitory nature of thalidomide and its hydrolytic products on ABC transporter-mediated flux of CPT-11 and SN-38, we investigated their effects in MDR1-, MRP1-, and MRP2-MDCKII monolayers. The results are shown in Tables 4 and 5. In MDR1-MDCKII monolayers, thalidomide at 250 μM, its hydrolytic products at 10 μM, and PGA at 10 μM significantly inhibited MDR1-mediated CPT-11 BL to AP permeability by 13.6, 13.5, and 19.7%, respectively (P < 0.05) (Table 4). Such inhibition of MDR1-mediated BL to AP permeability by thalidomide and its hydrolytic products resulted in significant increase by 70.5 to 79.4% in AP to BL permeability of CPT-11. For SN-38, thalidomide at 250 μM, its hydrolytic products at 10 μM, and PGA at 10 μM significantly inhibited MDR1-mediated SN-38 BL to AP permeability by 28.0, 29.8, and 32.7%, respectively.
(P < 0.05) (Table 5). The inhibition of MDR1-mediated BL to AP permeability by thalidomide and its hydrolytic products also resulted in significant increase in AP to BL permeability of SN-38 (by 54.8–71.0%). Verapamil at 100 μM significantly inhibited MDR1-mediated CPT-11 and SN-38 BL to AP permeability by 56.7 and 63.9%, respectively (P < 0.01) (Tables 4 and 5). However, MK-571 and probenecid did not significantly affect the MDR1-mediated CPT-11 and SN-38 transport in MDR1-MDCKII cells (P > 0.05).

In MRP1-MDCKII cells, thalidomide at 250 μM, its hydrolytic products at 10 μM, and PGA at 10 μM significantly inhibited MRP1-mediated BL to AP permeability of CPT-11 by 16.5, 20.2, and 23.2%, respectively (P < 0.05) (Table 4). Such inhibition of MRP1-mediated BL to AP permeability of CPT-11 by thalidomide and its hydrolytic products resulted in significant increase in AP to BL permeability of CPT-11 by 31.7–51.2%. As for SN-38, thalidomide at 250 μM, its hydrolytic products at 10 μM, and PGA at 10 μM significantly inhibited MRP1-mediated BL to AP permeability of SN-38 by 15.2, 22.8, and 21.9%, respectively (P < 0.05) (Table 5). In addition, the inhibition of MRP1-mediated BL to AP permeability of SN-38 by thalidomide and its hydrolytic products resulted in significant increase in AP to BL permeability of SN-38 by 61.9 to 100.0% (P < 0.01). MK-571 at 100 μM and probenecid at 200 μM significantly inhibited MRP1-mediated BL to AP permeability of CPT-11 by 40.8 and 58.1%, respectively (P < 0.01) (Table 4). The values for SN-38 were 50.2 and 65.4% by MK-571 and probenecid, respectively (P < 0.01) (Table 5). Interestingly, verapamil at 100 μM also significantly inhibited the MRP1-mediated CPT-11 and SN-38 permeability by 40.8 and 50.2%, respectively (P < 0.01), in MRP1-MDCKII cells, suggesting that verapamil is not only a PgP inhibitor but also an inhibitor for MRP1.

In MRP2-MDCKII monolayers, thalidomide at 250 μM, its hydrolytic products at 10 μM, and PGA at 10 μM significantly inhibited MRP2-mediated BL to AP permeability of CPT-11 by 19.9, 28.8, and 30.6%, respectively (P < 0.05) (Table 4). The inhibition of MRP2-mediated BL-AP permeability of CPT-11 by thalidomide and its hydrolytic products resulted in significant increase in AP to BL permeability of CPT-11 by 27.3 to 40.9% (P < 0.05). For SN-38, thalidomide at 250 μM, its hydrolytic products at 10 μM, and PGA at 10 μM significantly inhibited MRP2-mediated BL to AP permeability of SN-38 by 17.8, 33.5, and 33.8%, respectively (P < 0.05) (Table 5). In addition, the inhibition of MRP2-mediated BL to AP permeability of SN-38 by thalidomide and its hydrolytic products resulted in significant increase in AP to BL permeability of SN-38 by 43.8 to 71.8% (P < 0.05) (Table 5). The values for SN-38 were 38.2 and 34.2% by MK-571 and probenecid, respectively (P < 0.05) (Table 5). However, verapamil at 100 μM insignificantly affect the MRP2-mediated CPT-11 and SN-38 transport in MRP2-MDCKII cells.

The inhibitory effects of thalidomide and its hydrolytic products on transporter-mediated CPT-11 and SN-38 flux may have limited clinical implication as reversing agents of multidrug resistance. Tumor cells generally exhibit high levels of expression of PgP and MRPs. Coadministration of thalidomide might enhance the anticancer effects of CPT-11 by inhibiting these transporters and enhance cellular uptake of CPT-11 and SN-38, but very high concentrations (≥250 μM) of thalidomide or its hydrolytic products must be achieved, which are physiologically irrelevant. Notably, thalidomide at a low concentration (25 μM), which can be easily achieved in patients, did not significantly affect the AP to BL and BL to AP transport of CPT-11 and SN-38 in all of the monolayers examined, with the exception of MDR1-MDCKII monolayers, where it significantly increased the MDR1-mediated AP to BL permeability of CPT-11 by 32.3% (P < 0.05) (Table 4).

Discussion

In the present study, we demonstrated that the anti-TNF-α agent thalidomide significantly attenuated CPT-11-induced intestinal and blood toxicities in rats. Coadministered thalidomide significantly alleviated body weight loss, myelosuppression, diarrhea, intestinal inflammation, and histological damages caused by CPT-11. To address the underlyling mechanism for these protective effects of thalidomide on CPT-11 induced intestinal damage, we explored the roles of both pharmacodynamic and pharmacokinetic components.

Thalidomide pretreatment suppressed increased cytokines production, including IL-1β, IL-6, TNF-α, and IFN-γ but increased IL-2 protein level in the intestine and liver. Consistently, the TNF-α mRNA levels were also brought down by thalidomide pretreatment in these tissues. TNF-α plays a critical role in chemotherapy-induced intestinal lesions, and it can potentiate the immune response of many other proinflammatory cytokines, including IL-1β, IL-2, IL-6, and IFN-γ (Sonis, 2004). The combination of thalidomide reduced the TNF-α production at both protein and mRNA levels compared with the control group treated with CPT-11 only. This finding indicated that the protective effect of thalidomide on CPT-11-induced toxicity might be attributed to its down-regulatory effect on TNF-α expression. In addition, the inhibitory effect of thalidomide on TNF-α mRNA expression could be ascribed to the increased degradation of TNF-α mRNA, as indicated by our RT-PCR results. Because the production of TNF-α at protein levels was also suppressed by thalidomide as indicated by enzyme-linked immunosorben assay, thalidomide also acted on transcriptional level.

Modulation of other important cytokines may also play an important role in the anti-inflammatory and antiapoptotic effects of thalidomide. The inhibition of intestinal IL-1β, IL-6, and IFN-γ by thalidomide may contribute to the protective effect on CPT-11-associated intestinal inflammation due to the important roles of these cytokines in the signal amplification of CPT-11-induced intestinal injuries initiated by TNF-α. The inhibitory effect of thalidomide on IL-1β and IL-6 may be attributed to its inhibitory effect on TNF-α and IFN-γ in local mucosal immunocompetent cells. Reduced TNF-α expression may consecutively lead to a reduced synthesis of IFN-γ and a reduced infiltration of activated lymphocytes. Notably, the combined use of thalidomide alleviated the decrease in IL-2 expression caused by CPT-11, which may be involved in the attenuated effect of thalidomide on CPT-11-induced intestinal lesion.

Apart from the modulation effects on proinflammatory cytokines, thalidomide exhibited marked in vivo antiapoptotic
effects in our study, probably contributing to its protective effects on CPT-11-induced histological damages. Increasing evidence has shown that apoptosis represents an important event during gut inflammatory responses. The findings in our study also provided further support for this apoptosis-associated mechanism for intestinal lesion induced by chemotherapy. The inhibition of intestinal apoptosis by thalidomide might be attributed to the inhibition of TNF-α expression.

Another possible mechanism for the protective effect of thalidomide on CPT-11-induced intestinal toxicity is pharmacokinetic interactions between these two compounds. The pharmacokinetic study revealed that coadministered thalidomide significantly increased the AUC₀₋₅₀ of CPT-11 but decreased that of SN-38. Similar results were found in the 5-day multidosing study with greater modulating effect on the pharmacokinetic parameters of CPT-11 and SN-38 compared with the single-dose study. In addition, coadministration of thalidomide caused a significantly decreased elimination half-life of SN-38 in both 1- and 5-day protocols. The CL for CPT-11 was also significantly decreased with coadministered thalidomide in 5-day multidosing and single-dose studies. Exposure of intestines to SN-38 is believed to be the main reason for the occurrence of CPT-11-induced delayed-onset diarrhea. There is a clear correlation between the toxicity of CPT-11 and its pharmacokinetic parameters for plasma AUC and Cₘₐₓ of SN-38 in cancer patients. Thus, our findings may partially explain the protective effect of thalidomide against the gastrointestinal and blood toxicity of CPT-11.

We further explored the underlying reasons for the increased plasma CPT-11 concentration and AUC with decreased total clearance as well as reduced plasma SN-38 concentration and AUC with increased total clearance by thalidomide using bile duct-cannulated rats and several in vitro models, including rat liver microsomes, primary rat hepatocytes, and Caco-2 and MDCKII cells. Given that thalidomide is hydrolyzed to more than a dozen metabolites in vivo, it can be expected that these hydrolysis metabolites may have a modulating effect on the metabolism and transport of CPT-11 and SN-38. Coadministered thalidomide for 1 or 5 days significantly reduced the biliary excretion of CPT-11, SN-38, and SN-38 glucuronide. Consistently, coadministered thalidomide for 5 days, but not for a single dose, also significantly reduced the cecal concentrations of CPT-11, SN-38, and SN-38G. The reduced biliary excretion of CPT-11 and its major metabolites may partially explain the reduced cecal exposure of these compounds by thalidomide. The effects of coadministered thalidomide on the plasma, biliary, and intestinal pharmacokinetics of CPT-11 and its major metabolites (in particular, the highly cytotoxic SN-38) may provide partial explanation for the attenuated toxicity of CPT-11.

The formation of SN-38 from CPT-11 is via the CE-catalyzed hydrolysis with SN-38 being further converted into glucuronide via UGT1As. In vitro models, including rat liver microsomes and primary rat hepatocytes, were used to investigate the possible effects of thalidomide and its hydrolytic products on CPT-11 and SN-38 metabolism. In rat liver microsomes, a significant decrease in the hydrolysis of CPT-11 at 0.5 μM by the total hydrolysis products of thalidomide (10 μM), instead of thalidomide, was observed. Such inhibitory effect was dependent on the total concentration of thalidomide hydrolysis products. This inhibitory effect could also be seen for the hydrolysis of higher CPT-11 concentration (78 μM) but no significant effect. However, thalidomide and its hydrolysis products did not show any significant effects on CPT-11 hydrolysis and SN-38G formation when CPT-11 was incubated with primary rat cultured hepatocytes, and SN-38 glucuronidation was incubated from SN-38 in primary rat cultured hepatocytes. Not surprisingly, rat liver microsomes and primary rat hepatocytes generated differential results due to the great differences of these two in vitro models. Thus, the extrapolation of the these effects observed in vitro to in vivo situation may be difficult because of the discrepancies in drug concentrations, microenvironment, and intrinsic limitations of in vitro models.

The parental drug and its major metabolites are excreted into the bile via PgP and MRP1 and MRP2 (Gupta et al., 1994; Chu et al., 1998; Tian et al., 2005). Thus, we further investigated the effects of thalidomide and its hydrolytic products on the transport of CPT-11 and SN-38 in primary rat hepatocytes, Caco-2, and MDCKII overexpressing MDR1, MRP1, or MRP2. Interestingly, thalidomide at 250 μM, PGA at 10 μM, or thalidomide hydrolysis products at 10 μM significantly increased the intracellular accumulation of both CPT-11 and SN-38 when coincubated with the substrate or preincubated for 2 h in primary rat hepatocytes. The increased intracellular accumulation of CPT-11 and SN-38 into rat hepatocytes might increase their metabolism because of increased substrate availability. The increased accumulation cannot be attributable to damage of the cellular plasma membrane, which in turn could increase drug influx. The rat hepatocytes remained viable during drug accumulation studies over 120 min as measured using trypan blue exclusion.

The mechanism for the increased intracellular accumulation of CPT-11 and SN-38 by thalidomide and its hydrolysis metabolites is unknown, but their inhibitory effects on SN-38 transporters such as PgP and MRP2 are implicated. Thus, we further investigated the effects of thalidomide and its hydrolytic products on the transport of CPT-11 and SN-38 in Caco-2 and MDCKII cells overexpressing MDR1, MRP1, or MRP2. In both Caco-2 and wild-type MDCKII monolayers, thalidomide at 250 μM, its total hydrolytic products at 10 μM, and PGA at 10 μM similarly and significantly decreased the secretory transport of CPT-11 and SN-38. In addition, thalidomide at 250 μM, its hydrolytic products at 10 μM, and PGA at 10 μM significantly inhibited MDR1, MRP1, and MRP2-mediated CPT-11 and SN-38 transport. These findings suggest that both thalidomide and its hydrolytic products could inhibit the transport of CPT-11 and SN-38 via PgP, MRP1, and MRP2. PGA is considered the major contributor for such inhibitory effects from thalidomide hydrolytic products. The inhibitory effects of thalidomide and its hydrolytic products on PgP and MRP1/2 may partially explain the reduced biliary excretion and CPT-11 and its major metabolites in vivo in rats. Thalidomide itself is not a substrate for PgP, but its transport in Caco-2 monolayers was sodium-, temperature-, energy-, and pH-dependent. Moreover, coadministered thalidomide has been found to inhibit the MRP2-mediated biliary excretion of the glucuronides of the antivascular agent 5,6-dimethylxanthenone-4-acetic acid (Kestell et al., 2000). These results indicate that thalidomide and its hydrolytic products such as PGA are potential modulators of PgP and MRPs.

The results from the present studies have important im-
pecifications in drug development of thalidomide and its related agents. The protective effects of thalidomide on CPT-11-induced complications may provide a new treatment approach for chemotherapy-associated histological damages using anti-TNF-α agents through the inhibition of proinflammatory cytokine expression and intestinal epithelial cellular apoptosis. The achievement in abolition or alleviation of chemotherapy-induced complication may reduce morbidity and mortality caused by severe diarrhea and inflammation and avoid overall reduction in effectiveness of therapy caused by interruptions or dose reductions during treatment. Therefore, administration of higher dose chemotherapy regimens with improved tumor regression could be achieved.

When the gut and plasma concentrations of SN-38 (the major cytotoxic metabolite of CPT-11) were reduced by coadministered thalidomide, it would raise a concern that the efficacy of CPT-11 therapy would be compromised due to reduced SN-38 levels in vivo. Interestingly, a recent pilot clinical study indicated that coadministered thalidomide enhanced the antitumor activity of CPT-11 in nude mice bearing colon cancer patients (Govindarajan et al., 2000). Our preliminary study also found that coadministered thalidomide markedly enhanced the antitumor effect of CPT-11 in nude mice bearing human colon cancer xenograft (S. F. Zhou, M. Huang, and A. L. Xu, unpublished data). Thus, the interactions between thalidomide and CPT-11 appear very complicated with multiple pharmacokinetic and pharmacodynamic pathways and molecular targets being involved.

In summary, we demonstrated that coadministered thalidomide significantly reduces CPT-11-induced intestinal and blood toxicity. Thalidomide inhibited the production of proinflammatory cytokines, including TNF-α, IL-6, IL-1β, and IFN-γ, and alleviated intestinal epithelial apoptosis. Coadministered thalidomide significantly increased the AUC of CPT-11 but decreased the systemic exposure of SN-38 and their biliary excretion, which might be attributed to the inhibitory effects on the hydrolysis of CPT-11 and enhanced effects in the intracellular accumulation of SN-38 by thalidomide hydrolytic products. Thalidomide and its hydrolytic products such as PGA are novel modulators for PgP, MRPl, and MRP2. These results highlight that both pharmacodynamic and pharmacokinetic components play important roles in the protective effects of thalidomide against CPT-11-induced intestinal toxicity.

References

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