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Relationship between Development of Diarrhea and the Concentration of SN-38, an Active Metabolite of CPT-11, in the Intestine and the Blood Plasma of Athymic Mice Following Intraperitoneal Administration of CPT-11

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Severe diarrhea occurred during daily intraperitoneal administration of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) at a dose of 50 mg/kg in athymic mouse. Serial determination of CPT-11 and 7-ethyl-10-hydroxycamptothecin (SN-38), with the use of an on-line solid extraction HPLC system, demonstrated that much higher levels of the compounds are retained in the intestine and the blood plasma after five consecutive daily injections than after a single injection. Histologic examination of the gastrointestinal tract showed hemorrhagic colitis on day 7 and later after five consecutive daily injections of CPT-11. The direct cause of diarrhea associated with CPT-11 administration is considered to be enterocolitis caused by high levels of SN-38 and/or CPT-11 retained for a long period in the intestine.

Key words: SN-38 — Athymic mouse — Diarrhea — Hemorrhagic colitis

7-Ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11), a semisynthetic derivative of camptothecin (CPT), has been found to have significant activity against a variety of experimental tumors[1-3] through the inhibition of DNA topoisomerase I.[4,5] Phase II studies were carried out in patients with leukemia, lymphoma,[6] carcinoma of the cervix, the ovarium[7] and the lung[8] and the drug was shown to be effective. Major toxic effects of this drug were reported to be myelosuppression and gastrointestinal toxicity, especially severe diarrhea characterized by a delay in onset.[6]

The aim of the experiment described here was to determine the concentrations of CPT-11 and its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) in the intestine and the blood plasma and to study the mechanism of the gastrointestinal toxicity of CPT-11, using the athymic mouse as a model.

MATERIALS AND METHODS

Drugs CPT-11, CPT and SN-38 were synthesized and kindly provided by Daiichi Pharmaceutical Co. Ltd., Tokyo, and Yakult Honsha Co. Ltd., Tokyo.

Animals Male athymic (nu/nu) mice (BALB/c) weighing 18–20 g (CLEA Japan, Tokyo) were housed in plastic cages with sterilized woodchip bedding. They were fed CMF pellets (Oriental Yeast Co. Ltd., Tokyo) and allowed to drink sterilized water *ad libitum*. All the experiments were performed in an animal laboratory at a controlled temperature (25°C).

Administration of drugs and sample collection The drugs were dissolved in distilled water. The mice were divided into two groups, one for the serial determination of CPT-11 and SN-38 in the intestine and the blood plasma and the other for histologic study of the tissues of the gastrointestinal tract after the administration of CPT-11. For the histologic study of the gastrointestinal tract, CPT-11 was intraperitoneally administered at a daily dose of 25 mg/kg or 50 mg/kg for 14 days and the animals were examined histopathologically.

For the time-course study of the tissue and blood concentration of CPT-11 and SN-38, specimens were taken from three mice each before and 0.5, 1, 3, 6, 15, 24 and 48 h after a single injection or after the final injection of five days' successive administration of 50 mg of CPT-11/kg/day. Mice were killed under ether anesthesia by exsanguination. The abdomen was opened through a midline incision and the gastrointestinal tract was removed. One ml of blood was taken from venae cavae and 0.5 μmol of bis-*p*-nitrophenylphosphate was immediately added as an inhibitor of carboxylesterase. Blood plasma was diluted 10-fold with 0.01 *N* HCl and left standing for 30 min before centrifugation at 18,500*g*.

Prior to washing in cold 0.07 *M* phosphate buffer pH 7.2, the intestinal surfaces were examined for bleeding and/or ulceration and then the intestines were fixed in formalin (Sakura Unifix, Sakura Seiki, Tokyo) for histologic study. After fixation, each specimen was divided

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into strips about 3 mm wide, by making perpendicular cuts to the middle line. Two-micrometer thick sections were stained with hematoxylin-eosin and examined under a light microscope for histologic changes. For the quantitative determination of CPT-11 and SN-38, 7 cm of the gastrointestinal tract was cut out and placed on a metal plate, 10×8×2 cm, pre-chilled in dry-ice acetone. The tissue was pressed with another metal plate, pre-chilled in the same way, and the frozen tissue was broken up, put into a screw-capped plastic tube and kept at −80°C until analysis.

Extraction and determination of drugs For determination of CPT-11 and SN-38 in the tissue homogenates, one ml of 0.01 *N* HCl was added to frozen tissue and the mixture was treated with a sonicator (Sonifier-450, Branson, Danbury, CT) for 30 s. The homogenate was frozen and quickly thawed again. This procedure was repeated three times. The supernatant of this homogenate obtained by centrifugation at 18,500*g* for 5 min was injected into an HPLC apparatus.

HPLC analysis On-line solid extraction and an AS-4000 autosampler (Hitachi, Tokyo) were controlled by a PT-8000 (Tosoh, Tokyo) with an L-6000 pump (Hitachi). A Model L-6200 pump (Hitachi) was used for chromatographic separation. Detection was performed with a fluorescence detector F-1050 (Hitachi). A reversed-phase TSKgel ODS-80T_M column, 250×4.6 mm I.D. (Tosoh) with a precolumn TSKgel ODS-80T_M, 15×3.2 mm I.D. (Tosoh) was used for chromatography. The injection volume was 50 μl. The mobile phases consisted of 0.1 *M* phosphate buffer, pH 4.0, containing 3 m*M* 1-heptane sulfonic acid sodium salt/methanol (45/55, v/v) and acetonitrile/water (1/2, v/v) for CPT-11 and SN-38, respectively. Flow rates were 0.8 ml/min for CPT-11 and 1.0 ml/min for SN-38. A fluorospectromonitor was set at an excitation wavelength of 370 nm and an emission wavelength of 430 nm for CPT-11 and at 380 nm and 555 nm for SN-38, respectively. CPT-11 and SN-38 gave relative retention times (with respect to CPT) of 0.67 and 0.86, respectively. The recoveries of CPT-11 and SN-38 added to the tissue homogenates were 95% and 98%, respectively. The lower limits of detection for CPT-11 and SN-38 were 16.0±1.5 ng/ml and 1.7± 0.2 ng/ml, respectively.

RESULTS

Gastrointestinal toxicity Daily i.p. administration of 50 mg of CPT-11 per kg induced diarrhea in two of 12 mice on day 5 (Fig. 1). From day 8 severe watery diarrhea associated with moderate intestinal bleeding occurred in all the mice. The intestinal wall was extremely thin. Hemorrhagic contents were found from the small intestine to the rectum but no ulcerative or necrotic lesions were observed on the surface of the stomach or the intestine.

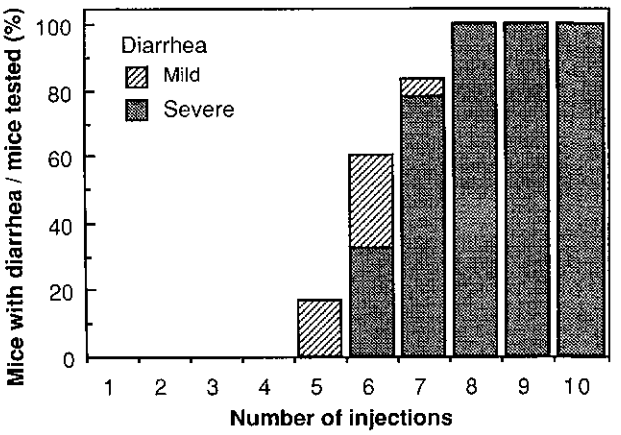


Fig. 1. Occurrence of diarrhea following intraperitoneal administration of CPT-11 at a dose of 50 mg/kg.

Histologic changes in the intestine following the administration of CPT-11 Daily administration of 25 mg of CPT-11 per kg induced an inflammatory infiltrate and slight fibrosis in the submucosa of the colon in one of three mice on day 10. Loss of epithelial cells was observed in some parts of the colon on day 14 in all the mice. Daily administration of 50 mg of CPT-11 per kg induced hemorrhagic colitis from day 7.

Day 7: Partial loss of villi and slight infiltration of inflammatory cells in the submucosa of the colon were observed in all the mice given 50 mg of CPT-11 per kg (Fig. 2-A).

Day 10: The changes in the gastrointestinal tract following administration of CPT-11 were most severe in the cecum, but changes varying in intensity were also observed at other sites of the colon. Flattened villi and glandular disruption changed the architecture of the crypt. The lumina propria mucosa contained a severe inflammatory infiltrate in two of three mice. Edema and scattered crypt abscesses were observed in some parts of the colon. Marked submucosal fibrosis and intraepithelial polymorphonuclear cells were also found (Fig. 2-B).

Day 14: Erosive changes in the colon were slight compared to those at day 10. The inflammatory infiltrate was scant and hyperplasia of the glandular tubules was observed, probably as regenerative hyperplasia after the erosive change. Some glandular tubules had transmigrated into the lymph follicles and folded under the lamina muscularis mucosae (Fig. 2-C).

Time course study of the concentrations of CPT-11 and SN-38 in the tissue of intestine Changes in the concentrations of CPT-11 and SN-38 in the intestine after a single or five consecutive daily injections of CPT-11 at a

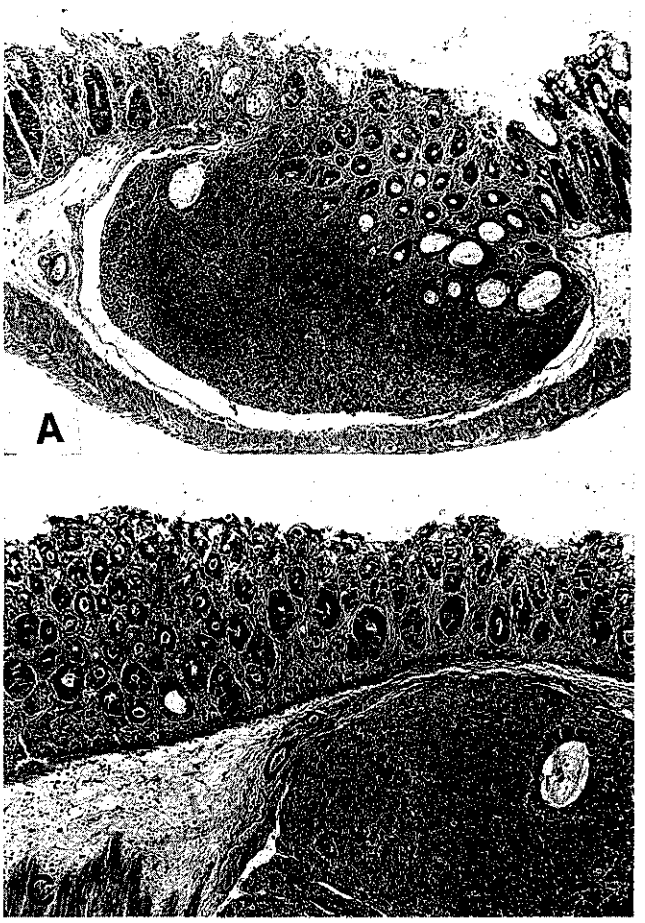


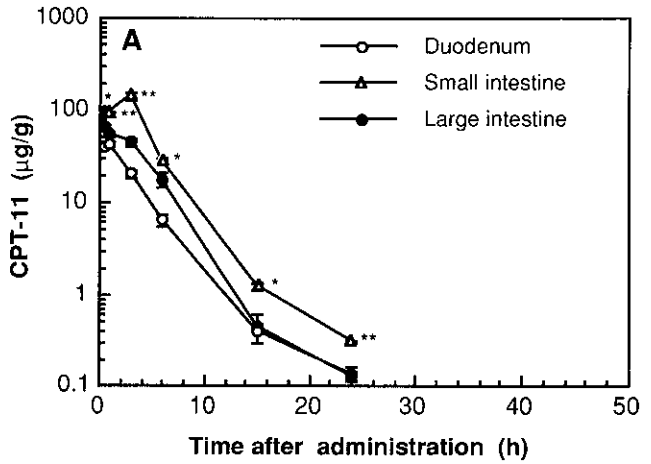
Fig. 2. Histologic changes in the intestine after administration of CPT-11. (H & E: original magnification ×100) (A) The colonic mucosa after seven consecutive daily injections of 50 mg of CPT-11 per kg. The colonic mucosa shows superficial erosion and regenerative changes. Transmigration of regenerated glandular tubules into an enlarged lymph follicle can be seen. (B) The colonic mucosa after 10 consecutive daily injections of CPT-11. Hyperplasia of glandular tubules, scattered crypt abscesses and submucosal fibrosis are observed. (C) The colonic mucosa after 14 consecutive daily injections of CPT-11. This photograph shows superficial erosion and regenerative changes with slightly atypical nuclei of the gland.

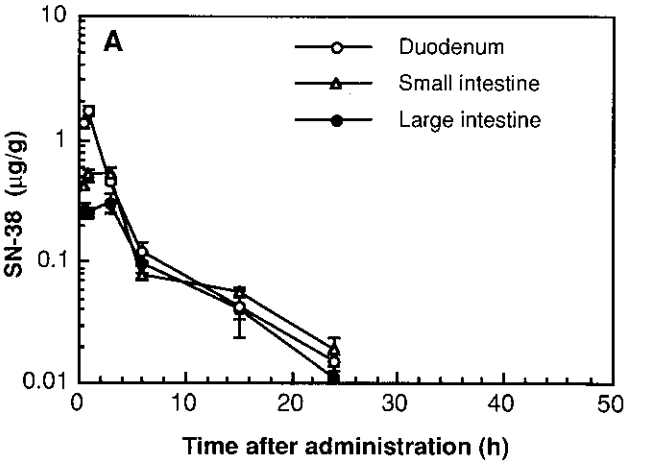
dose of 50 mg/kg are shown in Figs. 3 and 4. In both administration schedules, the concentration of CPT-11 in the small intestine was significantly higher than in the duodenum and the large intestine, and the maximum concentration of CPT-11 in the small intestine was more than 100 μg/g wet weight of tissue, recorded 3 h after administration (Fig. 3). There was no difference in concentration of SN-38 between the segments of the intestine (Fig. 4). The decline in the intestinal concentrations of CPT-11 and SN-38 were rapid in the first 6 h after the single injection and these metabolites were not detected 48 h later. On the other hand, they decreased slowly and were detected even at 48 h after five consecutive daily injections. The levels of CPT-11 and SN-38 in the tissue homogenates of each segment obtained 6 h or more after five consecutive daily injections were higher than those after the single injection.

Time course study of CPT-11 and SN-38 in the blood plasma Serial determinations of the concentrations of CPT-11 and SN-38 in the blood plasma after single injection or five consecutive daily injections of CPT-11 at a dose of 50 mg/kg are shown in Fig. 5. Twenty-four hours after the single injection, the level of CPT-11 was below 0.01 μg/ml, while that of SN-38 was 2.4 ng/ml. On the other hand, after five consecutive daily injection, CPT-11 and SN-38 disappeared gradually and the levels at 48 h were 0.04 μg/ml and 4.5 ng/ml, respectively.

DISCUSSION

Among the toxic side effects of CPT-11 and its analogues, persistent diarrhea is the most serious problem forcing cessation of therapy. Our main objective is to study the mechanism of diarrhea associated with clinical administration of CPT-11. Man and experimental animals seem to differ in their sensitivities to mucosal injury. In our initial study, we failed to produce diarrhea in conventional mice, such as BALB/c mice, even by high-dose administration of CPT-11. In this study, however, we succeeded in causing athymic nude mice to produce bloody diarrhea by daily administration of 50 mg/kg CPT-11, five days or more after treatment, al-





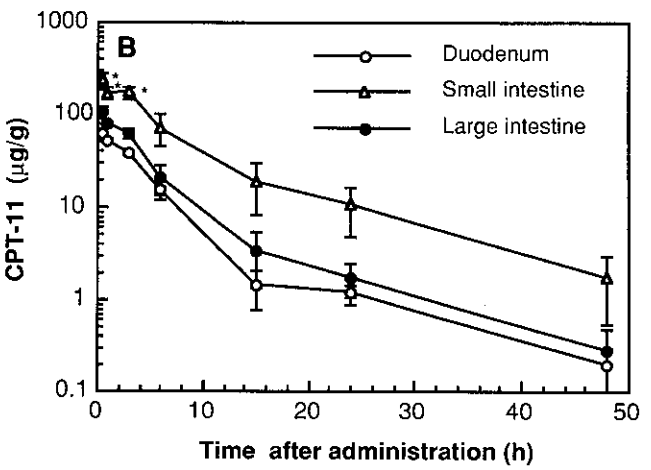


Fig. 3. Serial determination of the concentrations of CPT-11 in the intestine after a single injection (A) and five consecutive daily injections (B) of CPT-11 at a dose of 50 mg/kg. All values are means±SE. * $P<0.05$ and ** $P<0.01$ for the difference in concentration between the small intestine and the large intestine or duodenum.

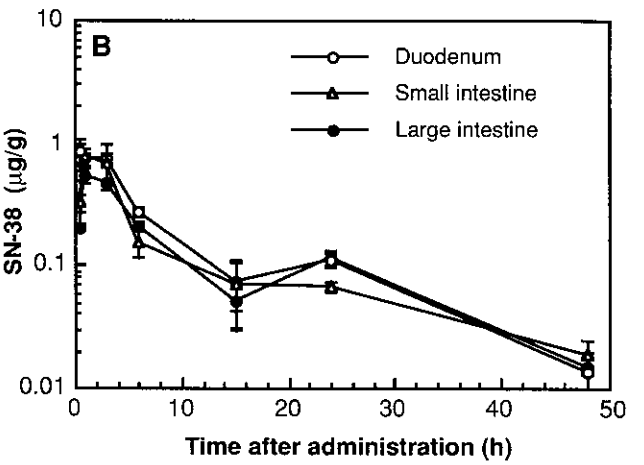


Fig. 4. Serial determination of the concentrations of SN-38 in the intestine after a single injection (A) and five consecutive daily injections (B) of CPT-11 at a dose of 50 mg/kg. All values are means±SE.

though a daily dose of below 25 mg/kg of CPT-11 failed to produce diarrhea.

Recently, Ohe and coworkers,[9] in a phase I study with a 5-day continuous infusion of CPT-11, showed that a daily dose of 40 mg/m^2 of CPT-11 produced grade 3 or 4 diarrhea in four of six patients. Compared with clinical doses known to produce diarrhea, the dose of 50 mg/kg of CPT-11 used to produce diarrhea in nude mice is high, showing low sensitivity of the intestine to mucosal injury. In cancer patients, severe diarrhea associated with CPT-11 administration is known to be watery and only a few patients produce bloody diarrhea or melena. In nude mice, however, watery diarrhea with moderate bleeding was observed. CPT-11 and SN-38 showed much greater accumulation in the duodenum, the small intestine and the large intestine after five consecutive daily injections of CPT-11 than after single treatment. Blood concentrations of SN-38 may be the sum of SN-38 formed from CPT-11 and the efflux of SN-38 from the tissues. Levels of CPT-11 and SN-38 in blood plasma gradually decreased but maintained low values after five consecutive daily injections, showing the same trend as intestinal tissues. Accumulation of CPT-11 and SN-38 at 48 h after five consecutive daily injections was higher in the intestinal wall than in blood plasma, and the levels of CPT-11 and SN-38 in the wall of the large intestine amounted to seven and four times those in the blood plasma per unit, respectively.

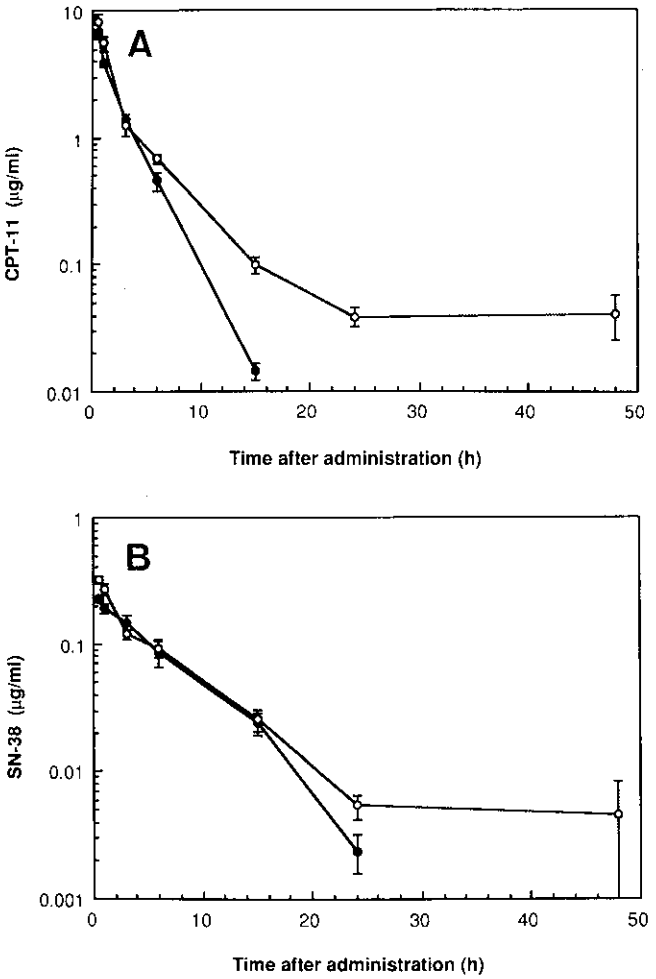


Fig. 5. Serial determination of the concentrations of CPT-11 (A) and SN-38 (B) in the blood plasma after a single (●) and five consecutive daily injections (○) of CPT-11 at a dose of 50 mg/kg. All values are means±SE.

Higher concentrations of CPT-11 were accumulated in the wall of the small intestine compared with those of the duodenum and the large intestine after a single injection or five consecutive daily injections of CPT-11, while the most severe injuries were found in the large intestine. The results show that the mechanisms underlying mucosal injury may differ essentially in the duodenum, small intestine and large intestine. The cytocidal effect of CPT-11 has been reported to be due to SN-38, a product formed by carboxylesterase. Enzymes converting CPT-11 to SN-38 *in vivo* have been found in the blood serum and the liver of mice.[10]

The causal role of CPT-11 and SN-38 accumulated in the intestinal wall in producing diarrhea is uncertain from our present study. In a study of the antiproliferative effect of CPT-11 on murine leukemia P388, Kawato and coworkers showed that the major intracellular activity of CPT-11 is due to SN-38, while weak activity of CPT-11 itself has been demonstrated.[11] Ohe and coworkers[9] showed that the frequency of diarrhea correlated with the AUC of CPT-11 and not with that of SN-38. The extrapolation of their results to nude mice is not straightforward, since drug metabolism in the intestinal mucosa differs from species to species.

Histologic study after administration of a high dose of CPT-11 revealed multiple erosion and inflammation in the colon on day 7 and later. We speculate that the diarrhea that developed under the present administration schedule is mediated by hemorrhagic enterocolitis. Destruction of the intestinal mucosa owing to the accumulation of SN-38 and/or CPT-11 may have resulted in discharge of mucus and blood into the intestinal lumen and severe impairment of absorption of water and electrolytes. A noteworthy feature in the histologic study was that regeneration of the epithelium was observed on day 14, when the high dose of CPT-11 was still being administered.

Individual differences in the occurrence of diarrhea are often observed after clinical administration of CPT-11, but all the mice in this study developed diarrhea after eight consecutive daily injections of CPT-11. Mechanisms underlying individual differences in the occurrence of diarrhea in humans remain to be studied.

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