Intestinal Alkalization As a Possible Preventive Mechanism in Irinotecan (CPT-11)-induced Diarrhea

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ABSTRACT

The therapeutic efficacy of irinotecan (CPT-11), a DNA topoisomerase inhibitor, is often limited by the induction of severe late-onset diarrhea. This prodruk and its active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), have a labile α-hydroxy-lactone ring that undergoes pH-dependent reversible hydrolysis. At physiological pH and higher, equilibrium favors the less toxic carboxylate form, whereas at acidic pH, the more potent lactone form is favored. We have reported previously that the initial uptake rate of CPT-11 and SN-38 by intestinal cells was significantly different between the respective lactone and carboxylate form. Results from the present study in HT-29 cells further demonstrate the correlation between the CPT-11/SN-38 initial uptake rate and the induced toxicity, cell cycle alteration, apoptosis, and colony-forming efficiency. The exposure of HT-29 cells to SN-38 for a limited period of time (<2 h) was sufficient to induce these events. Because the decreased initial uptake of SN-38 carboxylate resulted in a reduced cellular toxicity, we postulated that the CPT-11-induced diarrhea was preventable by influencing the equilibrium toward the carboxylate form and, thus, reducing its intestinal uptake. In the golden Syrian hamster model, p.o. sodium bicarbonate supplementation (5 mg/ml in drinking water) led to alkalization of the intestinal contents. In addition, this alkalization resulted in the reduction of the histopathological damage to the mucosa of the small and large intestine, as well as a 20% reduction of the intestinal SN-38 lactone concentration of animals receiving CPT-11 (20–50 mg/kg × 7 days). Taken together, these results from in vitro and in vivo studies support intestinal alkalization by sodium bicarbonate supplementation as a preventive mechanism against CPT-11-induced diarrhea. In addition, this provides a strong rationale for the usage of this measure as an adjunct to CPT-11 treatment.

INTRODUCTION

CPT-11 is a water-soluble derivative of camptothecin, an anti-tumor alkald isolated from Camptotheca acuminata, and presents a wide spectrum of antitumor activity through the inhibition of DNA topoisomerase I (1). This chemotherapeutic agent is broadening its clinical impact because it has shown clinical responses for many malignancies, such as advanced colon cancers, for which CPT-11 therapy has been approved worldwide, but also lung, gastric, pancreatic, cervical, ovarian, leukemia, and lymphoma (2–7); and survival benefits for these patients (8). Leukopenia and diarrhea are the two major side effects of patients receiving CPT-11. Leucopenia duration can be minimized and, therefore, controlled by administration of granulocyte colony stimulating factor (9). However, delayed diarrhea occurs in the majority of the treated patients. Several studies have reported this side effect to be grade 3 (severe) or 4 (life threatening), according to the National Cancer Institute Common Toxicity Criteria, in ≤40% of the patients (10), underlining diarrhea as the major limitation in the therapeutic use of CPT-11 (11, 12).

CPT-11/SN-38-induced diarrhea may occur in one or both of two different temporal settings. The first setting, early acute, is observed immediately after CPT-11 infusion. This phenomenon could be related to the early metabolism of the drug and is short lasting and prevented or rapidly suppressed with atropine administration (13). The second setting, late onset, occurs usually after an average period of 6 days, the mechanism of which is unknown. Recent reports have underlined the significant role of diarrhea and dehydration in the early death of patients treated with CPT-11 (11).

Drugs like loperamide, atorvastatin, and budesonide have been used to slow intestinal motility and decrease water and electrolyte movement through the bowel (14–16). High dose of loperamide is considered as standard treatment in Europe and the United States but presents a limited level of success (17). Additional approaches are being explored to overcome the intestinal toxicity of CPT-11, including the use of antibiotics or bacterial lipopeptide (JBT3002) to stimulate mucosal immunity and maintain mucosal architecture (18), as well as fish oil and glutamine supplementation to decrease the intestinal side effect (19, 20). Furthermore, although the mechanism of action is less clear, Kampo, the Chinese herbal medicine, has been used successfully in reducing diarrhea induced by irinotecan (14, 21).

CPT-11 is hydrolyzed by hepatic carboxylesterase to SN-38 (22). SN-38 has at least a 1000-fold more potent antitumor effect than CPT-11, as shown in vitro (1). In the liver, a portion of SN-38 undergoes subsequent conjugation by UDP-glucuronyltransferase to SN-38G (23). CPT-11, SN-38, and SN-38G are excreted into bile (23). Although there are no known quantitative reports in human, studies in the rat model suggest that the proportion of CPT-11, SN-38, and SN-38G secreted in bile is 83, 4.5, and 12.5%, respectively (24). Once in the intestine, SN-38G can be deconjugated in the cecum and colon to SN-38 by bacterial β-glucuronidase (25). CPT-11, SN-38, and SN-38G are believed to be reabsorbed into the enterohepatic circulation to a certain extent by intestinal cells (25).

Among the different CPT-11 metabolites, SN-38 has been considered not only as the most potent anticancer agent but also to be the cause of the treatment-related diarrhea (26). It has been suggested that enterocolitis caused by high levels of SN-38 and/or CPT-11 retained for long periods of time in the intestine was the direct cause of diarrhea associated with CPT-11 administration in athymic mouse (27). Furthermore, in patients with diminished or no UDP-glucuronyltransferase activity, such as in those with either Crigrler-Najjar syndrome I or Gilbert’s syndrome, the incidence of CPT-11-induced diarrhea is greater (26, 28), suggesting that decreased hepatic glucuronid conjugation results in an increased secretion of unconjugated SN-38 with, as a consequence, an increased concentration of luminal SN-38. This in turn could induce tissue injury and, therefore, diarrhea.

CPT-11, SN-38, and SN-38G have a labile α-hydroxy-3-lactone

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3 The abbreviations used are: CPT-11, irinotecan hydrochloride; SN-38, 7-ethyl-10-hydroxy-camptothecin; SN-38G, 7-ethyl-10-hydroxy-camptothecin glucuronide; PI, propidium iodide; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PS, phosphatidylserine; BW, body weight; HPLC, high-performance liquid chromatography.
ring, which undergoes reversible hydrolysis at a rate, which is mainly pH dependent (29). While under acidic conditions, formation of the lactone form is favored; at physiological pH and higher, the lactone form is unstable, and the equilibrium favors hydrolysis to open the lactone ring and yield the carboxylate form. The carboxylate form is a less potent inhibitor of topoisomerase I and has much weaker antitumor activity than its lactone counterpart. Recent studies from our laboratory have shown that CPT-11 and SN-38 lactone were both passively transported, whereas their respective carboxylate forms were actively transported in isolated intestinal cells (30). The uptake rate of CPT-11 and SN-38 lactone and carboxylate, respectively, is similar from duodenum to colon (31). However, the respective intestinal uptake rate of CPT-11 and SN-38 lactone is $\sim$10 times greater than that of the carboxylate form.

The principle role of topoisomerase I is the relaxation of DNA required for transcription and replication. As reported previously for camptothecin, active replication is a requirement for SN-38-induced cell toxicity (32–34). The stabilization by SN-38 of the topoisomerase I complex and its collision with the DNA replication fork may lead to the generation of permanent strand breaks thought to be responsible for the induction of cell death (35).

The aim of the present study was to clarify in vitro the relationship between the respective uptake of CPT-11, SN-38, and SN-38G lactone and carboxylate and the series of events associated with cell death, such as cell cycle alteration, apoptosis, and growth inhibition. The human colon adenocarcinoma HT-29 cell line was used to allow for long-term studies that are not possible using the isolated enterocyte model. In order to further test our hypothesis drawn from our in vitro studies, we investigated the effect of intestinal alkalization by p.o. sodium bicarbonate supplementation on CPT-11-induced diarrhea and intestinal cell injury in vivo in the golden Syrian hamster model.

**MATERIALS AND METHODS**

**Cell Culture Conditions.** The human colon adenocarcinoma HT-29 cell line (American Type Culture Collection, Manassas, VA) was grown in DMEM containing 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin at pH 7.4. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

**Reagents.** CPT-11, SN-38, and SN-38G were kindly provided by Yakult Honsha (Tokyo, Japan). Radiolabeled SN-38 (1°C) SN-38 was kindly provided by Daiichi Pharmaceutical (Tokyo, Japan) and Yakult Honsha. SN-38 and SN-38G were dissolved in DMSO while CPT-11 was dissolved in PBS as described previously (30). To prepare the respective lactone and carboxylate form, CPT-11, SN-38, and SN-38G were incubated overnight at room temperature in 50 mM PBS at pH 3.0 and 9.0, respectively. GFP-labeled Annexin-V and PI staining.

**Determination of the Cellular Uptake of [14C] SN-38.** The uptake of [14C] SN-38 by HT-29 cells was studied as described previously (30) using 24-well tissue culture plates (Corning, Inc., Corning, NY). The initial rate of uptake of SN-38 was derived from the linear regression analysis of the respective regression line obtained from the plot of the uptake as a function of time (30, 36). The respective initial rate of uptake was plotted against the corresponding concentration of the agent, and the data were fitted by least squares nonlinear regression analysis using the equation $V = (V_{max} \times S) / (K_m + S) + K_d \times V_{max}$, where $V$ represents the initial rate of uptake, $V_{max}$ is the maximum rate of uptake, $K_m$ is the apparent Michaelis constant, $K_d$ is the rate of diffusion, and $S$ is the respective concentration of SN-38.

**Cell Cycle Analysis.** For flow cytometric analysis of DNA content, 1 $\times$ 10⁶ HT-29 cells in exponential growth phase were treated with increasing concentrations (0.1–5 μM) of SN-38 lactone or carboxylate for 2 h. After removal of the drug, the cells were further incubated for 24 h and then washed three times with PBS. The cells were harvested and fixed with 70% ethanol for 10 min, then diluted with PBS and filtered through a 0.5% Triton X-100 for 10 min at 4°C. The supernatant collected by centrifugation at 15,000 rpm for 20 min was successively incubated with 0.4 mg/ml RNase A and 0.4 mg/ml proteinase K at 37°C for 1 and 2 h, respectively. The DNA was electrophoresed in a 1.5% agarose gel in Tris-borate buffer at 20 V for $\sim$4 h, stained with ethidium bromide (1 mg/ml), and visualized using an Eagle Eye II transilluminator (Stratagene, La Jolla, CA).

**GFP-labeled Annexin-V and PI Staining.** The apoptotic HT-29 cells were determined using GFP-labeled annexin-V and PI in accordance with the manufacturer’s instructions (Clontech). In brief, after treatment, the cells were harvested by trypsinization, and 1 $\times$ 10⁶ cells were stained with 1 μg/ml annexin-V-GFP and 50 μg/ml PI. Binding affinity for each dye was determined by flow cytometric analysis using flow cytometry. Excitation was 488 nm, and the emission filters used were 530 nm (FL1) for GFP and 620 nm (FL2) for PI, respectively. The cell population having the lower PI intensity but the higher GFP intensity was defined as apoptotic. The cell population with both high PI and GFP intensity was defined as necrotic. Fluorescence cutoff for the FL1 and FL2 channel was defined using HT-29 cells permeabilized with 0.1% Triton X-100-containing PBS. The percentage of cells in the S, G₀-G₁, and G₂-M regions were determined using GFP-labeled annexin-V and in accordance with the manufacturer’s instructions (Clontech). CPT-11 and SN-38 Determination. The SN-38 and CPT-11 lactone and carboxylate concentration in both culture medium and intestinal content was determined by HPLC equipped with a fluorescence detector and according to the method described by Rivory and Roberts (37) with minor modifications (38). Using an excitation and emission wavelength of 355 and 515 nm, respectively, the concentration for the respective lactone and carboxylate form of SN-38 and CPT-11 was determined and quantified as a percentage of the total. The CPT-11 and SN-38 solutions were 98% lactone form at pH 3.0 and 97% carboxylate form at pH 9.0. At pH 6.2 and 7.6, the respective lactone form of SN-38 was $\sim$70% and 25%.

**Animal Model Study.** Eight-week-old male golden Syrian hamsters (body weight: 100–120 grams) were fed a standard rodent chow diet and maintained on a 12:12 light-dark cycle at constant temperature and humidity. One group of hamsters was administered 5 mg/ml sodium bicarbonate in a 3% sucrose solution while the control group received only the sucrose solution for 2 days before daily i.p. injection of 100 μl of either CPT-11 (20 mg/kg) or the vehicle for a 7-day period. CPT-11 was dissolved in sterilized distilled water and incubated at 95°C for 10 min, then diluted with PBS and filtered through a 0.22-μm pore-size Millex-GV syringe filter unit (Millipore, Bedford, MA). As control, one group of hamsters only received the vehicle (100 μl of PBS) used to solubilize CPT-11. At the end of the experimental period, the animals were weighed and anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the

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**PREVENTION OF CPT-11-INDUCED DIARRHEA**

**Cytotoxicity Assay.** Rapid colorimetric assay for mitochondrial dehydrogenase activity (MTT assay) was used to estimate the drug-induced cytotoxicity as described previously (30).

**DNA Fragmentation.** HT-29 cells, at a density of 1 $\times$ 10⁶ cells in 60-mm dishes, were treated with increasing concentrations (0.1–2 μM) of SN-38 lactone or carboxylate for 2 h and then further incubated for 72 h in fresh culture medium that was changed three times and then collected by trypsinization and centrifugation. The cell pellets were lysed in 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100 for 10 min at 4°C. The supernatant collected by centrifugation at 15,000 rpm for 20 min was successively incubated with 0.4 mg/ml RNase A and 0.4 mg/ml proteinase K at 37°C for 1 and 2 h, respectively. The DNA was electrophoresed in a 1.5% agarose gel in Tris-borate buffer at 20 V for $\sim$4 h, stained with ethidium bromide (1 mg/ml), and visualized using an Eagle Eye II transilluminator (Stratagene, La Jolla, CA).

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and the cell-associated radioactivity was determined using a 2–9 (carboxylate) was analyzed by Kruskal-Wallis test (*, period. The comparative initial uptake rate of SN-38 prepared at pH 3 (lactone) and pH determined from the linear slope of the cellular uptake over the initial 90-s incubation

**RESULTS**

**Intestinal pH and Water Determination.** The pH of the jejunal, ileal, cecal, and colonic contents were determined after p.o. administration of increasing concentrations [0 (CTL), 0.5, 5, and 20 mg/ml] of sodium bicarbonate in 3% sucrose-containing water for 7 days. The pH was determined after homogenization of the samples in 0.9% saline. Homogenization of fecal samples was accomplished by pulverization with a mortar. The pH was determined using a micro pH meter (HM-17MX; Toa Electrics, Ltd., Tokyo, Japan). Each determination was performed at least three times to confirm the reproducibility.

The colonic content of the hamster was collected in a preweighed glass beaker and dried in a vacuum oven at 80°C for 48 h. The percentage of colonic water content was calculated from the difference between the respective wet and dry weight of the colonic content. During the experiment, the animals were humanely treated according to the guidelines for animal handling at the George Washington University.

**Statistics.** Except as otherwise indicated, results were expressed as mean ±SE. The statistical significance of the means was determined by either one-way ANOVA or Student’s t test.

**RESULTS**

**Initial Cellular Uptake Rate of SN-38.** The respective initial cellular uptake rate of SN-38 lactone and carboxylate by HT-29 was plotted as a function of the respective concentration and reported in Fig. 1. The uptake rate of SN-38 lactone was higher than that of SN-38 carboxylate at every concentration tested (P < 0.05 at 0.5 μM, and P < 0.005 at 1 and 2 μM). In addition, the uptake of SN-38 carboxylate was saturable and characterized by a V_{max} of 0.169 pmol · 10^6 cells⁻¹ · min⁻¹ and a K_{m} of 0.383 μM. Under the same conditions and within the range of concentrations tested, the uptake of SN-38-lactone was not saturable.

**Cytotoxicity of CPT-11 and its Metabolites.** Previously, the cell toxicity was determined 24 h after incubation of the HT-29 cells with SN-38 (30). However, while under these experimental conditions, SN-38 was added to the cells as the lactone or carboxylate form; it is possible that the relative proportion of these two forms could equalize during the 24-h incubation period studied. Therefore, we investigated the short-term cytotoxic effect of CPT-11, SN-38, and SN-38G in a period of time in which the respective lactone and carboxylate remains predominant (22, 39). HT-29 cells were incubated with the respective carboxylate and lactone form of CPT-11 and its metabolites for 20 min to 2 h. After three successive washes with PBS, the cells were further incubated for 72 h under control conditions. At the end of this period, the cellular toxicity was determined by the MTT assay. As shown in Fig. 2, 100 μM CPT-11 lactone induced a maximum of 40% cell death after 30-min exposure, whereas the same concentrations of the carboxylate counterpart required 60–120 min to induce a similar level of cell death. On the other hand, 0.5 μM SN-38 lactone induced an increased cell death from 35% after 30 min to ~50% after 120-min exposure, whereas the percentage of cell death induced by 0.5 μM SN-38 carboxylate reached a maximum of 30% after 30-min exposure and plateaued thereafter. In addition, 0.5 μM SN-38G was not cytotoxic even after 2-h incubation under either its lactone or carboxylate form. Furthermore, whereas concentrations ≥50 μM CPT-11 lactone were required to obtain 40% cell death, only 0.2 μM SN-38 lactone was sufficient to induce a similar cytotoxic effect. Under these conditions, SN-38G ≤5 μM remained without a significant toxic effect (data not shown).

As described in “Materials and Methods,” the respective lactone and carboxylate forms of either SN-38 or CPT-11 were prepared by overnight incubation in PBS buffered at pH 3 and 9, respectively. The addition of these agents at these pHs to the cell cultures did not significantly change the pH of the culture medium (pH 7.2–7.4). However, the pH of the intestinal lumen has been reported to range from 6.2 to 8.0 (40). Therefore, in an additional series of experiments, SN-38 was prepared at pH 6.2 and 7.6, respectively, and incubated with HT-29 cells maintained at pH 7.4. The results from Fig. 3A show that 0.1 μM SN-38 lactone incubation for only 2 h followed by washing and further incubation for 96 h was sufficient to induce 40%
of HT-29 cell death. This was significantly greater ($P < 0.01$) than that induced by its respective carboxylate counterpart.

To further study the importance of extracellular pH on SN-38-induced cellular toxicity, increasing concentrations of SN-38 lactone were prepared at pH $\leq 6.2$ and added for 2 h to HT-29 cells maintained at either pH 6.2 or 7.4. As shown in Fig. 3B, adjustment of the culture conditions to pH 6.2 or 7.6 for 2 h did not significantly affect cell viability. Furthermore, the level of toxicity of SN-38 lactone was similar, independent of the pH of the extracellular medium, suggesting for limited conversion of the lactone to the carboxylate form of SN-38 during the 2-h period of incubation and pH tested. These results were further supported by the determination of SN-38 lactone and carboxylate concentrations in the culture medium at pH 6.2 and 7.6. The respective SN-38 lactone concentration determined by HPLC was calculated to be $70\%$ at pH 6.2 and $\leq 25\%$ at pH 7.4–7.6 with little change over the 2-h incubation period.

**Effect of SN-38 on HT-29 Cell Cycle.** As shown in Fig. 4, A and B, significant alteration of the cell cycle was observed after treatment of the HT-29 cells with SN-38 lactone and carboxylate, respectively. These alterations in cell cycle were observed 24 h after removal of SN-38. The alteration of cell cycle in SN-38-treated HT-29 cells was characterized by a dose-dependent decrease in the percentage of cells in G$_1$-G$_0$ phase, a parallel increase of the cells in S phase, and an increase of those in G$_2$-M phase (Fig. 4A). The differential effect with a marked cell cycle arrest in S phase was observed with concentrations of SN-38 lactone $>0.1$ μM when compared with its carboxylate counterpart (Fig. 4B).

**SN-38/SN-38G-induced Apoptosis.** Preliminary results from the present study showed that 0.5–4 μM SN-38 lactone induced DNA fragmentation in HT-29 cells after 24–48-h incubation (data not shown). Thus, a quantitative determination of apoptosis was performed by assessing the SN-38-induced Annexin-V binding to HT-29 cells using flow cytometry. PS molecules are normally confined to the inner leaflet of the plasma membrane but are exposed to the outer plasma membrane leaflet during apoptosis to serve as a trigger for recognition of apoptotic cells by phagocytes. This PS externalization can be detected by staining with Annexin V, a protein that binds naturally to PS. As shown in Fig. 5A, SN-38 lactone induced apoptosis in a time-dependent manner with $\sim 30\%$ apoptotic cells observed 72 h after SN-38 addition. SN-38 lactone presented a dose-dependent apoptotic effect with a maximum effect observed at 1 μM (Fig. 5B). Furthermore, 1 μM SN-38 lactone was $>30$-fold more potent than its corresponding carboxylate ($P < 0.005$). As found by the MTT assay (data not shown), 5 μM SN-38G did not significantly induce apoptosis in HT-29 cells (Fig. 5B). These results are supported by other studies that suggest CPT-11 induces apoptosis in various cell lines, including mouse fibroblasts and human hepatoma PLC cells (41, 42). In addition, CPT-11-induced characteristic mucosal injury resulting from apoptosis and disruption of the intestinal epithelium has also been reported in the mouse model (19, 43).

**Comparative Effect of CPT-11, SN-38, and SN-38G on HT-29 Cell Proliferation.** To study the correlation between cytotoxicity and inhibition of proliferation in HT-29 cells, we compared the ability of CPT-11, SN-38, and SN-38G lactone and carboxylate to affect the colony formation of HT-29 cells. As shown in Fig. 6, among the agents tested, SN-38 lactone again showed the most potent dose-dependent inhibitory effect on colony formation. SN-38 (0.5 μM) lactone inhibited the growth of HT-29 colonies by $>60\%$, whereas at the same concentration, the inhibitory effect of SN-38 carboxylate was $\sim30\%$. Interestingly, 5 μM SN-38G had a significant growth inhibitory effect comparable with that observed with 0.5 μM SN-38 carboxylate. This is in contrast to the absence of effect of SN-38G on both cell toxicity and apoptosis.

All of these parameters associated to successive cellular events were well correlated with the initial rate of uptake of SN-38 (S phase accumulation versus uptake: $R^2 = 0.964$, $P < 0.01$; apoptosis versus uptake: $R^2 = 0.970$, $P < 0.01$; and colony formation versus uptake: $R^2 = 0.804$, $P < 0.05$, by quadratic regression).

**Preventive Effect of Sodium Bicarbonate Supplementation on CPT-11-induced Diarrhea in Golden Syrian Hamster Model.** To determine the effect of alkalization of the intestinal lumen on the prevention of CPT-11-induced diarrhea in vivo, sodium bicarbonate was administered p.o. in the drinking water to golden Syrian hamsters. Sodium bicarbonate supplementation for 7 days resulted in an increased intestinal pH in a dose-dependent manner with a maximum pH increase observed with 5 mg/ml (Fig. 7). While under these conditions, the hamster body weight was not significantly different (113.5 ± 3.9 grams), and the water consumption was decreased by $\sim40\%$ when 20 mg/ml sodium bicarbonate were administered. This decreased water consumption observed in the 20 mg/ml sodium bicarbonate group could be responsible for the decreased cecal and colonic pH level compared with control. In addition, and although not shown, those doses of bicarbonate did not significantly affect the hamster serum pH level.

We studied the effect of sodium bicarbonate on CPT-11-induced diarrhea. The hamsters were administered 5 mg/ml bicarbonate for 2 days before and during daily i.p. injection of CPT-11 for a 7-day period. From observation, all the animals receiving CPT-11 alone had diarrhea by days 3–5. In addition, while control animals gained body weight by $\sim0.3$ grams/day over the 7-day period, the animals receiving CPT-11 lost weight in a CPT-11 dose-dependent manner with a respective loss of 20 and 31% with CPT-11 20 and 50 mg/kg. Finally, whereas bicarbonate administration itself did not affect the body.
weight gain as compared with control, it reduced the loss of weight of the hamsters receiving CPT-11 (20–50 mg/kg) by ~50%.

The histological analysis of the section of the colon from the hamsters treated with CPT-11 showed severe, acute, and ulcerative colitis. There was necro-inflammatory debris in small foci within the epithelial cell layer with focal loss of cells. Numerous neutrophils were seen among epithelial cells and edematous lamina propria (Fig. 8A). On the other hand, the sections of colon from the hamster treated with CPT-11, in which bicarbonate was administered p.o. throughout the CPT-11 treatment, showed a moderate acute colitis with focal cryptitis that was less than that seen with CPT-11 alone. There was only occasional neutrophil aggregate in a crypt in the epithelial cell layer (Fig. 8A). These changes were not present in the group receiving the sodium bicarbonate supplementation alone.

This increased intestinal damage induced by 20–50 mg/kg CPT-11 was associated with a 70–75% increase in colonic water content. Although bicarbonate alone did not alter the hamster’s colonic water content, it significantly (P < 0.05) reduced that induced by CPT-11 by ~30% (Fig. 8B). From observation, while the feces of hamsters receiving CPT-11 were liquid, those of hamsters treated with CPT-11 and receiving bicarbonate supplementation closely resemble the feces of the control hamsters. Since following CPT-11 treatment alone as compared with CPT-11 coadministration with bicarbonate, the hamsters had a loss of body weight, the intestinal damage could, at least in part, be responsible for the decreased intestinal nutrient uptake in the CPT-11-treated group.

We also studied the effect of bicarbonate supplementation on the intestinal SN-38 and CPT-11 concentrations. Bicarbonate administration (5 mg/ml) in the drinking water resulted in ~20% decrease in intestinal SN-38 lactone concentration, whereas CPT-11 carboxylate was increased by ~15% when compared with control hamsters receiving a daily dose of 20 mg/kg CPT-11 alone. These results support the above described decreased SN-38 lactone-induced cell injury and diarrhea after bicarbonate administration.

DISCUSSION

The present study is the first to clearly demonstrate that an increased intestinal pH after bicarbonate p.o. administration was asso-
ciated with a reduction in the intestinal SN-38 lactone concentration, as well as cellular damage and diarrhea induced by CPT-11 in the golden Syrian hamster model. A daily injection of CPT-11 at a dose of 20–50 mg/kg BW induced diarrhea by days 3–5 and was lethal by day 5 at a dose ≥100 mg/kg BW. Under these conditions, p.o. administration of 5 mg/ml sodium bicarbonate in the drinking water significantly reduced both the fecal water content, as well as the intestinal tissue damage induced by 20–50 mg/kg BW CPT-11. These results are further supported by a Phase II clinical trial, suggesting a beneficial effect of sodium bicarbonate supplementation against CPT-11-induced severe delayed diarrhea (44).

SN-38 derived from the prodrug CPT-11 is suggested to exert its antitumor effect well before its elimination from the systemic circulation and secretion into bile (23, 45). Once secreted into bile, however, SN-38 has been shown in rats to accumulate intracellularly in intestinal epithelial cells (46). It is believed to be responsible for diarrhea attributed to CPT-11 administration in nude mice (27). These studies suggest that reduced accumulation of SN-38 in intestinal cells can diminish the severity of diarrhea and, consequently, increase the clinical usefulness of CPT-11 (19).

The present study supports CPT-11/SN-38 lactone-induced diarrhea to be the result of intestinal injury rather than increased intestinal secretion as reported previously in humans (47). Our results are in agreement with recent reports both in animal models (27, 43), as well as in cancer patients (48), in which lethal small-intestinal injury was associated to CPT-11-induced diarrhea. Therefore, one of the proposed mechanisms for bicarbonate action is to reduce both the intestinal concentration of SN-38 lactone and its induced tissue damage, resulting in reduced diarrhea.

The protective mechanism of bicarbonate p.o. administration involves, at least in part, a pH-dependent increased conversion of
CPT-11/SN-38 lactone to carboxylate, as shown in the present study. It is generally accepted that the α-hydroxy lactone ring is an absolute requirement for CPT-derived molecules to have in vitro and in vivo activities (49–51). However, although circumventing the opening of the lactone ring is one of the strategies proposed in order to optimize the antitumor activity of this drug (52), the lactone ring is unstable at physiological pH and above and can open to the much less active carboxylate form. Therefore, it is our hypothesis that the reduced incidence and severity of CPT-11-induced diarrhea is attributable, at least in part, to the opening of the lactone ring in the intestinal lumen. The rationale for this theory is based on our findings in both HT-29 cells and in enterocytes isolated from hamster small and large intestine (30). Results of these studies show that the initial uptake rate of the lactone form of SN-38 by HT-29 cells was significantly greater than that of its carboxylate counterpart (Fig. 1). Furthermore, in contrast to the lactone form, for which uptake remained unchanged, the transport of SN-38 carboxylate was carrier mediated and could specifically and significantly be inhibited by the addition of dinitrophenol (30). Finally, the initial uptake rate presents a good correlation with the cytotoxicity of SN-38 in HT-29 cells (see “Results” and Ref. 30).

This difference in the cytotoxicity of CPT-11 and SN-38 in HT-29 cells can probably be attributed to the respective differential rate of conversion between the lactone and carboxylate form of these agents. Indeed, Akimoto et al. (53) have reported that the constant of hydrolysis of SN-38 (50% conversion from lactone to carboxylate) was 13.5 h and 33 min at pH 6 and pH 7.4, respectively. Furthermore, Rivory et al. (39) have reported that conversion of CPT-11 lactone to carboxylate in vivo was rapid, whereas SN-38 was present predominantly as the lactone form at all times. The HPLC determination in the cell culture medium indicated that even after 2-h incubation at pH 7.4, SN-38 prepared at pH 6.2 and 7.6 remained ~70 and ≤25% as lactone, respectively. This limited conversion of the lactone to the carboxylate form was further supported by the fact that similar toxicity was observed independently of the pH of the incubation medium during the 2-h incubation period of the cells with SN-38 lactone.

As reported in the present study, the lactone form of SN-38 was more potent to induce both S and G2–M phase cell cycle arrest and apoptosis than its carboxylate counterpart. These results are consistent with what has been reported previously with camptothecin (33, 34). The lack of G1 arrest is probably attributable to the p53 mutation in the HT-29 cells and the loss of the p53-modulated G1 check point suggested previously by Goldwasser et al. (32). The delay or blockage of, at least, G2 phase after SN-38 treatment may allow for cellular DNA repair (32, 54). This has been suggested previously by various authors including Shao et al. (54), who have shown that UCN-01, which abrogates the G2-M checkpoint, increased camptothecin-induced cell toxicity. It is also worthwhile to mention that the HT-29 G2-M checkpoint is more sensitive to SN-38 than the S checkpoint, because the G2-M phase arrest is observed at lower SN-38 concentrations than those required to induce an arrest in S phase (Fig. 4B).

These results may underline some interregulatory mechanism(s) between these two checkpoints and support the observations with camp-
tothecin reported previously by Goldwasser et al. (32). Therefore, our present results demonstrate that the number of apoptotic cells induced by the lactone form of SN-38 was also greater than that induced by its carboxylate counterpart. This suggests that SN-38 can induce cell death in a DNA damage-dependent fashion overcoming any possible cellular rescue attributable to cell cycle arrest and increased DNA repair.

Furthermore, it was recently reported in this cell line that the replication protein A2 (RPA2) is an important protein of the replication complex for initiation and maintenance of S phase. In addition, this protein, which plays a role in signaling mechanisms that coordinate DNA replication and cell cycle, can be phosphorylated by pharmacological concentrations of camptothecin (55). Because these changes are related to the induction of DNA damage (replication fork collisions) in replicating DNA (34, 35), it is speculated that the specific changes in cell cycle can be a reflection of the magnitude of the DNA damage induced by CPT-11/SN-38. Therefore, the cell cycle analysis could be a convenient parameter to assess the pharmacological activity of CPT-11/SN-38, as far as the replication-arresting DNA damage is concerned.

Interestingly, in the present study, 5 μM SN-38G showed a significant growth inhibitory effect, even though no effect was observed using either the MTT or Annexin-V binding assay. Because it is believed that SN-38G does not induce any significant cytotoxic effect (56), these findings suggest the possible deconjugation of the glucuronide group from SN-38 during the prolonged culture period in HT-29 cells. In addition, it remains possible that short-term assays, such as the MTT assay and apoptosis determination, may not be sensitive enough to determine the cumulative cellular effect of SN-38G. The possibility that SN-38G can exhibit cellular toxicity after a prolonged period of culture raises the question of the clinical involvement of SN-38G in CPT-11-induced diarrhea.

The bicarbonate-induced improvement of tissue damage in the large and also in small intestine is somewhat puzzling, in light of the in vitro data showing no effect on overall toxicity of SN-38 with changes in pH of the incubation medium from 6.9 to 7.4 for 2 h. Indeed, whereas the transit time in human large intestine has been reported to be around 10 h, that of the small intestine averages 2–4 h (57), and these values should be shorter in the hamster model (58).

Therefore, the period allowed for the conversion of the SN-38/CPT-11 from lactone to carboxylate in the small intestine should have been too short to observe any protective effect against tissue damage. This, therefore, raises the question of possible additional effects of increased luminal bicarbonate besides increasing the conversion of the drug from the lactone to its carboxylic form.

In summary, the toxic effect of SN-38 lactone was maximum even after exposure of the cells for a period of time as short as 2 h. Furthermore, the pH-dependent opening of the SN-38 α-hydroxy lactone ring to its carboxylic form resulted in a decreased HT-29 apoptosis and growth inhibition, which were well correlated with the initial cellular uptake rate of this drug. These findings can explain, at least in part, the protective effect of intestinal alkalization on CPT-11-induced diarrhea in vivo.

However, the present study raises an important issue of the role that increased intestinal alkalization may have on the potency of CPT-11 on colorectal cancer therapy. Although this question cannot be fully addressed at the present time, without additional studies, different elements of a response can be proposed. First, CPT-11 therapy is generally limited to advanced colorectal cancer patients with the goal to ablate metastasis associated to internal organs, including liver and kidney (8, 59). Furthermore, colon carcinoma is a rigid tumor and, therefore, only the cells on the surface of the tumor can absorb the drug from the luminal side. Thus, this emphasizes the importance of the drug delivery from the blood supply. Under these conditions, elevated pH in the colonic lumen may not play a major role in the alteration of the drug absorption by the tumor cells. Therefore, it is the hypothesis of the authors that the efficacy of CPT-11 should not be particularly affected in colorectal cancer by the administration of bicarbonate.

In conclusion, it is believed that the control of late-onset diarrhea holds the key to the effective use of CPT-11, which, in turn, will improve survival benefit and quality of life for patients with malignancies (10). p.o. administration of bicarbonate appears to be a novel and effective protective mechanism against CPT-11/SN-38-associated tissue injury and side effects. Although the mechanism by which intestinal alkalization abrogates CPT-11-induced side effects needs further clarification in humans, results from our in vitro and in vivo studies suggest a pH-dependent facilitation of SN-38 carboxylate formation and a decreased intestinal epithelial cell accumulation. Finally, this approach may prove to be inexpensive, safe, and useful to increase the efficacy of CPT-11 in cancer patient therapy.

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