Metabolism of irinotecan and its active metabolite SN-38 by intestinal microflora in rats

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Abstract. One of the dose-limiting toxicities of irinotecan (CPT-11) is delayed-onset diarrhea, which is the greatest barrier to treatment with CPT-11-containing regimens. CPT-11 is converted to its active metabolite, SN-38, which is conjugated by hepatic uridine diphosphate glucuronosyl transferase to SN-38 glucuronide (SN-38G). SN-38G, once excreted in the intestinal lumen via bile, is extensively deconjugated by bacterial ß-glucuronidase with the regeneration of SN-38 in the intestinal lumen, which may cause diarrhea. However, the metabolism of CPT-11 and its metabolites by intestinal microflora are yet to be reported. This study was carried out to investigate the microbial transformation of CPT-11 and SN-38 using an anaerobic mixed culture of rat cecal microorganisms. No reaction in the mixed cultures was observed when CPT-11 or SN-38 lactone was added to the culture medium. When CPT-11 was added to the culture broth, a significant amount of water-soluble CPT-11 was detected in the spent culture medium. In contrast, only a slight amount of SN-38 was found in the supernatant when SN-38 lactone was added to the broth. A significant quantity of SN-38 was found in the sediment. In conclusion, these results strongly suggest that SN-38 produced from SN-38G by the action of bacterial ß-glucuronidase is rapidly adsorbed by the intestinal bacterial cell walls in the sediment because of the hydrophobic and lipophilic nature of SN-38, and a small amount of SN-38 remains in the intestinal luminal fluid. Thus, we need to reconsider the role of SN-38 in the intestinal lumen in CPT-11-induced late-onset diarrhea.

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Introduction

Irinotecan (CPT-11) is a water-soluble camptothecin derivative that inhibits topoisomerase I by stabilizing the enzyme-DNA 'cleavable complex', thus causing single-strand DNA breaks (1). CPT-11 has been demonstrated to show significant antitumor activity against various human malignancies, and the therapeutic role of CPT-11 as a single agent or in combination with other drugs has been investigated in several solid tumors, including colorectal, small and non-small cell lung, gastric, cervical and ovarian cancer (2). CPT-11 sometimes causes unpredictable and severe neutropenia and/or diarrhea, which constitute the drug's dose-limiting toxicity (3). High-dose loperamide can ameliorate CPT-11-induced diarrhea, but is not always adequate. In a randomized trial of small-cell lung cancer, grade 3/4 diarrhea occurred in 16.0% of the patients (4). The hydrophobic active metabolite, SN-38, is generated from CPT-11 by carboxylesterase, and then conjugated to SN-38 glucuronide (SN-38G) by hepatic uridine diphosphate glucuronosyltransferase. SN-38G is the inactive metabolite and is secreted in the duodenum (5-7). Many authors consider that the intestinal bacterial microflora are responsible for the damage to the intestinal mucosa, because intestinal bacterial ß-glucuronidase can deconjugate SN-38G into active SN-38, which causes mucosal damage in the small intestine thus causing toxic diarrhea (8-13).

However, there is no study, thus far, on the biotransformation of CPT-11 and SN-38 by the intestinal microflora, even though this knowledge is essential for the understanding of the precise mechanism by which CPT-11 induces late-onset diarrhea. Therefore, we report on our study of the transformation of CPT-11 and its metabolite, SN-38, using mixed microbial cultures of the rat cecal contents.

Materials and methods

Animals. Male wistar rats (12-week-old, weighing respectively 294 and 308 g) were obtained from Japan SLC (Hamamatsu, Japan). The animals were reared in cages with a wire-mesh bottom in a temperature- and humidity-controlled animal facility under a 12-h light-dark cycle. The animals

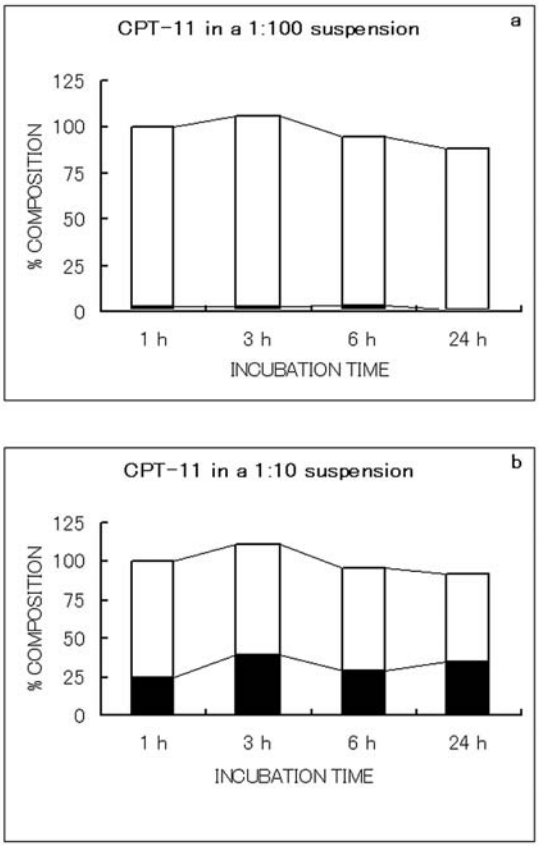

Figure 1. Changes in the percent composition of CPT-11 during the course of incubation. A 1:100 suspension (a) or 1:10 suspension (b) of the cecal contents grown in broth containing CPT-11 for up to 24 h. Samples were removed for assay at the indicated times. White, CPT-11 in the supernatant and black, CPT-11 in the sediment.

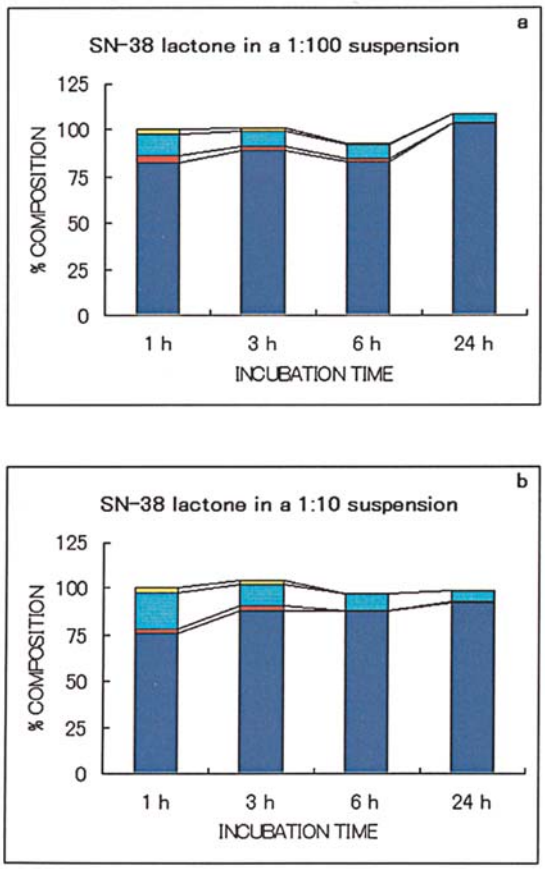

Figure 2. Changes in the percent composition of SN-38 during the course of incubation in the 100-fold (a) or 10-fold diluted (b) cecal contents. The lactone form of SN-38 was added to the culture medium, and incubated anaerobically under N_2 for up to 24 h. Dark blue, SN-38 lactone in the sediment; light blue, SN-38 lactone in the supernatant; yellow, SN-38 carboxylate in the supernatant and orange, SN-38 carboxylate in the sediment.

received a steam-sterilized commercial diet, and had free access to feed and water during the acclimated period.

Cecal contents. Two rats were sacrificed by exsanguination under pentobarbital anesthesia. The cecum was removed with its contents which were collected and transferred to an anaerobic chamber (Coy Laboratories, Grass Lake, MI) within 5 min. Cecal contents (5 g) were diluted 10-fold in 45 ml of sterile pre-reduced peptone-yeast extract broth (see below) under flushing with N_2. After vortex-mixing briefly, the cecal contents were further diluted to 100-fold (volume/volume) in peptone-yeast extract broth.

Culture experiment. The basal medium used in this experiment was peptone-yeast extract broth, which consisted of 1 g polypeptone (Daigo-Eiyo Chemical Co.); 1 g yeast extract (Difco Laboratories); 4 ml of the salt solution recommended by Holdeman *et al* (14); and l-cysteine-HCl, 0.05 g per 100 ml in 0.02 M phosphate buffer at pH 7.5, sterilized at 120˚C for 15 min. A 1:10 or 1:100 suspension of the cecal contents in the peptone-yeast extract broth was incubated for a specified period of time under an atmosphere of pure N_2 after adding CPT-11 or its metabolite SN-38. The culture medium was then analyzed.

Camptothecin-containing medium. CPT-11 was dissolved in distilled water at 10 mg/ml and an SN-38 lactone form was prepared by dissolving SN-38 in dimethylsulfoxide at 1 mg/ml. After sterilization by passage through a 0.45-μm membrane filter, 0.1 ml of CPT-11 solution or 0.1 ml of the solution of SN-38 lactone form was added to 10 ml of the cecal content suspension (1:10 or 1:100). The medium was then dispensed in 1.5-ml aliquots and in four 15x105-mm test tubes.

Incubation. For anaerobic growth, the tubes containing CPT-11 or its metabolite, SN-38, were inoculated under flushing with N_2, tightly stoppered and incubated for 1, 3, 6, or 24 h at 37˚C. The N_2 gas was used after removal of the residual oxygen by passing over a heated copper gauze.

Extraction and HPLC analysis of CPT-11 and its metabolites. The inoculated tubes were assayed for CPT-11 or its metabolite at various time-intervals during incubation under N_2 for up to 24 h. For the whole culture medium assay, 0.1 ml of the spent culture medium was mixed with 0.4 ml of methanol and centrifuged at 15,000 rpm for 1 min at 0˚C. For the assay of the supernatant fluid, 1 ml of the spent culture medium was centrifuged at 15,000 rpm for 1 min at 0˚C; 0.05 ml of each

supernatant was diluted with 0.2 ml of 0.15 M H_3PO_4 and 0.25 ml of the internal standard solution containing 1 μg/ml of camptothecin. The concentrations of CPT-11 and SN-38 were determined using a high-performance liquid chromatographic (HPLC) method with a fully automated on-line solid phase extraction system (Prospect, Spark Holland, Emmen, The Netherlands), as previously described (15). The quantification limit for CPT-11 and SN-38 was 0.5 and 0.05 mg/ml, respectively.

For the SN-38 lactone and carboxylate form analysis, 0.3 ml of the culture medium or its supernatant was added to 0.3 ml of methanol chilled in a dry-iced isopropanol bath. The mixture was then vortexed for 10 sec, and immediately centrifuged at 15,000 rpm for 1 min at -10˚C. The supernatant obtained was rapidly filled in the vial, set on an autosampler at 4˚C and analyzed in an HPLC system. The concentrations of the SN-38 lactone and carboxylate forms were determined using HPLC by the methods of Kaneda *et al* (16). The lower limits of quantification for the SN-38 lactone and carboxylate forms were 1 and 10 ng/ml, respectively.

Chemicals. CPT-11 (Lot no. 115A100) and SN-38 (Lot no. 300917R) were kindly donated by Yakult Honsha Co. Ltd. (Tokyo, Japan). Camptothecin was purchased from Sigma, St. Louis, MO. Other chemicals were of the highest commercially available grade.

Results

Adsorption of CPT-11 by bacterial cells. The 10- or 100-fold diluted cecal contents were grown anaerobically in broth in the presence of CPT-11. No metabolites of CPT-11 were found even after 24 h of incubation. In the 100-fold dilution broth, almost all of the CPT-11 was found in the supernatant but was barely detectable in the sediment (Fig. 1a). Even in the more densely re-suspended culture (10-fold dilution), the percentage of CPT-11 in the supernatant was ~80%, and ~20% of CPT-11 was adsorbed by the bacterial cells from the solution during the period of the study, reflecting the water-soluble nature of CPT-11 (Fig. 1b).

Disappearance of the lactone form of SN-38 from the culture solution. When the lactone form of SN-38 was added to the culture medium, a significant amount of SN-38 disappeared very rapidly from the supernatant fluid (Fig. 2a and b). This SN-38 was apparently adsorbed mainly onto the bacterial cells in the sediment. The adsorption of SN-38 on the bacterial cells was extremely rapid and achieved a plateau within 1 h. The plateau remained stable for up to 24 h. Since the lactone form of SN-38 was highly water-insoluble, SN-38 was rapidly and tightly bound to the hydrophobic bacterial cell wall, resulting in sparse free SN-38 remaining in the lactone form, either in the supernatant of the 10- or the 100-fold diluted culture.

Discussion

One of the major dose-limiting toxicities of CPT-11 is diarrhea, which may be early- or late-onset diarrhea (<24 or >24 h after CPT-11 administration, respectively) (2,17). Early-onset diarrhea is observed soon after CPT-11 infusion, and is presumably due to the inhibition of acetylcholinesterase activity by CPT-11. Late-onset diarrhea has a median onset of 5 and 11 days in the 3-weekly and weekly dosing schedule of CPT-11 (11), and has the potential to cause life-threatening dehydration and electrolyte imbalance. Since SN-38G, once excreted in the intestinal lumen, is extensively hydrolyzed by bacterial ß-glucuronidase to free SN-38, it was thought that direct intestinal damage by the free intestinal luminal SN-38 was responsible for the delayed-onset diarrhea. Takasuna *et al* (10,18) reported on the good correlation between the fecal concentration of SN-38 and the anatomic damage of the intestinal mucosa in mice, as well as a greater degree of histological damage in the intestinal segments where the bacterial ß-glucuronidase activity was prominent (caecum and colon). However, our knowledge of the precise mechanism involved in the latter is far from complete.

No metabolic conversion of CPT-11 or SN-38 occurred in the mixed cecal cultures under anaerobic growth conditions. Since CPT-11 is a water-soluble derivative of camptothecin, a significant quantity of CPT-11 was detected in the supernatant (Fig. 1). It is noteworthy that insufficient free SN-38 was found in the cell-free supernatant when the cecal contents were grown in broth in the presence of the lactone form of SN-38 (Fig. 2). A significant amount of SN-38 was detected in the bacterial cell pellets. Due to the highly hydrophobic and lipophilic nature of SN-38, it appears to get sufficiently adsorbed onto the hydrophobic areas of the cell wall and membrane, as well as onto the dietary fibers in the cecal contents. It has been reported that ~14% of the lipid content of the *E. coli* cell wall is readily extractable, and that the greater the amount of lipid present in the bacterial cell wall, the greater the uptake of the hydrophobic drug (19). Bacterial cell walls can adsorb SN-38 produced from SN-38G by the action of bacterial ß-glucuronidase. This may potentially reduce the availability of the active metabolite, SN-38, to the intestinal mucosa, thereby preventing SN-38 from acting directly on the intestinal tissue. Furthermore, the amount of SN-38 absorbed into the bloodstream would be reduced and so decrease the probability of tissue damage. More SN-38 would also be expected to be excreted in the feces. Khanna *et al* (20) reported that CPT-11 is converted to SN-38 by the carboxylesterase present in the small intestine. Taking these results into consideration, the local conversion of CPT-11 to SN-38 in the intestinal cells, especially the crypt cells, may be more important in determining the severity of diarrhea caused by the administration of CPT-11 than the amount of SN-38 in the intestinal lumen.

In conclusion, we demonstrated that a significant quantity of SN-38 was adsorbed onto intestinal microflora, but a small amount of SN-38 was detected in the supernatant *in vitro*. Further studies are needed to identify the exact role of the intestinal microflora in CPT-11-induced late-onset diarrhea.

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