Stability of UC-781, in Intestinal Mucosal Homogenates of the Rat, Rabbit, and Pig

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INTRODUCTION

Among the several classes of HIV inhibitors which have been developed, a number of molecules, commonly referred to as non-nucleoside reverse transcriptase inhibitors (NNRTIs), inhibit the HIV RT by a non-competitive interaction with an allosteric site of the enzyme (1). The thiocarboxanilides rank among the most potent NNRTIs (2–6). Several members of this group are able to inhibit HIV-1 replication *in vitro* in the lower nM concentration range. They display a favorable resistance spectrum, and proved markedly inhibitory to the Ile-100, Asn-103, Ala-106, Lys-138 and Cys-181 mutant virus strains (4). Two candidate compounds have been selected for potential future clinical trials, the oxime ether thiocarboxanilide UC-10 (2), and the pentenyloxy ether thiocarboxanilide UC-781 (4) (Fig. 1).

It is generally recognized that factors limiting the oral bioavailability are i) low aqueous solubility and/or low dissolution rate, ii) low membrane permeability, iii) binding to compounds in the gastro-intestinal (GI) tract, and iv) metabolism in the liver, the GI lumen, or in the GI mucosa (cytosol or membrane-related). A potential obstacle in the development of an oral dosage form for UC-781 is therefore its extremely low water solubility and dissolution rate, and its susceptibility to intestinal mucosal degradation (7). The objective of this study was to investigate the site-dependent metabolic degradation of UC-781 in mucosal homogenates of the GI tract of commonly used laboratory animals (rat, rabbit and pig), in order to determine the site for optimal oral drug delivery.

MATERIALS AND METHODS

Materials

UC-781 was supplied by Uniroyal Chemical Ltd. (Guelph, Ontario, Canada). NADPH, Folin's reagent, glucose, bovine serum albumine, and sodium potassium tartrate were from Sigma (Bornem, Belgium); Hanks' balanced salt solution (HBSS) and N-(2-hydroxyethyl)piperazine-N-ethanesulfonic acid (Hepes) were from Gibco BRL (N.V. Life Technologies, Paisley, Scotland); Cupper(II)sulfate was from Baker (Deventer, Holland); sodium carbonate was from Acros (Geel, Belgium); Hydroxypropyl-β-cyclodextrin (HPβCD) was from Fluka (Buchs, Swizerland). All other reagents and organic solvents were of analytical or HPLC grade. The water used for HPLC was purified with an Elgastat ion-exchange system (Elga Ltd., Bucks, UK).

Analysis of UC-781

UC-781 was analyzed using an HPLC system equipped with a L-7000 Lachrom pump, a L-7400 Lachrom UV detector set at 297 nm, and a D-7000 interface (all from Merck-Hitachi, Darmstadt, Germany), and a model 7125 Syringe loading sample injector (Rheodyne Inc., Cotati, US). UV signals were monitored and peaks were integrated using the D-7000 HSM software. The column used was a LiChrospher 60 RP Select B (5μm) (Merck, Darmstadt, Germany), the flow rate was 1 ml/min and the volume injected was 20 μl. The mobile phase consisted of acetonitrile 60% and phosphate buffer 40% (pH 4; 0.05 M).

The absorbance was linear in a concentration range between 0.25 and 5 μM ($R^2 > 0.999$). Concentrations of UC-781 were calculated using calibration curves made up from standards of known concentrations. Intra-day and inter-day variability expressed as relative standard deviation was less than 6%.

Degradation of UC-781 in Intestinal Homogenates of the Rat, Rabbit, and Pig

Duodenum and jejunum (combined segments), ileum and colon of the rat (male wistar), rabbit (New Zealand white), and pig (domestic) were rapidly excised after sacrificing the animals. Segments of each part were cut along the longitudinal axis, and washed with ice-cold HBSS to remove the intestinal contents. The intestinal mucosa of the various segments was removed by scraping with a glass microscope slide. The scrapings were homogenized using a Potter-Elvehjem glass homogeniser at 4°C in 5 ml of cold HBSS containing glucose (25 mM) and hepes (10 mM). After centrifugation of the crude intestinal tissue homogenate at 14,000*g* for 5 min, the supernatants were harvested and kept at 4°C. Protein concentration of all homogenates was determined according to the method of Lowry et al. (8) using bovine serum albumin as a standard. Prior to their use in the degradation studies, protein content of all preparations was adjusted to 1 or 2 mg/ml. NADPH was added to the homogenates to a final concentration of 1 or 2 mM. Degradation of UC-781 in the homogenates was determined by adding a prewarmed (37°C) stock solution of the drug made up in HPβCD solution (used to increase the drug solubility) to the crude extract at 37°C. The final concentration of UC-781 and HPβCD in the homogenate was 4 μM and 2% (w/w), respectively. Samples (200 μl) were taken at predetermined time points, added to 200 μl of ice-cold methanol and vortexed to immediately arrest enzymatic activity. The experiment was stopped after 180 min or when the drug concentration was

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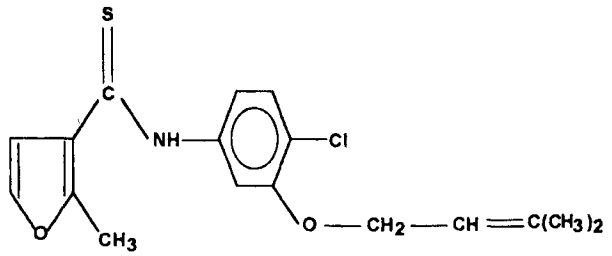

Fig. 1. Chemical structure of UC-781.

below the detection limit. The samples were centrifuged for 5 min at 14,000*g* and the supernatant was analyzed by HPLC to determine the UC-781 concentration.

Preliminary experiments had shown that UC-781 was stable in aqueous solutions containing 2 mM of NADPH and 2%(w/w) of HPβCD at 37°C for at least 24 hours.

RESULTS AND DISCUSSION

In a recent publication, Balimane and co-workers (7) reported the metabolism of UC-781 in rat jejunal homogenates. They concluded that the low oral bioavailability is due to its low aqueous solubility and presystemic metabolism, despite adequate intestinal permeability. It has been described that the activity of various enzymes (such as cytochrome P450) shows a gradual decrease from the duodenum to the colon (9). Therefore, we investigated the stability of UC-781 in homogenates of different segments of the GI tract of commonly used laboratory animals, in order to explore the site for optimal oral drug delivery.

Figure 2 shows the influence of NADPH on the stability of UC-781 in homogenates of jejunum and duodenum of the rabbit (total protein concentration is 2 mg/ml). In the absence of NADPH, the remaining drug concentration after as long as 3 h incubation was higher than 95%, while the addition of NADPH (1 mM) to the homogenates provoked a significant degradation: the remaining drug concentration decreased to 9.2% after 60 min incubation. When the concentration of NADPH was increased to 2 mM, the remaining drug concentration was 41% after 20 min. Since this value was statistically not significantly different (T-test; $p > 0.05$) from that with 1 mM of NADPH, all further experiments were performed with 1 mM of the cofactor. Similar observations were made in jejunum and duodenum homogenates from the pig and rat (data not shown), although a slightly higher breakdown decrease was observed in the rat homogenate of the combined jejunum and duodenum without addition of NADPH (the remaining concentration was 80% after 180 min). These results suggest that UC-781 may be a substrate for the enzymes of the GI cytochrome P450 system. Phase I metabolism by intestinal cytochrome P450 enzymes has long been considered to have a relatively minor impact on oral drug bioavailability because concentrations of individual cytochrome P450 enzymes averaged for the entire intestine are estimated to be approximately 20- to 200-fold lower than those found in the liver. However, this traditional view on intestinal metabolism has recently been criticized in the light of the observations that enzymes of the cytochrome P450 IIIA sub-family, which are considered to be the main phase I drug-metabolizing enzymes in humans and that account for approximately 70% of the cytochrome P450 content in human enterocytes, are expressed at high levels in the mature villus tip enterocytes of the small intestine (10). A study by de Waziers et al. (11) demonstrated that cytochrome P450 IIIA4, which is by far the most important of these enzymes, is present in all human tissues, except for the kidneys. Moreover, they showed that, after the liver, the small intestine contained the highest specific content of cytochrome P450 IIIA4; the amount in the ileum was approximately 50% of that of the jejunum and approximately one third of that of the duodenum. Low enzyme concentrations were also found in the colon.

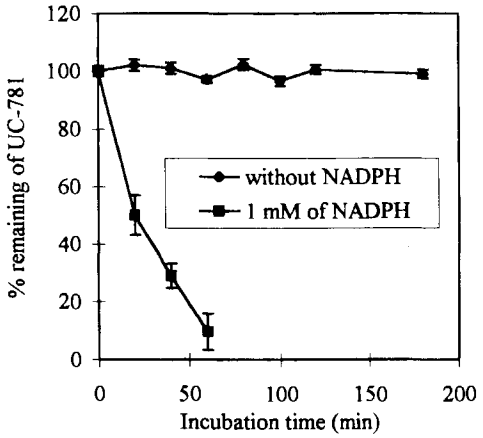

Fig. 2. Influence of NADPH concentration on the stability of UC-781 in the homogenate of the jejunum and duodenum (combined segments) of the rabbit. The total protein concentration was 2 mg/ml. Error bars indicate standard deviations; n = 3.

Figure 3, panels A, B, and C show the concentration-time profiles of UC-781 during incubation in intestinal homogenates of the rat, rabbit, and pig, respectively (the concentration of NADPH was 1 mM and the total protein concentration was 2 mg/ml). The results show that the enzymatic stability of the drug is site-dependent, and increases from the duodenum/jejunum to the ileum and colon. In all animals at all time points, the difference between the remaining drug concentration in the homogenates of the duodenum and jejunum was significantly lower than in those of the ileum or colon (ANOVA, $p < 0.05$). At the end of the experiment (180 min), the remaining concentration of UC-781 in homogenates from the pig intestine amounted to 46, 102, and 100% of the initial values in the combined segments of the duodenum and jejunum, the ileum and the colon, respectively. In homogenates of combined segments of duodenum and jejunum from the rabbit, the experiment was stopped after 60 min, since at this time the drug concentration had dropped to less than 10% of the initial value. In homogenates of the ileum and colon, the remaining concentration after 180 min was 86 and 98%, respectively. Incubation in combined jejunal and duodenal homogenate of the rat led to a decrease of more than 70% after 40 min, while the remaining drug concentration was 84 and 100% of the initial value after 180 min in the ileal and colonic homogenates. The results obtained, further demonstrate that the metabolism in the homogenates is dependent on the animal species used. The rate and extent of UC-781 degradation in the combined jejunal and duodenal homogenates of the rat and rabbit were significantly higher than in those of the pig (ANOVA, $p < 0.05$).

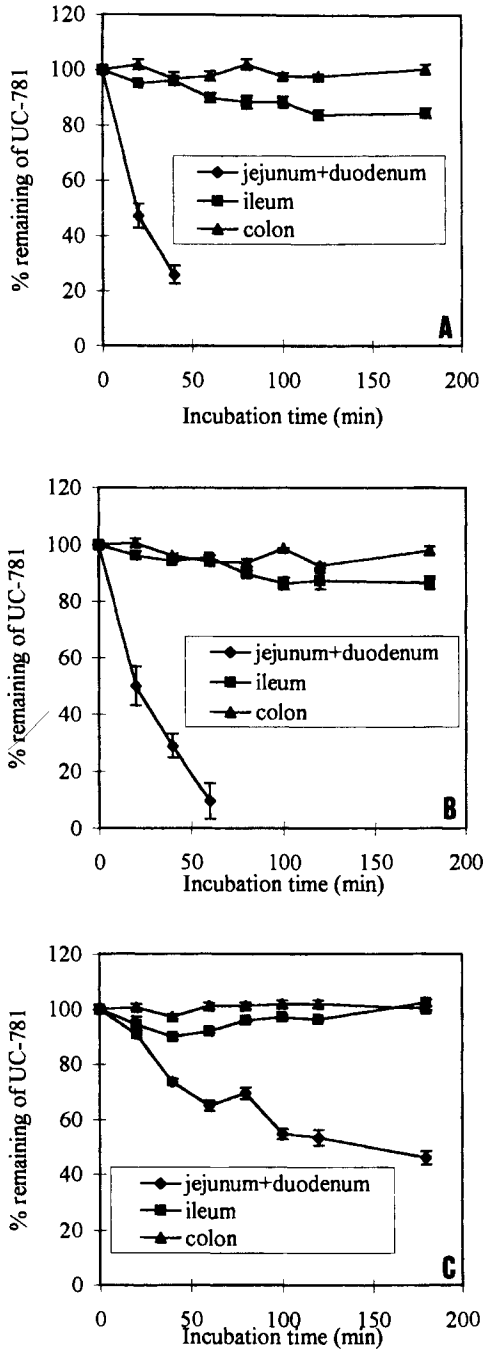

Fig. 3. Concentration (expressed as % of initial value) of UC-781 in homogenates of duodenum and jejunum (combined segments), ileum, and colon of the rat (A), rabbit (B), the pig (C). The concentration of NADPH in the homogenates was 1 mM and the total protein content was 2 mg/ml. Error bars indicate standard deviations; n = 3.

HPLC analysis revealed the formation of a metabolite of UC-781 during incubation in intestinal homogenates of the duodenum and jejunum from the three species. The retention time of this metabolite was 2.66 min, while UC-781 eluted after 5.62 min. Although the identity of this metabolite is still unknown, the HPLC data indicate that UC-781 is converted to a polar metabolite, and the similarity in the retention times suggests the likeliness that the metabolite is the same in rats, rabbits and pigs.

At this moment, no data are available on the metabolism of UC-781 in the human GI tract, but the results obtained in this study suggest that drug delivery to the proximal part of the GI tract is likely to compromise its oral bioavailability.

CONCLUSIONS

In this study we demonstrated the site-dependent metabolic degradation of UC-781 in intestinal homogenates of the rat, rabbit, and pig. In the combined segments of the duodenum and jejunum, degradation to a polar metabolite was observed, while in the ileum and the colon, the drug was relatively stable. The metabolism of the drug was more pronounced in the rat and rabbit GI homogenates than in those of the pig. The increase in metabolism in the presence of NADPH suggests that UC-781 is metabolized by cytochrome P450 enzymes. The information obtained in this study suggests the usefulness to develop a delivery system to release UC-781 in the ileum or colon, in order to avoid significant presystemic metabolism.

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