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RESEARCH ARTICLE

Synthesis and evaluation of sulfate conjugated metronidazole as a colon-specific prodrug of metronidazole

Hyunjeong Kim[1], Yonghyun Lee[1], Hansun Yoo[1], Jihye Kim[1], Hyesik Kong[2], Jeong-Hyun Yoon[1], Yunjin Jung[1], and Young Mi Kim[1]

[1]*Laboratory of Biomedicinal/Medicinal Chemistry, College of Pharmacy, Pusan National University, Busan, Korea and* [2]*Urologic Oncology Branch, National Cancer Institute, Bethesda, MD, USA*

Abstract

For an effort to improve therapeutic property of metronidazole (MTZ) which is a drug of choice for protozoal infections such as luminal amoebiasis, sulfate conjugated metronidazole (MTZS) was prepared and evaluated as a colon-specific prodrug of MTZ. The apparent partition coefficient of MTZ was greatly reduced by the sulfate conjugation. While (bio) chemically stable in the contents of the upper intestine, MTZS was rapidly cleaved to liberate MTZ on incubation with the cecal contents of rats. MTZ liberated from MTZS metabolized quickly at least partly by a microbial nitroreductase, suggesting the relevance of the metabolism to bioactivation of MTZ for antimicrobial action. Consistent with the hypothesis, MTZS elicited antibacterial activity in the cecal contents, which was as potent as free MTZ. The systemic absorption of MTZS was very low after oral administration of MTZS. In parallel with this, whereas MTZ disappeared mostly during the transit of the proximal small intestine, a substantial amount of MTZS remained in the small intestine, moving down to the large intestine where it metabolized rapidly. In addition to the efficient colonic delivery of MTZS, MTZS markedly reduced the systemic absorption of MTZ. Taken together, MTZS may be a potential colon-specific prodrug of MTZ which possesses improved therapeutic and toxicological properties.

Keywords: Controlled drug delivery, drug delivery, drug targeting, oral drug delivery

Introduction

Intestinal amebiasis, an infectious disease caused by *Entamoeba histolytica*, a single-celled protozoan parasite, is one of major public health problems in developing countries. The trophozoite form of *E. histolytica* invades the colonic epithelium and causes consequent amebic colitis or amebic abscesses in the brain, which are dreadful complications of *E. histolytica* infection(Stanley, 2003). World Health Organization (WHO), in its recent estimates, has placed the death toll from amebiasis at 40,000–100,000 lives annually (Petri, 2003).

Metronidazole (MTZ), an antibiotic of which chemical structure is classified as nitro 5-imidazoles, is a drug of choice for the treatment of intestinal amebiasis and other anaerobic protozoal and bacterial infections (Freeman et al., 1997). In addition, MTZ is being used for the treatment of inflammatory bowel disease (Egan and Sandborn, 1998). Pharmacologically, MTZ enters microbial cells and goes through the reduction of the nitro group of it by low redox potential electron transport proteins. A product generated by this process forms a covalent adduct with guanine or cytosine in the DNA strand and/or functionally essential proteins in the microbes, resulting in antimicrobial effects (Ludlum et al., 1988; Leitsch et al., 2007).

The pharmacokinetic profile of MTZ indicates that greater than 90% of the dose is rapidly absorbed after oral administration with conventional tablet formulation (Lau et al., 1992; Lamp et al., 1999). As a result, the amount of MTZ that reach the large intestine where trophozoites reside is minimal. Although small amount of MTZ reached the target site can still promote the

Address for Correspondence: Professor Young Mi Kim, College of Pharmacy, Pusan National University, Busan, Korea 609-735. Tel: 051-510-2807. Fax: 051-513-6754. E-mail: ymikim@pusan.ac.kr Dr. Yunjin Jung, College of Pharmacy, Pusan National University, Busan, Korea 609-735. Tel: 051-510-2527. Fax: 051-513-6754. E-mail: jungy@pusan.ac.kr

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elimination of amebiasis, it is not without unwanted systemic adverse effects such as nausea, vomiting, headache, glossitis, and in few cases convulsions (Kapoor et al., 1999). For these reasons, it is an attractable therapeutic strategy to deliver MTZ specifically to the infected target site, maximizing therapeutic efficacy and minimizing adverse effects attributed to systemically absorbed drug. Therefore, researches on colonic delivery of MTZ with pharmaceutical and prodrug approaches have been widely carried out (Krishnaiah et al., 2001; Krishnaiah et al., 2002; Mundargi et al., 2007; Kim et al., 2009).

In designing colon-specific prodrug, the drug molecule is coupled with a promoiety such as non-absorbable polymers or hydrophilic small molecules by a linkage which is stable in the upper intestine and labile in the colon. As a result, absorption of drug-promoiety compound in the upper intestine is very limited and a substantial amount of it is delivered to the colon, where an active drug is released by enzymes produced by intestinal microorganisms (Sinha and Kumria, 2001; Jung and Kim, 2010).

Van Eldere et al. reported on the isolation and identification of intestinal steroid-desulfating bacteria from rats and humans (Van Eldere et al., 1988). Huijghebaert et al. reported that microbial estrone sulfatase activity in the intestinal contents of rats was high in the cecum, but very low in the small intestine (Huijghebaert et al., 1984). These reports suggested that sulfatase in the gastrointestinal tract is of microbial origin and its activity is high in the cecum, but very low in the small intestine. Therefore, it is very likely that sulfate moiety satisfies the aforementioned requirement for a colon-specific promoiety. In fact, we previously reported that sulfated steroids, prednisolone and dexamethasone 21-sulfate sodium, act as a colon-specific prodrug of the steroids *in vivo* and *in vitro* (Jung et al., 2003; Kim et al., 2006). In this study, sulfate moiety was introduced as a colon-specific promoiety for colonic delivery of MTZ, which should provide another chemical option for colonic delivery of the anti-amebic agent using a prodrug approach. Recently, a colon-specific prodrug of MTZ has been reported, where *N*-nicotinoyl-(2-acetoxy)-*D,L*-glycine is adopted as a colon-specific promoiety (Kim et al., 2009). Sulfate conjugated MTZ (MTZS) was prepared in high yield, which is an advantage for industrialization. The antimicrobial activity in the cecal contents and biodisposition of MTZS were investigated to evaluate the conjugate as a colon-specific prodrug of MTZ with improved therapeutic and toxicological properties.

Material and methods

Materials

MTZ and sulfatrioxide triethylamine complex (STT) were purchased from Sigma Chemical Co. (St. Louis, MO) and were used as received. Solvents for NMR and HPLC were obtained from Merck Inc. (Darmstadt, Germany). All other chemicals were reagent grade, commercially available products. IR spectra were recorded on a Bomem MB 100 FT-IR spectrophotometer (Bomem, Ontario, Canada). ^{1}H-NMR spectra were taken on a Varian AS 500 spectrometer (Santa Clara, CA) and the chemical shifts were in ppm downfield from tetramethylsilane. Elemental analysis was carried out by an Elemental Analyzer System (Profile HV-3, Manchester, UK). A Polytron PT 3100 homogenizer (Kinematica, Bohemia, NY) was used for homogenization of the tissue of the gastrointestinal tracts of rats. A Taitec microincubator M-36 (Tokyo, Japan) was used for incubation. The animal protocol used in this study has been reviewed and approved by the Pusan National University–Institutional Animal Care and Use Committee (PNU-IACUC) on their ethical procedures and scientific care. (Approval number: PNU-2008-0010).

Preparation of MTZS

To the solution of MTZ (1.71 g, 10.0 mmol) in 64 mL of anhydrous benzene and pyridine (1/1), STT (3.80 *g*, 17.0 mmol) was added in portions with stirring at 56–60°C for 20 min. The reaction mixture was evaporated under reduced pressure to remove the solvent and obtained metronidazole sulfate triethylammonium (MTZST) as oily residue. After purification by silica gel open column chromatography, MTZST was dissolved in minimum amount of water and suspended into a solution of 10% NaCl with mechanical stirring for 1 h. The resulting precipitates, MTZS, were collected by suction filtration and recrystallized from absolute ethanol (yield 80%). mp: 194–195 (dec). IR (nujol) cm^{-1}: 1613, 1545, 1279, 1055, 830. ^{1}H-NMR (D_2O): 2.7 (s, 3H), 4.4 (t, 2H), 4.8 (t, 2H), 8.4 (s, 1H). EA for $C_6H_8N_3SO_6Na$: C, H, N, S.

HPLC analysis of drugs

The HPLC system consisted of Model 305 and 306 pumps, a 117 variable UV detector, a Model 234 autoinjector, a Model 805 manometric module, and a Model 811C dynamic mixer from Gilson (Middleton, WI). A symmetry C18 column (Waters, Seoul, Korea; 4.6 × 250 mm, 5 μm) with a guard column (Waters; 3.9 × 20 mm) was used. The mobile phase consisted of 0.05 M, pH 4.5 phosphate buffer/acetonitrile (9/1) and was filtered through a 0.45 μm membrane filter before use. The mobile phase was eluted at a rate of 1 mL/min. The eluate was monitored by measuring the absorbance at 313 nm at a sensitivity of AUFS 0.01. Gilson 712 software was employed for the data analysis. The retention time of MTZ and MTZS was 8.7 and 6.3 min, respectively. Concentration of MTZ and MTZS in the sample was calculated from the calibration curve. The calibration curve was constructed at the concentration range of 1–50 μg/mL. All the calibration curves were constructed by preparing the standard samples using biological media corresponding to each *in vitro*/*in vivo* experiment (the correlation coefficients of the calibration curves were between 0.999–0.996). In the case of cecal contents, the autoclaved cecal contents

were used. The detection limit was about 0.5 and 0.8 μg/mL for MTZ and MTZS, respectively, in our experimental condition.

Apparent partition coefficient, solubility and chemical stability

To measure solubility, MTZ or MTZS (50 mg equivalent of MTZ) was placed in a microtube containing 1 mL of pH 6.8 isotonic phosphate buffer solution. It was shaken for 24 h at 25°C. After centrifugation, a 20 μL portion of the supernatant was analyzed by HPLC. For measurement of apparent partition coefficients, to a 20 mL portion of MTZ (1.0 mM) or MTZS solution (1.0 mM) in 0.1 M, pH 7.4 phosphate buffer solution saturated with 1-octanol, was added 20 mL of 1-octanol saturated with 0.1 M, pH 7.4 phosphate buffer solution. The mixture was shaken for 24 h at 37°C. Concentration of MTZS or MTZ in the aqueous phase was analyzed by HPLC. The apparent partition coefficient was calculated by using the equation (Co–Cw) % Cw where *C*o and *C*w represent the initial and equilibrium concentration, respectively, of the drug in aqueous phase. The pH stability was determined by incubating the solution of MTZS (1.0 mM) in pH 1.2 hydrochloric acid buffer, pH 4.5 acetate buffer and pH 7.4 phosphate buffer at 37°C for 24 h. At a predetermined time interval, a 20 μL portion of the solution was removed and analyzed the concentration of MTZ and MTZS by HPLC.

Incubation of drugs with the contents of rat small intestine

A male Sprague–Dawley rat (210–275 g, 7–8 weeks old) was anesthetized by diethyl ether and a midline incision was made. The contents of the small intestine was collected and diluted to half concentration with 0.05 M, pH 6.8 isotonic phosphate buffer. A 2 mL of the contents suspension was mixed with 2 mL of MTZ or MTZS solution (200 ppm equivalent of MTZ) dissolved in pH 6.8 isotonic phosphate buffer. The mixture was incubated at 37°C in a shaking incubator. At an appropriate time interval, 200 μL portion of the sample was taken and centrifuged at 14,000 rpm for 3 min. To a 100 μL portion of the supernatant, was added 900 μL of methanol to precipitate protein in the sample, vortexed for 2 min, and centrifuged for 5 min at 14,000 rpm. The concentration of MTZ and MTZS in a 20 μL portion of the supernatant was determined by HPLC.

Incubation of drugs with the cecal contents of rat

The cecal segment of the intestine was cut open and the contents were collected. The cecal contents were diluted with 0.05 M, pH 6.8 phosphate buffer solution to the concentration of 20% (w/v) and filtered through a piece of medical gauze to remove the fibrous mass. A 2 mL portion of MTZ or MTZS solution (at an appropriate concentration) in 0.05 M, pH 6.8 isotonic phosphate buffer was mixed with 2 mL of the cecal contents, which was incubated at 37°C in a shaking incubator. In separate experiments, MTZ was incubated with the cecal contents obtained from rats with TNBS-induced colitis or pretreated with 0.2 mM of dicoumarol. The whole process was carried out in a glove box, which was previously displaced by nitrogen. At an appropriate time interval, 200 μL portion of the sample was removed and centrifuged at 14,000 rpm for 3 min. To a 100 μL portion of the supernatant, was added 900 μL of methanol to precipitate protein in the sample, vortexed for 2 min, and centrifuged for 5 min at 14,000 rpm. The concentration of MTZ and/or MTZS in a 20 μL portion of the supernatant was determined by HPLC.

Antimicrobial activity of MTZS in the cecal contents

The cecal contents were diluted with Lysogeny broth (LB) broth to afford the 20% (w/v) suspension. A 7.5 mL portion of MTZ or MTZS solution (equivalent to 500 ppm MTZ) in LB broth was mixed with 7.5 mL of the cecal contents immediately followed by immersion of a dialysis tube (Slide-A-Lyzer® MINI Dialysis Unit, Pierce, Rockford, IL) containing *E. Coli* (XL1-Blue, Stratagene, Santa Clara, CA; 200 μL, O.D.: 0.18–0.25) in each cecal suspension, which was incubated at 37°C in a shaking incubator. All the processes were performed in a nitrogen glove bag. For comparison, the same experiment was done without the cecal contents. The antimicrobial activity of MTZ and its prodrug was determined by measurement of O.D. of the *E.Coli* 8 h after incubation.

Incubation of MTZ and MTZS with the liver homogenates and the plasma

To determine the metabolic stability of MTZ and MTZS in liver, the rat liver was homogenized and diluted with saline to 20% (w/v). A 2 mL portion of MTZ or MTZS (200 ppm equivalent of MTZ) in pH 6.8 phosphate was mixed with 2 mL of the 20% liver homogenate. The mixture was incubated at 37°C in a shaking incubator. To determine the metabolic stability in plasma, a 200 μL portion of MTZ or MTZS (200 ppm equivalent of MTZ) in pH 7.4 phosphate buffer and an 800 μL portion of the rat plasma were mixed and incubated at 37°C in a shaking incubator. At an appropriate time interval, a 200 μL portion of the liver homogenates sample was removed and centrifuged at 14,000 rpm for 5 min. To a 100 μL portion of the supernatant or the plasma sample, was added a 900 μL portion of methanol to precipitate protein in the samples. It was vortexed for 2 min, and centrifuged for 5 min at 14,000 rpm. The concentration of MTZ or MTZS in a 20 μL portion of the samples was determined by HPLC.

Analysis of MTZ and/or MTZS in the blood and various segments of rat gastrointestinal tract after oral administration of drugs

Male SD rats 250–260 g were maintained with free access to a stock diet and water. These rats were fasted overnight (16 h) before administration of MTZ or MTZS. Water bottles were removed from the cages at least 30 min before drug administration to assure that stomach would be empty. MTZ or MTZS (4 mg equivalent of MTZ/kg) in

isotonic saline solution was orally administered to rats. After an appropriate time interval, the animals were sacrificed by ether anesthesia. Blood was collected by intra-cardiac puncture through a heparinized syringe before death. After blood collection and the contents of various segments of gastrointestinal tract were obtained (Kim et al., 2009). Blood samples were immediately centrifuged for 5 min at 14,000 rpm and the concentration of MTZ and/or MTZS in the plasma samples was determined by HPLC. The contents of proximal small intestine (PSI), distal small intestine (DSI), cecum and colon were collected separately, and the concentration of MTZ and/or MTZS by HPLC was analyzed.

Recovery of MTZS and MTZ in feces and urine after oral administration of drugs

Male SD rats (250–260 *g*) were placed in a metabolic cage and starved for 24 h prior to use for the experiments but had free access to water. MTZ or MTZS (4 mg equivalent of MTZ) was orally administered. The fecal and urinary samples were collected separately at a 2-h interval and stored immediately in a freezing cabinet at −20°C until analysis. The fecal or urinary samples were 10-fold diluted with 0.05 M pH 6.8 isotonic phosphate buffer solution (Kim et al., 2009). The samples were vortexed and centrifuged at 5000 rpm for 3 min. To a 100 μL portion of the supernatant, was added 900 μL of methanol to precipitate protein in the samples, vortexed for 2 min, and centrifuged for 5 min at 14,000 rpm. The concentration of MTZ and/or MTZS in a 20 μL portion of the supernatant was analyzed by HPLC.

Statistical analysis

The results are expressed as the mean ± SE. One-way ANOVA followed by Tukey's (HSD) test was used for testing the difference between data. Differences with $p < 0.05$ were considered significant. The XLSTAT® Software (Addinsoft, Inc, Suite 503, NY) was used for the statistical analysis.

Results

Preparation of MTZS

MTZS was prepared by the synthetic method as shown in Figure 1. Sulfate was easily conjugated with the hydroxyl group in MTZ to afford MTZS in high yield. Structure of MTZS was identified from IR, ^{1}H-NMR spectra and elemental analysis. Data from the elemental analysis were in good agreement with the calculated values for C, H, N and S (within 0.5% from the calculated values). Infrared spectra showed two strong absorption band originating from -S=O asymmetric and symmetric stretching of sulfate ester around 1279 and 1055 cm^{-1}, respectively. The most characteristic change in ^{1}H-NMR spectra was the downfield shift of the two protons on α-carbon to 0.5 ppm by the introduction of sulfate ester group.

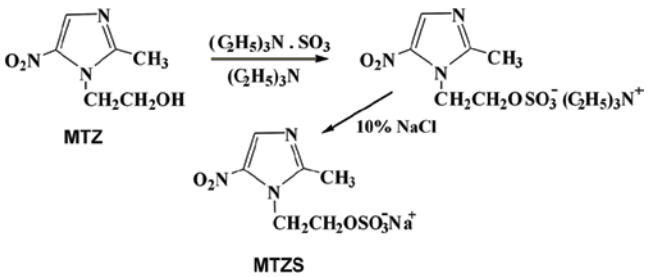

Figure 1. Synthesis of metronidazole sulfate. MTZS was prepared as described under "Material and Methods". MTZ, metronidazole; MTZS, sulfate conjugated metronidazole.

Apparent partition coefficient, solubility and chemical stability

The apparent partition coefficients in 1-octanol/water system for MTZ and MTZS were 0.95 and 0.15, respectively. While the solubility of MTZ was 10.5 mg/mL, MTZS was freely soluble. To examine the chemical stability of MTZS during the transit through the GI tract, MTZS was incubated in pH 1.2, 4.5 and pH 7.4 buffer solutions. Degradation of MTZS and generation of MTZ was negligible up to 10 h.

Incubation of MTZS in the contents of various segments of the rat GI tract

We examined whether MTZS could be delivered to the large intestine without significant loss in the upper intestine and liberate MTZ at the target site. MTZS (100 ppm equivalent of MTZ) was incubated with the contents of various segments of the rat GI tract monitoring the change of the MTZS level. As shown in Figure 2A, after 10 h incubation, the level of MTZS decreased to 82, 90 and 10% of the dose in the contents of PSI, DSI and cecum, respectively, indicating that, while relatively stable in the contents of the upper intestine, MTZS is degraded rapidly in the cecal contents. To examine the effect of the concentration of the cecal contents on the disappearance of MTZS, MTZS was incubated in the cecal contents at various concentrations. As shown in Figure 2B, MTZS disappeared during the incubation, which was accelerated as the concentration of the cecal contents increased. Although the sulfate conjugate was degraded extensively, MTZ was not detected in the cecal contents. We hypothesized that MTZ liberated from MTZS also metabolizes in the cecal contents, the rate of which is greater than that of the liberation of MTZ from MTZS, resulting in no accumulation of MTZ. To test this hypothesis, MTZ was incubated in the cecal contents at various concentrations and the rates of disappearance were compared with those of MTZS. As shown in Figure 2C, consistent with our hypothesis, MTZ disappeared much faster than did MTZS in the cecal contents. To see MTZ liberated from MTZS in the cecal contents, MTZS at elevated concentrations (250 and 500 ppm) was incubated in 5% cecal contents. While hardly detected on incubation of a 250 ppm concentration of MTZS (data not shown), MTZ was detected at a maximal level of 3.3 ppm on incubation of a 500 ppm concentration of MTZS (Figure 2D).

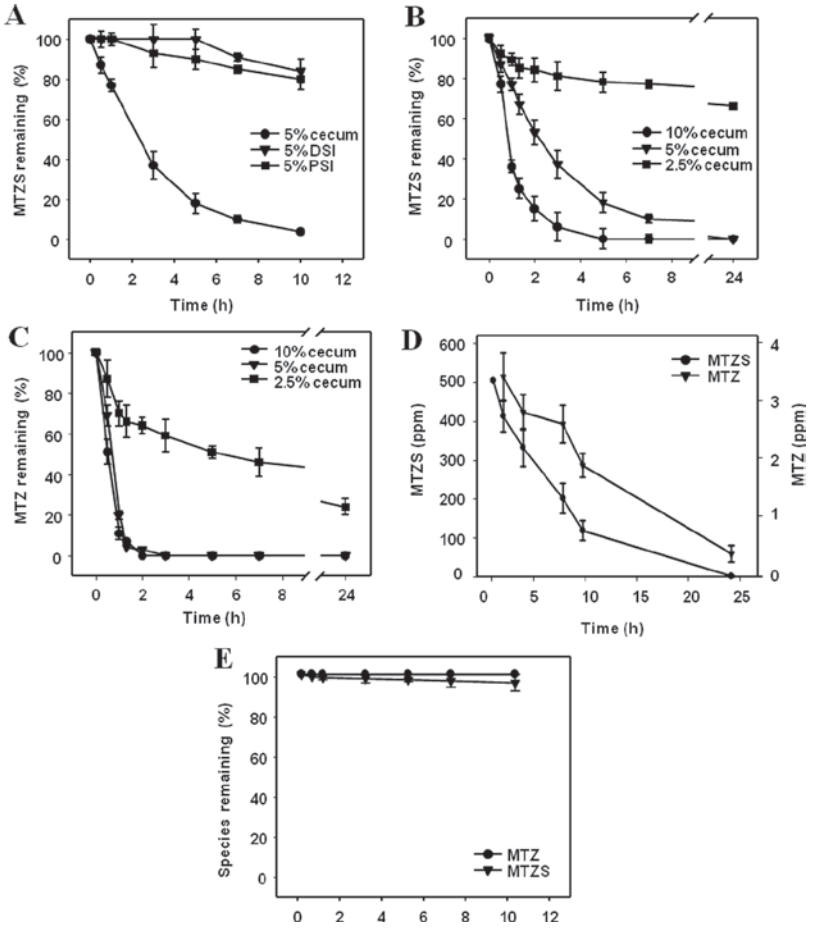

Figure 2. Stability of MTZS in the contents of various segments of the GI tract of rats. (A) Metronidazole sulfate (MTZS, 100 ppm equivalent of MTZ) was incubated with the 5% contents of various segments of the GI tract of rats. The concentration of MTZS was determined at appropriate time intervals. (B) MTZS (100 ppm equivalent of MTZ) was incubated with the cecal contents at various concentrations. The concentration MTZS was determined at appropriate time intervals. (C) Metronidazole (MTZ, 100 ppm) was incubated with varied concentrations of the cecal contents of rats. The concentration of MTZ was determined at appropriate time intervals. (D) Metronidazole sulfate (MTZS, 500 ppm) was incubated with 5% cecal contents of rats. The concentrations of MTZ and MTZS were determined at appropriate time intervals. (E) MTZ (100 ppm) or MTZS (100 ppm equivalent of MTZ) was incubated in 5% cecal contents which was autoclaved at 121°C for 20 min. The concentration of either MTZ or MTZS was determined at appropriate time intervals. The data in "A", "B", "C", "D" and "E" are mean ± SE ($n = 5$).

We examined whether the disappearance of MTZS and MTZ was dependent on microbial enzymes. The cecal contents was autoclaved to inactivate microbial enzymes (Kim et al., 2005) followed by addition of MTZS or MTZ and the change in their levels was monitored. No significant change was detected up to 10 h (Figure 2E). Since amoebic infection causes colitis, and MTZ is also used for the treatment of inflammatory bowel disease, we examined whether inflammation affected the metabolism of MTZS and MTZ. To do this, the metabolic change of MTZS and MTZ was monitored in the cecal contents at various concentrations obtained from normal rats or rats with TNBS-induced colitis. As shown in Figure 3A and 3B, no significant difference was observed.

Cecal metabolism of MTZ is relevant to the bioactivation for antimicrobial action

Since MTZ enters protozoans or bacteria and the nitro group is reduced to give an amoebicidal and bactericidal metabolite (Ludlum et al., 1988; Lamp et al., 1999), it was examined whether the disappearance of MTZ in the cecal contents was relevant to the metabolism to the active metabolite. We first tested whether MTZ metabolized by microbes in the cecal contents. To do this, the cecal contents were centrifuged followed by filtration of the supernatant using a filter membrane with 0.22-μm pore size and MTZ was incubated in the filtered supernatant. No change of the MTZ level was observed up to 10 h. To further examine the relevance of the disappearance of MTZ with the bioactivation of MTZ, MTZ was incubated with the cecal contents pretreated or left untreated with dicumarol, a nitroreductase inhibitor (Kinouchi and Ohnishi, 1983), and the change of MTZ level was monitored. As shown in Figure 4A, the reductase inhibitor delayed the disappearance of MTZ. To experimentally prove that the cecal metabolisms of MTZ and MTZS were associated with antimicrobial activity, MTZS or MTZ was treated in the cecal contents diluted with LB broth where a dialysis tube containing *E. Coli* was immersed and antimicrobial activity of the drugs was determined

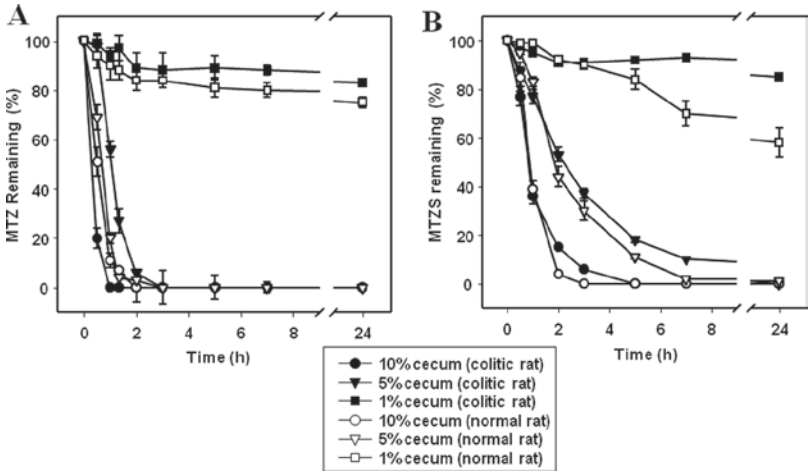

Figure 3. The effect of colonic inflammation on the cecal metabolism of MTZ and MTZS. (A) Metronidazole (MTZ, 100 ppm) was incubated with varied concentrations of the cecal contents obtained from normal or colitic rats. The concentration of MTZ was determined at appropriate time intervals. (B) The same experiment was done with MTZS (100 ppm equivalent of MTZ). The data in "A" and "B" are mean ± SE ($n = 5$).

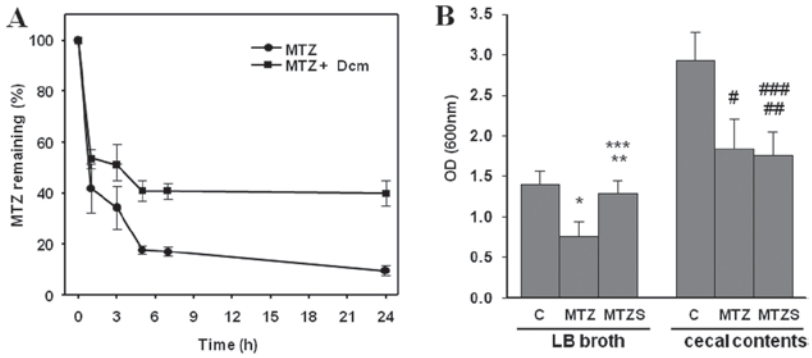

Figure 4. The cecal metabolism of MTZ is relevant to bioactivation of MTZ. (A) Metronidazole (MTZ, 200 ppm) was incubated with the 10% cecal contents of rats in the presence of dicuomarol (Dcm). The concentration of MTZ was determined at appropriate time intervals as described under. The data are mean ± SE ($n = 5$). (B) The cecal contents were diluted with LB broth to afford the 20% (w/v) suspension. 5 mL portion of MTZ or MTZS solution (equivalent to 500 ppm MTZ) in LB broth was mixed with 5 mL of the cecal contents immediately followed by immersion of a dialysis tube in each cecal suspension, which was incubated at 37°C in a shaking incubator in a nitrogen glove bag. For comparison, the same experiment was done without the cecal contents. The antimicrobial activity of MTZ and its prodrug was determined by measurement of O.D. of the *E.* Coli 8 h after incubation. Control (C): group untreated with either MTZS or MTZ (*$p < 0.001$ vs. control; **$p > 0.05$ vs. control; ***$p < 0.005$ vs. MTZ; #$p < 0.01$ vs. control; ##$p < 0.05$ vs. control; ###$p > 0.05$ vs. MTZ).

by measurement of O.D. of the *E. Coli*, which is one of methods to evaluate bacterial growth, 8 h after the incubation. For comparison, the same experiment was done without the cecal contents. As shown in Figure 4B, MTZS exhibited an antibacterial activity as potent as MTZ in the cecal contents whereas the antibacterial activity of MTZS was negligible compared to that of MTZ in the LB broth (without the cecal contents).This result strongly suggests that MTZS is microbially converted to MTZ, which is subsequently bioactivated in colonic microbes to exhibit antibacterial activity.

Systemic absorption of orally administered MTZS

Although the low apparent partition coefficient of MTZS suggests limited systemic absorption upon oral administration of the prodrug, we wanted to examine the notion *in vivo*. MTZS was administered orally and plasma concentration of MTZS was determined. For comparison, the same experiment was done with MTZ. As shown in Figure 5A, while oral administration of MTZ gave a significant amount of the drug in the blood (C_{max}: 7.8 μg/mL, T_{max}: 1.6 h), no MTZS in the plasma was detected throughout the whole experimental period after oral administration of MTZS. To further confirm the limited systemic absorption of MTZS, the concentration of MTZS in the urine collected for 24 h was determined after oral administration of MTZS. For comparison, the same experiment was done with MTZ. As shown in Figure 5B, the urinary recovery of MTZS was about 3.3% of the dose after oral administration of MTZS. After oral administration of MTZ, about 11.2% of the dose was excreted as an intact form and a small amount of metronidazole sulfate, one of the liver metabolites of MTZ (Loft and Poulsen, 1989), was observed in the urine. To exclude the possibility that the low urinary recovery of MTZS is due to metabolic changes of MTZS in the blood and/or the liver, MTZS was

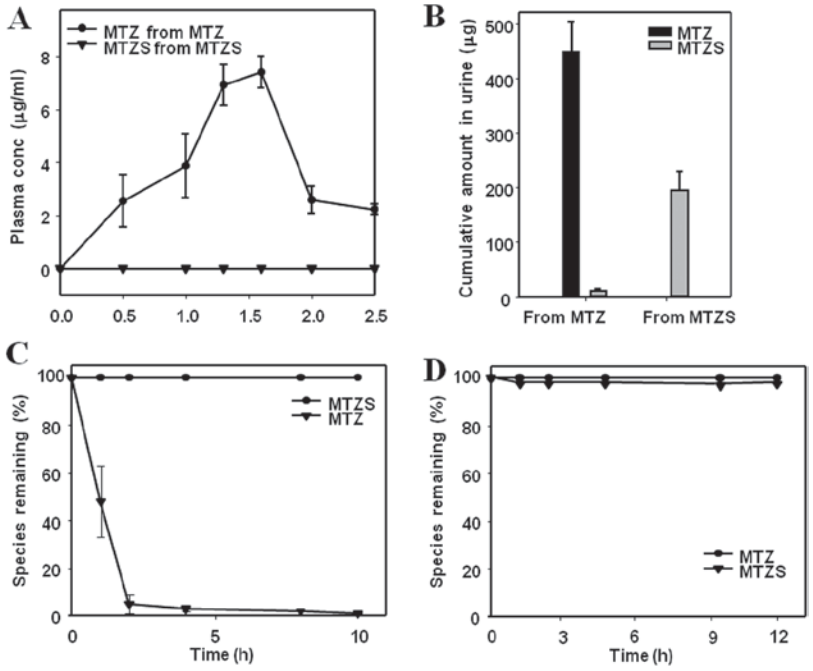

Figure 5. The systemic absorption of orally administered MTZS. (A) MTZS (4 mg equivalent of MTZ) was administered orally to rats (250–260 g) and the plasma concentration of MTZS was determined at appropriate time intervals. For comparison, the same experiment was done with MTZ (4 mg). (B) The same experiment was done with MTZS (4 mg equivalent of MTZ) as described in "A" except for monitoring MTZS level in the urine. (C) MTZS (100 ppm equivalent of MTZ) was incubated in the 10% liver homogenates of rats. The concentration of MTZS was determined at appropriate time intervals. The same experiment was done with MTZ (100 ppm). (D) MTZS (100 ppm equivalent of MTZ) was incubated in the 80% plasma of rats. The concentration of MTZS was determined at appropriate time intervals. The same experiment was done with MTZ (100 ppm). The data in "A", "B", "C" and "D" are mean ± SE ($n = 5$).

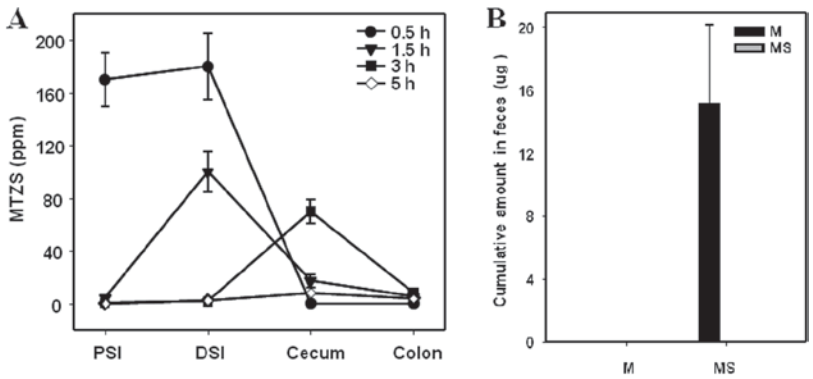

Figure 6. Distribution of MTZ and MTZS in the GI tract and fecal recovery after oral administration of MTZS. (A) MTZS (4 mg equivalent of MTZ) was administered orally to rats (250–260 g). The concentration of MTZS and MTZ was determined in the lumens of the proximal small intestine (PSI), distal small intestine (DSI), cecum and colon of rats at appropriate time intervals. For comparison, the same experiment was done with MTZ (4 mg). (B) MTZS (4 mg equivalent of MTZ) was administered orally to rats (250–260 g). The fecal samples were collected separately at a 2-h interval. The concentration of MTZS and MTZ was analyzed. For comparison, the same experiment was done with MTZ (4 mg). The data in "A" and "B" are mean ± SE ($n = 5$).

incubated with the 80% plasma and the 10% liver homogenates. For comparison, the same experiment was done with MTZ. While MTZ metabolized rapidly in the liver homogenates, the level of MTZS was not changed up to for 12 h (Figure 5C). The levels of MTZS and MTZ were not changed in the plasma (Figure 5D).

Colonic delivery and systemic absorption of MTZ following oral administration of MTZS

The results of *in vitro* incubation and the systemic absorption of MTZS suggest that a large fraction of orally administered MTZS can reach the large intestine followed by liberation of MTZ at the target site. To verify this *in vivo*, MTZS was administered orally to rats and MTZS and MTZ were monitored in PSI, DSI, cecum, colon and feces at various time intervals. For comparison, the same experiment was done with MTZ. While MTZ was not detected anywhere in the GI tract 1 h after oral administration of MTZ (data not shown), as shown in Figure 6A, MTZS, which passed the PSI, was accumulated up to about 0.7 mM (175 µg/mL) in the DSI and then moving down to the large intestine. While MTZS was detected very low in

the colon, the maximal concentration of MTZS reached about 0.28 mM (70 μg/mL) at the cecum 3 h after administration. Moreover, after oral administration of MTZS, MTZ was not detected at all in the upper intestine and hardly detected in the large intestine probably resulting from rapid colonic metabolism of MTZ liberated from MTZS (data not shown). On the other hand, as shown in Figure 6B, MTZS was not observed and a little amount of MTZ (15 μg) was recovered in the feces collected for 24 h after administration of MTZS. The fecal recovery was 0.4% of the dose. On the contrary, either MTZ or MTZS was not detected at all in the feces after administration of MTZ (Figure 6B). The systemic absorption of MTZ is related to its adverse effects. We examined whether the prodrug of metronidazole was able to reduce the systemic absorption of MTZ in addition to the efficient colonic delivery of MTZ. MTZS was administered orally to rats and the plasma concentration and urinary excretion of MTZ was monitored. MTZ was not detected in the plasma and urine throughout the whole experimental period (24 h) upon oral administration of MTZS (data not shown).

Discussion

In this study, we *in vitro/in vivo*-evaluated MTZS as a potential colon-specific prodrug of MTZ. MTZS was (bio)chemically stable and non-absorbable in the upper intestine resulting in the efficient colonic delivery of MTZ. Furthermore, MTZS was converted to MTZ in the large intestine followed by rapid disappearance of MTZ probably resulting from the metabolism to an active metabolite. In addition to the efficient colonic delivery of MTZ, MTZS reduced the systemic absorption of MTZ, which is related to its adverse effects.

In our previous papers, sulfate conjugated steroids were developed as a colon-specific prodrug of the steroids (Jung et al., 2003; Kim et al., 2006). We applied the delivery system to MTZ, a drug of choice for luminal amoebiasis. As expected, the hydroxyl group in MTZ was readily sulfated to afford MTZS. The delivery system conferred a greater hydrophilicity to MTZ as indicated by the lowered apparent partition coefficient, suggesting that systemic absorption of MTZS would be limited. This was verified by the *in vivo* data demonstrating that MTZS was not detected in the blood and was recovered at less than 5% of the dose in the urine after oral administration of MTZS. Since MTZS was stable in the plasma and the liver homogenate, it would be reasonable to assume that the MTZS in the urine mainly comes from MTZS absorbed systemically from the upper intestine. MTZS was likely to be undetectable in the blood because of low systemic absorption and rapid excretion of MTZS, an anionic sulfate prodrug.

Most of the prodrug, which is not absorbed in the upper intestine, seems to be delivered to the large intestine. This argument is supported by the *in vitro* and *in vivo* data showing that (i) MTZS is stable not only in pH 1.2 and pH 7.4 buffers but also in the contents of the upper intestine; (ii) MTZ was not at all detected in the upper intestine although a large amount of MTZS was monitored after oral administration of MTZS. It would be natural that the level of MTZS in the large intestine be lower than that of MTZS in the DSI, considering rapid metabolism of MTZS in the cecal contents.

Although MTZ was not detected in the large intestine after oral administration of MTZS, it is very likely that MTZS delivered to large intestine is converted to MTZ and subsequently exerts an anti-protozoal action. This speculation is rationalized by the data demonstrating that (i) MTZ also metabolized as quickly as did MTZS in the cecal contents, (ii) dicumarol, a nitroreductase inhibitor, delayed the disappearance of MTZ during incubation in the cecal contents and nitroreduction of MTZ is a microbial metabolism that generates an active metabolite to kill microbes (Ludlum et al., 1988; Lamp et al., 1999), (iii) MTZS elicited antibacterial activity in the cecal contents, which was as potent as MTZ. This argument was further supported by showing that MTZS at an elevated concentration (500 μg/mL) disappeared as MTZ appeared although MTZ was produced at much lower amount than MTZS disappeared. Our data showing that MTZ did not at all disappear when incubated in the microbe-free cecal contents and the autoclaved cecal contents imply that such a microbial metabolism occurred to MTZ in the cecum.

Since our data showed that induction of inflammation, which reportedly affects the metabolism of drugs in the large intestine (Marteau et al., 2003), made no big difference in the metabolisms of MTZ and MTZS, MTZS is likely to exhibit similar therapeutic efficiency regardless of inflammation in the large intestine. In addition to a greater delivery of MTZ to the target site, MTZS should reduce adverse effects by systemic absorption of MTZ. We demonstrated that MTZ was not detected in the blood and the urine after oral administration of MTZS while MTZ administered orally gave a significant amount of MTZ in the blood and the urine. Taken together, our data suggest that MTZS is a promising colon-specific prodrug of MTZ which could improve therapeutic and toxicological properties.

Jung et al. has suggested that to have various colon-specific promoieties coupling with a functional group is one strategy to overcome obstacles in developing a practical colon-specific prodrug such as inter and intra-individual variation in prodrug activation and the colonic metabolism of parent drugs (Jung and Kim, 2010). The conversion of the colon-specific prodrugs (with different promoieties) to the drugs is carried out by different activation systems (bacterial enzymes) in the large intestine, which may provide an option minimizing variation in the prodrug activation. In addition, controlling the colonic distribution of the active parent drug liberated from a colon specific prodrug is also important for treatment of colonic diseases in which a pathological lesion may be present at various regions. In case, a parent drug is very susceptible to the colonic metabolism (as MTZ), the parent drug converted from its colon-specific prodrug

should be metabolized rapidly during moving down the colon and could not reach a pathological lesion distant from the conversion site. For this reason, the colonic distribution of the drug would depend mainly on the conversion rate of its prodrug in the large intestine, which may vary according to promoieties (Jung and Kim, 2010), Therefore, along with *N*-nicotinoyl-(2-acetoxy)-*D,L*-glycine that is previously reported as a colon-specific promoiety of MTZ (Kim et al., 2009), sulfate ester promoiety should make a contribution to development of a clinically useful colon-specific prodrug of MTZ. Moreover, the synthetic process of the sulfation is simple and usually gives high yield compared with those of other delivery systems such as azo bond, glycoconjugation, dextran conjugation, *N*-nicotinoyl-(2-acetoxy)-*D,L*-glycine and amino acid conjugation (Friend and Chang, 1985; McLeod et al., 1994; Jung et al., 2000; Lee et al., 2001; Jain et al., 2006). This may be of great advantage for industrialization of a colon-specific prodrug.

Declaration of interest

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