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Cyclooxygenase-2 Inhibition with Celecoxib Enhances Antitumor Efficacy and Reduces Diarrhea Side Effect of CPT-11

Ovidiu C. Trifan,[1] William F. Durham, Valerie S. Salazar, Jennifer Horton, Benjamin D. Levine, Ben S. Zweifel, Thomas W. Davis, and Jaime L. Masferrer

Oncology Pharmacology, Pharmacia Corp., Chesterfield, Missouri 63198

ABSTRACT

Combining anticancer drugs with different mechanisms of action has the potential to enhance antitumor effect. CPT-11 (Camptosar, irinotecan), a topoisomerase I inhibitor, has been shown to be highly effective in the treatment of a variety of cancers. However, its clinical usage is often complicated by late diarrhea. A number of studies have shown that cyclooxygenase (COX)-2 is overexpressed in many forms of human tumors, suggesting that COX-2 inhibition may be useful in the treatment of cancer. In this study, we used two mouse tumor models (HT-29 and colon-26 cells) to evaluate the effect of combining CPT-11 with celecoxib on tumor growth. We also assessed the involvement of COX-2 in the pathogenesis of CPT-11-induced late diarrhea using a rat model. Results indicate that celecoxib enhances the antitumor effect of CPT-11 and reduces the severity of late diarrhea in a dose-dependent manner. The extended benefits of combining celecoxib with CPT-11 may significantly improve the outcome of cancer patients.

INTRODUCTION

Combining antineoplastic agents has proven to be an effective method in cancer therapy. The increased efficacy of combination therapy results from added cytotoxic effects, especially if the drugs have nonoverlapping mechanisms of action. Additional reasons for this approach are the potential synergistic effects of certain antitumor drug combinations and the possibility to overcome multidrug resistance. Extensive experimentation is required to find suitable drug combinations because combining anticancer agents may have either a positive or a negative impact on the cytotoxic effects of the drugs involved.

CPT-11 (Camptosar, irinotecan) is a topoisomerase I inhibitor (1–3) that has been shown to be highly effective in treatment of colon (4–7), stomach (8, 9), pancreas (10), and non-small cell lung cancers (11, 12). Although efficacious, CPT-11 dosage is limited (particularly in debilitated patients) by toxicities such as diarrhea (13–15).

In both animal models and humans, CPT-11 administration induces two types of diarrhea: early and late. In humans, early diarrhea occurs within 24 h of CPT-11 administration and is a clinical component of a cholinergically mediated syndrome that induces colonic hyperstimulation (15). CPT-11 has been shown to mimic the effects of acetylcholine by inhibiting acetylcholinesterase and by binding to muscarinic receptors (16, 17).

Late diarrhea, which usually occurs more than 24 h after the CPT-11 injection, is National Cancer Institute grade 3 or 4 in up to 31% of the patients (18). The current preferred method of treatment involves nonspecific and supportive measures such as high doses of loperamide (13).

It has been reported that CPT-11 administration is associated with increased colon prostaglandin synthesis in both *in vivo* and *ex vivo* models. Both PGE_2[2] and TXA_2, shown to increase after CPT-11 treatment, have been reported to play a key role in water and electrolyte balances in the colon (19, 20). Led by these observations, we hypothesized that late diarrhea could be, at least in part, a consequence of COX-2 induction secondary to colonic mucosal damage after CPT-11 treatment. Thus, addition of a COX-2-specific inhibitor like celecoxib might be useful in decreasing the CPT-11-induced diarrhea.

Another reason for combining these drugs is the potential for enhanced anticancer efficacy. There is epidemiological evidence showing that NSAIDs could yield a 40–50% reduction in relative risk of death by colon and breast cancer (21–25). Many studies that followed this observation established that COX-2 (targeted by NSAIDs) is involved in the pathogenesis and evolution of a variety of cancers (26–28). COX-2-derived prostanoids were shown to modulate cytokine synthesis, to influence cell proliferation and apoptosis, and to modulate the nuclear translocation and function of tumor suppressor gene products (27–29). Another proposed mechanism for the antitumor effect of COX-2 inhibitors was that they inhibit the growth of newly formed blood vessels (30–32). The discovery of COX-2 involvement in angiogenesis has opened the very exciting possibility that COX-2 inhibition may be useful in the treatment of virtually all types of cancers rather than only malignancies characterized by high levels of COX-2 expression. In these circumstances, combinations of a COX-2-specific inhibitor such as celecoxib and chemotherapeutic agents such as CPT-11, 5-fluorouracil, or ionizing radiation would represent a logical advance in cancer treatment.

Given that the mechanisms of action of celecoxib and CPT-11 are different, combination of the two drugs could increase antitumor efficacy. Using the HT-29 human colon xenograft model (33) and the mouse syngeneic colon-26 model we assessed the effect of combining CPT-11 with celecoxib on tumor growth. Although both cell lines constitutively express COX-2 (34, 35), it has been reported that HT-29 cells contain an enzymatically inactive form of COX-2 (35). Because late diarrhea is usually undetected in mice, we also tested the involvement of COX-2 in the pathogenesis of CPT-11-induced late diarrhea using a rat model. We found an enhanced antitumor effect when CPT-11 was administered with celecoxib. Another finding was that COX-2 is overexpressed in the colon of animals treated with CPT-11 and is associated with higher levels of tissue PGE_2, known to affect the electrolyte and fluid balance in the colon. Celecoxib reduced the severity of late diarrhea in a dose-dependent manner, and this effect was associated with a reduction of colon PGE_2 levels. Taken together, these results support the possibility that the combination of CPT-11 and celecoxib could have an added benefit in cancer therapy.

MATERIALS AND METHODS

Animals. Male nude mice (4–6 weeks old; Harlan, Indianapolis, IN) were used to induce the HT-29 tumors, and male BALB/c mice (4–6 weeks old; Harlan) were used for the colon-26 tumors. Male Sprague Dawley rats (220–

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[1] To whom requests for reprints should be addressed, Oncology Pharmacology, AA5C, Pharmacia Corp., 700 Chesterfield Parkway North, Chesterfield, MO 63198. Phone: (636) 737-5270; Fax: (636) 737-6136; e-mail: ovidiu.c.trifan@pharmacia.com.

[2] The abbreviations used are: PGE_2, prostaglandin E_2; COX, cyclooxygenase; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; NSAID, nonsteroidal anti-inflammatory drug; BID, twice a day.

280 g; Harlan) were used to study the late diarrhea induced by CPT-11. Rats were housed on wire-bottom cages with paper underneath. Mice received a rodent meal diet (PMI Feeds, Inc.), and rats received standard rodent chow (8640 Harlan Teklad 22/5 Rodent Diet; Harlan) and water *ad libitum*. All animal care and husbandry were conducted in accordance with the Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. All animal use was reviewed and approved by the Institutional Animal Care and Use Committee.

CPT-11 Preparation. CPT-11 (PNU-101440E, lots 69063 and 67828) was obtained from Pharmacia Corp. R&D Global Distribution Center (Kalamazoo, MI). An injectable formulation of CPT-11 was obtained by dissolving CPT-11 (20 mg/ml), D-sorbitol (45 mg/ml; Sigma, St. Louis, MO), and D-lactic acid (0.9 mg/ml; Sigma) in Milli-Q water heated to 70–90°C for 5–10 min. The pH was adjusted to 3.5. The resulting solution was sterile-filtered and stored protected from light until the moment of administration.

Administration of Celecoxib, Indomethacin, and SC-560. Celecoxib and SC-560, a COX-1-specific inhibitor (36), were synthesized at Pharmacia. Indomethacin was purchased form Sigma. Celecoxib was administered mixed in the diet, by oral gavage in a solution of 0.5% methylcellulose (Sigma) and 0.025% Tween 20 (Sigma), or split equally between meal and one oral gavage. Indomethacin and SC-560 were administered by oral gavage. Plasma concentration of celecoxib was measured as described elsewhere (37).

HT-29 and Colon-26 Mice Tumors. HT-29 cells were maintained in McCoy's 5a medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin-glutamine (Invitrogen). Colon-26 murine colon adenocarcinoma cells were maintained in culture in DMEM (Invitrogen) containing 10% fetal bovine serum and 1× penicillin-streptomycin-glutamine (Invitrogen). Tumors were established in nude and BALB/c mice, respectively, by s.c. injection of a suspension of cells (1×10^6) in 30% Matrigel (BD Biosciences, Bedford, MA) in the right hind footpad. Mice were weighed on the day of injection (day 0), and body weight and tumor volume (measured using a plethysmometer) were measured at the indicated time points.

Effect of Celecoxib on HT-29 Tumor Growth. Starting 14 days after tumor initiation (tumor volume, ~100 μl), mice carrying HT-29 tumors ($n = 12$) received celecoxib mixed in the diet, at concentrations equivalent to 1.56, 6.25, and 25 mg/kg body weight/day.

Effect of Celecoxib/CPT-11 Combination on Tumor Growth. In the experiments using HT-29 tumors, starting on day 14 (tumor volume, ~ 100 μl), groups of mice ($n = 8$) received vehicle, 30 mg/kg body weight CPT-11 (i.p.) every 4 days, 25 mg/kg body weight/day celecoxib mixed in the meal, or a combination of 25 mg/kg body weight/day celecoxib (diet) and 30 mg/kg CPT-11 (i.p.) every 4 days. In the experiments using colon-26 tumors, groups of mice carrying tumors ($n = 12$), starting 13 days after initiation of tumors (tumor volume, ~500 μl), received vehicle, 50 mg/kg body weight/day celecoxib equally divided between the meal and one oral gavage, two i.p. injections of CPT-11 (100 mg/kg) on days 13 and 18, or both drugs combined.

Model of CPT-11-induced Late Diarrhea. To establish a model of CPT-11-induced diarrhea, groups of rats ($n = 6$–12) were treated with various doses and schedules of CPT-11 by i.v. injections in the tail vein. In Sprague Dawley male rats injected with 150 mg/kg body weight/day CPT-11 for 2 consecutive days, late diarrhea starts on day 4 (approximately 48 h after the final dose of CPT-11), becomes most severe by day 5, and gradually resolves toward day 7–8. Scoring of late diarrhea was conducted twice daily (a.m. and p.m. on days 4, 5, 6, and 7 (a total of eight times). Observations on day 8–10 were used to confirm animals' recovery from the adverse effects caused by the late diarrhea. Body weight was monitored daily throughout the study. The severity of the diarrhea was scored using a scale described by others (14): 0 (normal; normal stool or absent); 1 (slight; slightly wet and soft stool); 2 (moderate, wet and unformed stool with moderate perianal staining of the coat); and 3 (severe, watery stool with severe perianal staining of the coat).

Incidence of diarrhea scores 2 and 3, incidence of diarrhea score 3 only, average diarrhea score, and relative body weight at day 6 were used to evaluate the severity of late diarrhea for each animal.

Role of COX-2 and COX-1 in CPT-11-induced Diarrhea. Groups of rats ($n = 6$–10) were treated with CPT-11 (i.v., 150 mg/kg/day) for 2 consecutive days, with or without celecoxib coadministration (0.1, 0.3, 1, 3, 10, 30, 50, and 150 mg/kg/day, oral gavage, BID, for 8 days starting 1 day before the first CPT-11 injection). The experiment was repeated three times.

Groups of rats ($n = 7$–10) were treated with CPT-11 (i.v., 150 mg/kg/day) for 2 consecutive days, with or without coadministration of SC-560 (at 1, 3, and 10 mg/kg/day, oral gavage, BID), indomethacin (at 1, 3, and 10 mg/kg/day, oral gavage, BID), or celecoxib (30 mg/kg/day, oral gavage, BID) for 8 days starting 1 day before the first CPT-11 injection. The experiment was repeated three times. Additional animals ($n = 4$) were included in selected groups to measure colon prostaglandin content at day 5. The time course of biochemical changes in the colon induced by CPT-11 alone or in combination with celecoxib was studied in a separate experiment. Two groups of rats were treated with CPT-11. One of the groups received an additional 30 mg/kg/day celecoxib for 8 days (starting 1 day before the first CPT-11 injection). Randomly selected animals in each group ($n = 4$) were euthanized using CO_2 at the indicated time points. Three groups of rats ($n = 8$) received s.c. one or two 2 mg/kg doses of the 2B5 anti-PGE_2 antibody or nonimmune IgG (38).

Blood and Tissue Harvesting. Animals were euthanized using CO_2. Approximately 100 μl of blood from celecoxib-treated animals were collected by cardiac puncture and used to measure drug plasma concentration. The entire colon was harvested and snap-frozen in liquid nitrogen to be used for prostaglandin and protein measurement.

PGE_2 and TXB_2 Measurements. PGE_2 and TXB_2 content in tissue were measured using an ELISA-based assay. Briefly, homogenized tissue was suspended in prostaglandin extraction buffer [70% ethanol and 30% of 0.1 M sodium phosphate (pH 4.0)] and incubated on wet ice for 30 min. All samples were then centrifuged at 3800 rpm for 10 min, and the supernatant was collected. A fixed volume of each sample was dried under nitrogen at 37°C and resuspended in ELISA buffer. PGE_2 and TXB_2 levels were determined following the protocol recommended by Cayman Chemical Co. (Ann Arbor, MI). Anti-PGE_2 antibody (2B5) was supplied internally. All remaining reagents were from Cayman Chemical Co.

COX-2 and COX-1 Western Blotting. Frozen tissue (250 mg) was lysed in lysis buffer [100 mM TBS (pH 8.0), 1% NP40, 50 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)] using QiaShredder columns (Qiagen, Hilden, Gemany) according to the manufacturer's instructions. Protein (50 μg/lane) was separated by electrophoresis on 8% Tris-glycine polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed with anti-COX-2 and anti-COX-1 antibodies from Cayman Chemical Co.

Statistical Analysis. For each tumor volume study, a one-way ANOVA was performed on the tumor volumes and rank transformed percentage inhibition values. Percentage inhibition is defined for each tumor as 100 minus 100 times the change in volume from baseline divided by the average (median) change in the vehicle control group for the time period. For the CPT-11-induced diarrhea study, data from several independent experiments were combined and analyzed using a two-way (experiment and treatment group) ANOVA. The group means and SE presented are the least square means provided by a SAS Proc GLM analysis on the raw data values.

RESULTS

Effect on Tumor Size of Celecoxib in Combination with CPT-11. Celecoxib administered in the diet prevented, in a dose-dependent manner, the growth of HT-29 tumors in nude mice (Fig. 1*A*). Celecoxib administered at 1.56 mg/kg/day caused a 24.15 ± 6.4% ($P < 0.01$ compared with vehicle-treated animals) inhibition in tumor growth (mean ± SE estimated at the end of the study day 50). Celecoxib at 6.25 mg/kg/day caused a 49.1 ± 5.5% ($P < 0.001$ compared with vehicle-treated animals) inhibition, and celecoxib at 25 mg/kg/day caused a 62.2 ± 2.8% inhibition ($P < 0.001$ compared with vehicle). The same model was used to measure the effect of combining 25 mg/kg/day celecoxib with CPT-11 (30 mg/kg/4 days; Fig. 1*B*). In this setting, CPT-11 inhibited tumor growth 28.7 ± 14.2% ($P < 0.01$ compared with vehicle-treated animals) by day 48, whereas celecoxib inhibited tumor growth 72.3 ± 6.6% ($P < 0.001$ compared with vehicle). Interestingly, administering both agents at the same

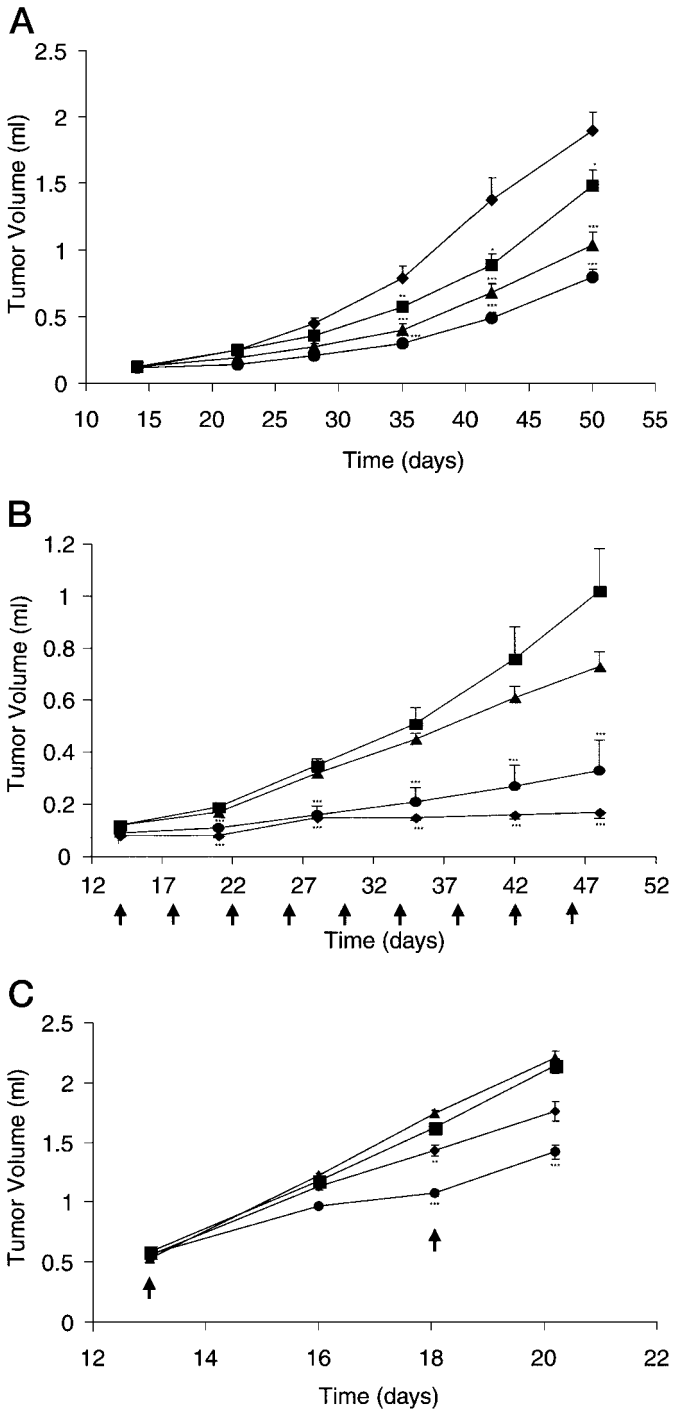

Fig. 1. Celecoxib enhances the antitumor properties of CPT-11. HT-29 and colon-26 tumors were generated by injecting 1×10^6 cells suspended in 30% Matrigel s.c. in the right hind footpad of nude or BALB/c mice, respectively. *A*, groups of 12 nude mice carrying HT-29 tumors were fed standard rodent chow containing vehicle (♦) or celecoxib at concentrations equivalent to 1.56 (■), 6.25 (▲), and 25 (●) mg/kg body weight/day. Drug treatment started 14 days after the tumor initiation and continued throughout the whole study. *B*, groups of eight nude mice carrying HT-29 tumors received vehicle (■), 25 mg/kg body weight/day celecoxib in the meal (●), an i.p. injection with 30 mg/kg CPT-11 every 4 days (▲), or a combination of 25 mg/kg body weight/day celecoxib and 30 mg/kg CPT-11 every 4 days (♦). Drug treatment was initiated on day 14, when tumors reached a volume of approximately 100 μl, and continued until day 48. *C*, groups of 12 BALB/c mice carrying colon-26 tumors 13 days after tumor initiation received vehicle (▲), 50 mg/kg body weight/day celecoxib in a split regimen (25 mg/kg/day in the meal and 25 mg/kg/day by oral gavage; ■), two i.p. injections of 100 mg/kg/day CPT-11 on days 13 and 18 (♦), or a combination of both drugs (●). Drug dosing was initiated when tumors reached approximately 500 μl. *Vertical arrows* (↑) indicate CPT-11 administration. Data are presented as mean ± SE. Statistical significance was calculated compared with the vehicle group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

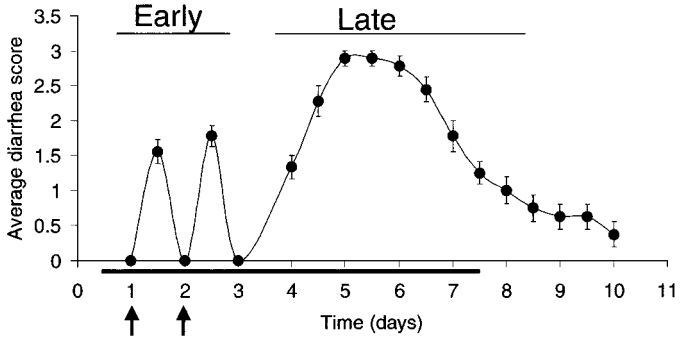

Fig. 2. CPT-11 induces early and late diarrhea. Sprague Dawley male rats received tail vein injection with two consecutive doses of 150 mg/kg/day. Diarrhea was scored twice per day (a.m. and p.m.) using the following scale: 0, normal (normal stool or absent); 1, slight diarrhea (wet and soft stool); 2, moderate diarrhea (wet and unformed stool with moderate perianal staining of the coat); and 3, severe diarrhea (watery stool with severe perianal staining of the coat). Data represent average daily scores ± SE calculated from individual animal scores. *Black horizontal bar* indicates the period of time rats received different treatments in conjunction with CPT-11.

time inhibited tumor growth by 91.4 ± 2.1% ($P < 0.001$ compared with vehicle; $P < 0.001$ compared with CPT-11). To further demonstrate the value of this drug combination, we tested it in a very aggressive tumor model (colon-26), in which therapy was initiated when tumors were at 0.5 ml (Fig. 1*C*). Under these conditions, celecoxib and CPT-11 given alone caused a modest reduction of tumor growth [day 20: 3.8 ± 7.5% and 22.6 ± 3.9% ($P < 0.001$ compared with vehicle), respectively]. When both drugs were used, there was a 38.6 ± 5.2% ($P < 0.001$ compared with vehicle; $P < 0.05$ compared with CPT-11; $P < 0.001$ compared with celecoxib) reduction in tumor growth.

Celecoxib administration also prevented the reduction in body weight caused by tumors. At the end of the study (day 20), normal animals reached 26.3 ± 0.2 g, whereas untreated tumor-bearing animals (vehicle) had an average body weight of 20.5 ± 0.6 g, animals receiving CPT-11 alone had an average body weight of 21.1 ± 0.5 g, animals receiving 50 mg/kg/day celecoxib had an average body weight of 23.1 ± 0.7 g ($P < 0.01$, comparative to vehicle), and animals receiving celecoxib combined with CPT-11 had an average body weight of 24.0 ± 0.7 g ($P < 0.001$ compared with vehicle).

CPT-11 Dose and Administration Schedule Affect the Severity of Late Diarrhea and Animal Survival. As shown in Fig. 2, CPT-11 induces both early and late diarrhea. Atropine administration (2 mg/kg body weight, s.c.) 15 min before CPT-11 injection completely prevented the early-phase diarrhea, with no effect on late diarrhea (data not shown).

We assessed the role of total dose and dosing regimen of CPT-11 on the severity of diarrhea and animal survival. As shown in Table 1, higher total doses of CPT-11 resulted in a more severe diarrhea. On the other hand, four consecutive daily doses of 80 mg/kg/day CPT-11 caused a less severe late diarrhea than two consecutive daily doses of 150 mg/kg/day, suggesting that the administration schedule is another critical determinant in the severity of late diarrhea. We sought to induce maximal diarrhea while minimizing the effect on the animal survival/recovery. Among different doses and schedules of CPT-11 assessed, we found that dosing CPT-11 at 150 mg/kg/day for 2 consecutive days is consistently associated with severe diarrhea and little or no mortality. Experiments aimed to test the effect of COX inhibition were conducted using this dose and schedule of CPT-11 as a means of generating late diarrhea.

Effect of Celecoxib on CPT-11-induced Diarrhea and Body Weight Loss. Animals in the control group (CPT-11 alone) had a high incidence of diarrhea scores 2 and 3 (Table 2). Cotreatment with

Table 1 *Effect of CPT-11 dose and administration schedule on the severity of late diarrhea and animal survival*

To establish a rat diarrhea model, groups of 6–12 animals received single or multiple daily doses of CPT-11 at the indicated concentrations. Scoring of late diarrhea was conducted twice a day on days 4, 5, 6, and 7 (a total of eight times). The severity of the diarrhea was scored using the following scale: 0, normal (normal stool or absent); 1, slight (slightly wet and soft stool); 2, moderate (wet and unformed stool with moderate perianal staining of the coat); and 3, severe (watery stool with severe perianal staining of the coat). Diarrhea results are reported as mean ± SE. Two consecutive doses of 150 mg/kg/day CPT-11 repeatedly yielded maximum diarrhea with no (or reduced) mortality. This dose and schedule were utilized in all subsequent experiments.

Total CPT-11 (mg/kg rat)	CPT-11 dose (mg/kg/day)	Dosing schedule	Incidence of diarrhea[a] (%)		Relative body weight at day 6[b] (%)	Mortality[c] (%)
			Score 2 & 3	Score 3		
120	120	QD[d] × 1 day	0.0	0.0	107.9 ± 1.3	0
150	150	QD × 1 day	0.0	0.0	109.0 ± 0.7	0
200	200	QD × 1 day	32.0 ± 15.0	0.0	96.2 ± 3.7	0
240	120	QD × 2 day	48.4 ± 10.6	12.5 ± 6.9	88.9 ± 3.2	0
270	135	QD × 2 day	73.2 ± 7.4	31.9 ± 7.8	80.5 ± 1.4	0
300	150	QD × 2 day	80.4 ± 7.1	38.9 ± 7.6	79.1 ± 1.5	0
320	80	QD × 4 day	50.0 ± 6.8	23.3 ± 12.0	83.9 ± 1.5	0
360	120	QD × 3 day	81.9 ± 7.5	30.6 ± 8.4	77.2 ± 1.3	0
400	100	QD × 4 day	84.1 ± 2.4	63.6 ± 7.6	79.7 ± 1.5	33
480	120	QD × 4 day	95.8 ± 1.8	88.9 ± 1.6	75.8 ± 0.9	92

[a] Incidence of diarrhea scores 2 and 3 and incidence of diarrhea score 3 were calculated for each animal by counting observations with that score from day 4 to 7 (total of 8 observations/animal).
[b] Relative body weight at day 6 was calculated for each animal relative to animal's weight at the beginning of the study (day 0).
[c] Mortality represents percentage of dead animals at the end of the study.
[d] QD, once a day.

celecoxib decreases the severity of late diarrhea in a dose-dependent manner, with a maximal effect obtained with celecoxib at 30 mg/kg/day. Similar to the previous observation, celecoxib reduced the incidence of diarrhea score 3 in a dose-dependent manner (Table 2). A statistically significant improvement was obtained with drug doses between 0.3 and 150 mg/kg/day (Table 2). The average diarrhea score for each group follows the same trend, with the incidence of scores 2 and 3 or the incidence of score 3 only.

In these experiments, animals treated with CPT-11 (control group) experienced a progressive decline in body weight, reaching 78.3 ± 1.2% of their original body weight (relative body weight) by day 6 (Table 2). Animals receiving celecoxib lost less body weight, and the effect was dose dependent. A statistically significant effect was obtained with celecoxib at concentrations higher than 1 mg/kg/day (83.8 ± 2.3%; $P < 0.05$ compared with the CPT-11 group), and the maximum effect was obtained with celecoxib 30 mg/kg/day (85.2 ± 1.2%; $P < 0.001$ compared with the CPT-11 group).

Celecoxib Effect on CPT-11-induced Diarrhea Could Be Mediated by a PGE_2-dependent Mechanism. CPT-11 administration led to an increase in COX-2 protein and of PGE_2 levels in rat colon. As shown in Fig. 3*A*, the colon COX-2 protein, as detected by Western blot analysis, increased by day 4 and reached a maximum at day 5. A similar trend could be observed for the PGE_2 content of the colon (Fig. 3*B*). In contrast, colon COX-1 protein levels showed little change after CPT-11 treatment (Fig. 3*A*).

Administering celecoxib at concentrations of 10, 30, and 50 mg/kg/day yielded plasma concentrations of 0.8 ± 0.1, 1.8 ± 0.3, and 2.4 ± 0.6 μg/ml, respectively, sufficient to restore colon PGE_2 to levels equivalent to those of vehicle-treated animals (Fig. 3, *B* and *D*). The effect of celecoxib on tissue levels of PGE_2 correlates with the antidiarrhea efficacy of celecoxib (Table 2).

We addressed the relative contribution of COX-1 and COX-2 to the production of colon PGE_2 using indomethacin and a COX-1-selective inhibitor, SC-560. Data in Fig. 3*D* and Table 3 show that inhibiting COX-1-derived PGE_2 in the colon with SC-560 had no effect on diarrhea. In addition, indomethacin that inhibited PGE_2 synthesis at all concentrations tested (Fig. 3*D*) caused a significant worsening of diarrhea when administered at 10 mg/kg/day (Table 3) and a major increase in mortality (100% mortality by day 7).

To further address the role of PGE_2 in CPT-11-induced diarrhea, we administered s.c. anti-PGE_2 antibody. One 2 mg/kg dose (on day 1 before the first CPT-11 injection) significantly reduced the inci-

Table 2 *Celecoxib reduces in a dose-dependent manner late diarrhea and body weight loss induced by CPT-11*

Animals were treated with CPT-11 at 150 mg/kg/day for two consecutive days and with vehicle or celecoxib at the indicated concentrations. Celecoxib was administered by oral gavage twice a day (the treatment column indicates the total daily dose), starting the day before the CPT-11 administration until day 7 (a total of 8 days). Scoring of late diarrhea was conducted twice a day on days 4, 5, 6, and 7. All numbers represent mean ± SE of combined results from three independent experiments. Statistical significance was calculated compared with control (CPT-11 only) group. For average score only, statistical significance was calculated on rank-transformed values.

Treatment	N[a]	Incidence of diarrhea[b] (%)		Average diarrhea score[c]	Relative body weight at day 6[d] (%)
		Score 2 & 3	Score 3		
Control (CPT-11)	29	89.1 ± 5.0	52.1 ± 4.6	2.4 ± 0.1	78.3 ± 1.2
Celecoxib, 0.1 mg/kg/day	9	83.7 ± 9.5	39.9 ± 8.6	2.2 ± 0.2	77.5 ± 2.3
Celecoxib, 0.3 mg/kg/day	28	72.3 ± 5.1[e]	30.5 ± 4.6[f]	2.1 ± 0.1[f]	81.5 ± 1.2
Celecoxib, 1 mg/kg/day	9	60.1 ± 9.5[f]	21.0 ± 8.6[f]	1.8 ± 0.2[f]	83.8 ± 2.3[f]
Celecoxib, 3 mg/kg/day	29	68.6 ± 5.0[f]	24.5 ± 4.6[g]	1.9 ± 0.1[g]	82.9 ± 1.2[e]
Celecoxib, 10 mg/kg/day	29	60.6 ± 5.0[g]	18.0 ± 4.6[g]	1.7 ± 0.1[g]	85.0 ± 1.2[g]
Celecoxib, 30 mg/kg/day	29	51.9 ± 5.0[g]	14.0 ± 4.6[g]	1.6 ± 0.1[g]	85.2 ± 1.2[g]
Celecoxib, 50 mg/kg/day	19	50.1 ± 6.4[g]	21.3 ± 5.8[g]	1.6 ± 0.1[g]	84.7 ± 1.5[f]
Celecoxib, 150 mg/kg/day	19	54.6 ± 6.4[g]	14.1 ± 5.8[g]	1.6 ± 0.1[g]	84.2 ± 1.5[f]

[a] N, the total number of animals accounted for that experimental condition.
[b] Incidence of diarrhea scores 2 and 3 and incidence of diarrhea score 3 were calculated for each animal by counting observations with that score from day 4 to 7 (total of 8 observations/animal).
[c] For each animal, the mean late diarrhea score was calculated by averaging the scores between days 4 and 7 (eight observations).
[d] Relative body weight at day 6 was calculated for each animal relative to animal's weight at the beginning of the study (day 0).
[e] $P < 0.05$.
[f] $P < 0.01$.
[g] $P < 0.001$.

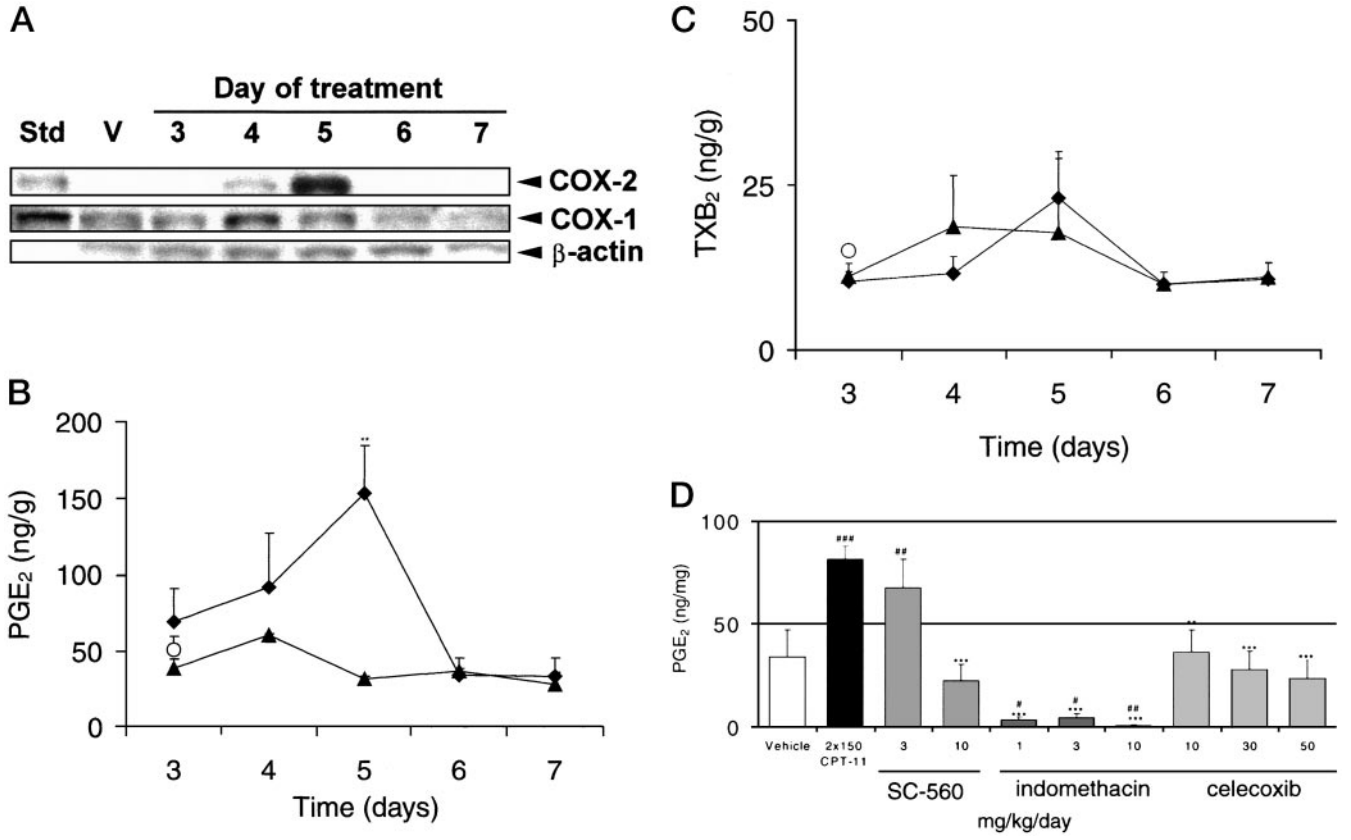

Fig. 3. CPT-11 induces COX-2 expression and PGE_2 synthesis in rat colon. Male Sprague Dawley rats that received two consecutive injected doses of 150 mg/kg/day were treated with or without celecoxib. On indicated days (days 3–7), groups of four animals were used to harvest the entire colon. Part of the tissue was used for protein extraction, and part was used to analyze the prostaglandin content. *A*, equal amounts of total protein of randomly selected samples from the CPT-11-treated group were used to determine COX-2 content by Western blot analysis. Membranes were stripped and reprobed with anti-COX-1 and *β*-actin antibody. *Std*, protein standard; *V*, normal animals sacrificed on day 3. *B*, colon PGE_2 content was determined by ELISA. Each time point represents the average ± SE of four samples. ♦, CPT-11-treated animals; ▲, animals treated with CPT-11 + 30 mg/kg/day celecoxib; ○, normal animals sacrificed at day 3. *C*, colon TXB_2 content was determined as described. Each time point represents the average ± SE of four samples. ♦, CPT-11-treated animals; ▲, animals treated with CPT-11 + 30 mg/kg/day celecoxib; ○, normal animals sacrificed at day 3. *D*, relative contribution of COX-1 and COX-2 to tissue PGE_2. Groups of four animals were sacrificed at day 5 and used to measure tissue PGE_2. Animals received vehicle (normal animals) or two doses of 150 mg/kg body weight/day CPT-11 alone (CPT-11, 2 × 150) or in combination with SC-560, indomethacin, or celecoxib at the indicated concentrations (mg/kg/day). #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ compared with vehicle-treated animals. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ compared with animals treated with CPT-11 alone.

dence of CPT-11-induced diarrhea score 3 (from 50.0 ± 4.7% in the control group to 25.0 ± 11.2%; $P < 0.05$). However, when animals received two doses (on days 1 and 3) of anti-PGE_2 antibody, the beneficial effect on CPT-11-induced diarrhea disappeared (incidence of diarrhea score 3 was 41.7 ± 9.5%), a trend resembling the response to the nonselective inhibitor indomethacin (Table 3).

TXB_2 has been linked to Cl^- secretion and has been reported to increase in colon mucosa in an *ex vivo* system (19, 30). Interestingly,

Table 3 *Effect of SC-560, indomethacin, and celecoxib on CPT-11-induced diarrhea and body weight loss*

Groups of 7–10 rats were treated with two consecutive tail vein injections of 150 mg/kg/day CPT-11 and twice daily gavages with vehicle (control group), 30 mg/kg/day celecoxib, or three different concentrations of SC-560 and indomethacin (the treatment column indicates the total daily dose). All COX inhibitors were administered starting the day before the CPT-11 injection until day 7 (a total of 8 days). Scoring of late diarrhea was conducted twice a day on days 4, 5, 6, and 7. The experiment was repeated three times. All numbers in the table represent mean ± SE of combined results from three independent experiments. Statistical significance was calculated compared with control (CPT-11 only) group. For average score only, statistical significance was calculated on rank-transformed values.

Treatment	N[a]	Incidence of diarrhea[b] (%)		Average diarrhea score[c]	Relative body weight at day 6[d] (%)
		Score 2 & 3	Score 3		
Control (CPT-11)	25	90.4 ± 4.0	55.7 ± 5.3	2.5 ± 0.1	78.7 ± 1.3
SC-560, 1 mg/kg/day	23	84.8 ± 4.1	58.6 ± 5.6	2.5 ± 0.1	79.8 ± 1.4
SC-560, 3 mg/kg/day	23	85.4 ± 4.0	57.2 ± 5.6	2.4 ± 0.1	80.7 ± 1.4
SC-560, 10 mg/kg/day	24	84.9 ± 4.0	52.5 ± 5.5	2.4 ± 0.1	80.2 ± 1.4
Indomethacin, 1 mg/kg/day	26	77.0 ± 3.9	42.7 ± 5.3	2.2 ± 0.1	82.1 ± 1.3
Indomethacin, 3 mg/kg/day	17	87.7 ± 4.9	61.0 ± 6.7	2.5 ± 0.1	80.7 ± 1.8
Indomethacin, 10 mg/kg/day	10	100.0 ± 0.0[e]	100.0 ± 0.0[f]	3.0 ± 0.2[f]	75.2 ± 2.6
Celecoxib, 30 mg/kg/day	26	51.0 ± 3.9[f]	24.0 ± 5.3[f]	1.7 ± 0.1[f]	88.2 ± 1.3[f]

[a] *N*, the total number of animals accounted for that experimental condition.
[b] Incidence of diarrhea scores 2 and 3 and incidence of diarrhea score 3 were calculated for each animal by counting observations with that score from day 4 to 7 (total of 8 observations/animal).
[c] For each animal, the mean late diarrhea score was calculated by averaging the scores between days 4 and 7 (eight observations).
[d] Relative body weight at day 6 was calculated for each animal relative to animal's weight at the beginning of the study (day 0).
[e] $P < 0.05$.
[f] $P < 0.001$.

in our system, the colon TXB_2 content did not change after CPT-11 treatment and was not affected by celecoxib (Fig. 3*C*). However, both indomethacin and SC-560 (a COX-1-specific inhibitor) decreased tissue TXB_2 (data not shown), suggesting that it is derived from COX-1 and that blocking its production has no apparent effect on diarrhea in this model.

DISCUSSION

A growing body of evidence has suggested the potential application of NSAIDs in cancer chemoprevention (39–41) and chemotherapy (30, 31, 34). Because NSAIDs are known to inhibit both COX-1 and COX-2, the basis for their antitumor effects is conceivably their ability to block the generation of prostaglandins from arachidonic acid. A number of studies showed that COX-2 is overexpressed in many forms of human cancers (29), whereas COX-1 is expressed constitutively at a low level in the normal tissues and does not increase during transformation. Recent studies revealed a direct link between COX-2 overexpression and tumorigenesis (30, 42). Several mechanisms by which COX-2 contributes to tumorigenesis have been identified, including inhibition of apoptosis, increased angiogenesis, increased invasiveness, modulation of inflammation/immunosuppression, and conversion of procarcinogens to carcinogens (27).

Consistent with the predicted qualities of combination therapy, in two mouse tumor models we observed an enhanced antineoplastic effect when celecoxib was used in combination with CPT-11. Making this drug combination even more attractive was the finding that celecoxib reduced the diarrhea side effect of CPT-11 in rats. Unlike mice, Sprague Dawley rats have been extensively used to study CPT-11-induced diarrhea for their similarity with humans in this respect (14).

Data presented here show that CPT-11 leads to an increase in COX-2 expression in rat colon, with a maximum in protein levels 5 days after the initiation of CPT-11 treatment. This increase in COX-2 is associated with an increase in tissue PGE_2 levels. Interestingly, this time course tightly follows the evolution of late diarrhea in this model, suggesting a causal relationship between COX-2 induction and late diarrhea, where PGE_2 is the mediator. Consistent with our observation, an earlier report (19) showed an increase in colon PGE_2 levels after CPT-11 administration.

In our study, celecoxib reduced the severity of late diarrhea and the body weight loss induced by CPT-11 in a dose-dependent manner. The effective doses of celecoxib used in this study (10–50 mg/kg/day) yielded plasma concentrations between 0.8 and 2.4 μg/ml, which were sufficient to inhibit tissue COX-2, as suggested by the decrease in the colon PGE_2 to normal levels. Celecoxib also had demonstrated antiangiogenic and antitumor activities in the same dose range (31, 43). In humans, these plasma concentrations (between 0.8 and 2 μg/ml) were achieved when celecoxib was administered at 400 mg BID (44).

In normal untreated animals, in the absence of COX-2, tissue PGE_2 is COX-1 derived. Belley and Chadee (45) have shown that PGE_2 stimulates rat and human colonic mucin exocytosis via the prostaglandin receptor EP(4) receptor, thus playing an important role in mucosal maintenance as well as in pathological conditions. Such a role for COX-1-derived PGE_2 was suggested by finding significant levels of this prostaglandin in the normal tissue and by worsening of diarrhea and overall condition of animals treated with indomethacin. However, additional studies will be required to fully explain this observation.

In pathological conditions associated with increased COX-2 expression, the resulting additional PGE_2, besides stimulating mucus secretion, may also act on the epithelial cells lining the mucosa, triggering Cl^- secretion (19), water loss, and subsequent diarrhea. It is plausible that such a mechanism has a protective role in irritant/infectious conditions, but it is difficult to assess its significance after CPT-11 treatment. It might represent a protective mechanism intended to help the recovery process of the intestinal mucosa or be just a deregulated response. In support of deregulation, it is important to note that selectively inhibiting COX-2-derived PGE_2 with celecoxib led to a substantially better and faster animal recovery after CPT-11 treatment.

A number of publications have suggested the involvement of TXA_2 in the pathogenesis of CPT-11-induced diarrhea (20, 46). The relationship between CPT-11, TXA_2, and Cl^- secretion has been established in an *ex vivo* system. The reported TXA_2 (respectively, TXB_2) increase, shortly after CPT-11 treatment, is more consistent with an early type of diarrhea and is probably a corollary of the antiacetylcholinesterase and/or acetylcholine-mimetic properties of CPT-11. In this context, the major source of TXB_2 could be COX-1 that is constantly expressed throughout the entire length of the rat gastrointestinal tract.

Our data, together with previous observations published in the literature (47), suggest an inflammatory component in the pathogenesis of CPT-11-induced late diarrhea. Here we presented data showing that COX-2 is induced in the rat colon after CPT-11 treatment and that this is concurrent with an increase in PGE_2 production. Histopathological analysis revealed glandular cell dysplasia, mucosal atrophy, and fused villi. There were also frequent foci of mononuclear cell infiltration with lymphocytes as a main component. Interestingly, the intensity of COX-2 expression varies throughout the length of the colon after CPT-11 treatment, suggesting that local conditions play a critical role in the mechanism of induction (data not shown).

Additional published observations convey circumstantial support to the hypothesis that CPT-11-induced diarrhea is, at least in part, mediated by a COX-2-dependent mechanism. Fujita *et al.* (48) showed that thalidomide, a compound with immunomodulatory and antiangiogenic properties (49), could inhibit the lipopolysaccharide-mediated induction of COX-2 in murine macrophages. The same mechanism could be involved in the observation that thalidomide may reduce the severity of CPT-11-induced diarrhea in human patients (50). Another drug considered for treatment of CPT-11-induced late diarrhea in humans is glutamine (51). Glutamine has been shown to reduce PGE_2 levels (52), and this might contribute to the described antidiarrhea and antitumor properties.

Additional experiments will be required to address the mechanism involved in depth. However, it is tempting to hypothesize that cellular damage, subsequent to CPT-11 treatment, could weaken the barrier properties of lining intestinal epithelium, resulting in exposure of the lamina propria to luminal contents, which is known to induce COX-2 in a variety of cell types (53–55).

The dual benefit of combining celecoxib with CPT-11 may provide significant improvement to the outcome of cancer patients. These data provide a strong rationale for performing clinical trials of CPT-11 in combination with celecoxib.

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Cyclooxygenase-2 Inhibition with Celecoxib Enhances Antitumor Efficacy and Reduces Diarrhea Side Effect of CPT-11

Ovidiu C. Trifan, William F. Durham, Valerie S. Salazar, et al.

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