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Research Paper

Significant Intestinal Excretion, One Source of Variability in Pharmacokinetics of COL-3, a Chemically Modified Tetracycline

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Purpose. This study was undertaken to examine the disposition of COL-3, a chemically modified tetracycline, in order to elucidate its major route of elimination as one possible source of the variability in pharmacokinetics of COL-3 *in vivo*.

Methods. The disposition profile of COL-3 *in vivo* was assessed by examining the urinary and fecal excretion of the unchanged drug and/or its metabolites in rats after single intravenous and oral administration. The biliary excretion of COL-3 administered orally in bile duct–cannulated rats was also examined. In addition, plasma protein binding and cytochromes P450–mediated metabolism were explored along with erythrocyte partitioning i*n vivo*. Furthermore, transport of COL-3 across Caco-2 monolayers was performed to elucidate the mechanism of intestinal excretion of COL-3 *in vivo*.

Results. COL-3 was extensively bound to plasma protein in rat (98%) and human plasma (95%). The affinity of rat blood cells for COL-3, as measured by the ratio of drug concentration in blood cells to that unbound in plasma, was about 36. Of the single intravenous and oral doses, less than 0.2% and 0.03% were excreted unchanged in rat urine, respectively; while 32.1 ± 9.9% and 38.8 ± 6.1% were recovered unchanged in rat feces, respectively, within 48 h postdosing. Of the oral dose, 1.36 ± 0.66% and 2.97 ± 0.88% were excreted in rat bile as the unchanged COL-3 and the total of COL-3 and its glucuronide conjugate, respectively, within 24 h after dosing. COL-3 had insignificant cytochrome P450–mediated metabolism but underwent phase II metabolism (i.e., glucuronidation) in a minor quantity. COL-3 was not a substrate of P-glycoprotein. Its transport across Caco-2 monolayers was significantly affected by protein binding and pH.

Conclusions. Intestinal excretion, a route different from biliary excretion, is the major route of elimination for COL-3 in rats. Variability in intestinal excretion, due to extreme variable intestinal contents (food and digestive fluids), could be one source of variability in COL-3 pharmacokinetics *in vivo* in addition to the dissolution rate–limited absorption.

KEY WORDS: Caco-2 cell; chemically modified tetracycline; COL-3; intestinal excretion; transport study; variability in pharmacokinetics.

INTRODUCTION

COL-3, 6-deoxy-6-demethyl-4-dedimethylamino-tetracycline, also known as CMT-3 (Metastat, CollaGenex Pharmaceuticals, Newton, PA, USA), is a non-antimicrobial chemically modified tetracycline (CMT). Since the first CMT was described in 1987 (1), more than 30 different CMTs, in which the 4-dimethylamino group is removed, have been developed. CMTs lose the antibacterial activity of tetracycline but retain or even enhance the inhibition activity of matrix metalloproteinases (MMPs). COL-3 potently inhibits MMPs (i.e., MMP-2, MMP-9, and MT1-MMP) (2–5), which are believed to be positive contributors in tumor growth and metastasis. COL-3 has undergone phase I clinical trials in patients with refractory metastatic cancers (6), AIDS-related Kaposi sarcoma (7), and other solid malignancies (8). It is currently being assessed in phase II clinical trials in patients with Kaposi sarcoma.

The modifications of tetracycline structure produce CMTs with different pharmacological properties, as well as different pharmacokinetic behaviors, from tetracyclines. COL-3 is the structurally simplest and the most lipophilic of the clinically tested tetracycline analogs to date (6). In rats, COL-3 shows an irregular absorption profile with a double-peak or absorption plateau after oral administration. The variable and slow dissolution rate-limited absorption, probably due to its high lipophilicity, has been demonstrated to be the main cause of the irregular absorption profile of COL-3 in rats (9). In cancer patients, COL-3 shows a even more erratic concentration-time profile with the first peak occurring at 2–12 h (median, 6 h) and the second peak occurring at 22–48 h (median, 25 h) (6); its pharmacokinetic profile is characterized by a substantial interpatient variability in oral clearance

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ABBREVIATIONS: AP, apical; BL, basolateral; BSA, bovine serum albumin; CMC, carboxylmethyl cellulose sodium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HBSS, Hank's Balanced Salt Solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; MEM, Minimum Essential Medium; MMP, matrix metalloproteinase; MWCO, molecular weight cutoff; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; P_{app}, apparent permeability coefficient.

(CL/F) (median, 0.13 ml min^{-1} kg^{-1}; range, 0.06–0.49 ml min^{-1} kg^{-1}) and terminal half-life (median, 56.7 h; range, 23.7–144.4 h) (6). Rudek and colleagues (10) thus attempted to elucidate possible sources, other than absorption, of variability of COL-3 pharmacokinetics *in vivo*, by exploring plasma protein binding, metabolism, renal excretion, and various body size measures besides age, gender, and dose level. All these factors tested, however, failed to account for the variable oral clearance and terminal half-life of COL-3 observed in patients (10). Details of the disposition process of COL-3 in the literature up to date are still lacking. Perhaps a major route of elimination for COL-3, which remains unclear and is yet to be explored, could be one possible and important source of variability in COL-3 pharmacokinetics *in vivo*.

Schach van Wittenau and colleagues (10) have shown that in rats, dogs, and probably in humans (patients with renal insufficiency in particular), intestinal excretion from the systemic circulation (blood) into the lumen of the small intestine is the major route of elimination for doxycycline, a highly lipophilic tetracycline analogue with chemical structure similar to COL-3. Hence, it was hypothesized that COL-3 undergoes a substantial intestinal excretion *in vivo*. The current study was undertaken to elucidate the major route of elimination for COL-3. Determination of the fecal, biliary, and urinary recoveries of COL-3 in rats was performed. Additionally, plasma protein binding, erythrocyte partitioning, and P-450–mediated metabolism were investigated. Furthermore, transport studies across Caco-2 monolayers were performed to elucidate the mechanism of intestinal excretion of COL-3 and also to provide an insight into the contribution of intestinal excretion to the variability of COL-3 pharmacokinetics *in vivo*.

MATERIALS AND METHODS

Chemicals and Reagents

COL-3 was a gift from CollaGenex Pharmaceuticals (Newtown, PA, USA). Carboxylmethyl cellulose sodium (CMC), polyethylene glycol 400 (PEG-400), β-glucuronidase (type B-3: from bovine liver, 4000 U/mg), sulfatase (type V: from Limpets, 7.6 U/mg), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), nicotinamide, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, Hank's balanced salt solution (HBSS) (without sodium bicarbonate and phenol red), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Minimum essential medium (MEM) Eagle (with Earle's salts, L-glutamine, and nonessential amino acids, without sodium carbonate), penicillin-streptomycin, fetal bovine serum (FBS), 2.5% trypsin-EDTA, ^{14}C-mannitol (50 mci/mmol) were bought from PerkinElmer Life Sciences (Boston, MA, USA). Transwell cell culture chamber insert (polycarbonate membrane and polystyrene plates, 12-mm diameter, 0.4-μm pore size) was obtained from Costar (Bedford, MA, USA). All other chemicals and reagents were commercially available and of the analytical grade or high-performance liquid chromatography (HPLC) quality.

Animals

Male Sprague-Dawley (SD) rats (200–250 g) were obtained from Laboratory Animal Center (National University of Singapore, Singapore), and housed in a temperature-controlled room (25 ± 1°C) with a 12-h light-dark cycle. The animals were fasted overnight prior to drug administration, and allowed access to the standard pellet food and water *ad libitum* 1 h postdosing. At the end of the experiment, the rats were euthanized using diethyl ester. The research adhered to the principles of laboratory animal care (NIH Publication No. 85-23, revised 1985).

In Vivo Studies

Urinary and Fecal Excretion

The urinary and fecal recoveries of COL-3 were determined in the rats following either oral administration or intravenous injection. For oral studies, a freshly prepared COL-3 suspension in 2% CMC at the concentration of 5 mg/ml was given to the rats (n = 5) by oral gavage (30 mg/kg). For intravenous studies, the right femoral vein of the rats was cannulated with polyethylene cannula (PE 10, I.D. 0.28 mm, O.D. 0.61 mm), as described previously (9), and the rats were allowed to recover overnight. On the day of experiment, a freshly prepared COL-3 solution at the concentration of 2.5 mg/ml in PEG-400/pH 7.6 phosphate buffer (4/6, v/v) was injected as intravenous bolus (10 mg/kg) via the veinal catheter to the rats (n = 5). The femoral venous catheter was flushed by 0.3 ml of 0.9% saline solution after the intravenous injection.

After oral or intravenous dosing, the rats were individually housed in the metabolic cages. Urine and feces were collected up to 48 h after dosing, and stored at –20°C until analysis. COL-3 in urine and feces were quantitated by a validated reversed-phase high-performance liquid chromatographic method with ultraviolet (UV) detector (11). The total of unchanged COL-3 and its glucuronide conjugate in rat urine was determined as previously described (9).

Biliary Excretion

Biliary excretion of COL-3 was determined in the bile duct–cannulated (BDC) rats after oral administration of COL-3 suspension at the dose of 30 mg/kg. The BDC rats (n = 5) were prepared as described previously (9). Total bile was collected up to 24 h after oral dosing. Unchanged COL-3 as well as the total of unchanged COL-3 and its glucuronide conjugate in rat bile was quantitated as described previously (9).

Erythrocyte Partitioning

To determine the erythrocyte partitioning of COL-3 in rat blood, COL-3 suspension (30 mg/kg) was administered to the rats (n = 3) by oral gavage. Blood samples were drawn, using orbital bleeding technique, prior to and 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 30, 35, and 48 h after dosing. At each time point, two blood samples were drawn: one (0.2 ml) was used for determination of COL-3 blood concentration (C_b), and the other one (0.4 ml) was centrifuged (1100 × *g*, 10 min, 4°C) to separate serum for measurement of COL-3 serum concentration (C_p). All blood and serum samples were stored at –20°C until analyzed.

The serum concentrations of COL-3 were determined using the validated HPLC method (11). The whole blood

concentrations of COL-3 were measured using the same HPLC system with modified sample pretreatment. Briefly, into 100 μl of the blood sample was added 200 μl of acetonitrile–10% $ZnSO_4$–0.5 M oxalic acid (75:15:5, v/v), followed by vortex mixing (30 s), and centrifugation (37,000 × *g*, 10 min, 4°C). The supernatant was collected, and an aliquot of 20 μl was injected into the HPLC.

In Vitro Studies

Rat/Human Plasma Protein Binding

A Microcon centrifugal filter device (Microcon YM-3, Millipore Cop., Bedford, MA, USA) with a molecular weight cutoff (MWCO) of 3000 was used to separate the free drug from its bound form in rat/human plasma. The nonspecific adsorption of COL-3 to the Microcon filter device was examined with 5–50 μg/ml of COL-3 in phosphate buffer (pH 7.4). The concentrations of COL-3 in the pre- and postcentrifuged phosphate buffer were determined by HPLC (11). The extent of nonspecific binding of COL-3 to the Microcon device was less than 2%.

Rat or human blank plasma (0.5 ml) was spiked with known amount of COL-3 to obtain final concentrations of 5, 10, 20, and 50 μg/ml. Each concentration was in quintuplicate. The samples were thoroughly mixed and equilibrated at 37°C in a shaking water bath for 1 h, after that each sample was transferred to a Microcon centrifugal filter device (Microcon YM-3), and centrifuged (8300 × *g*, 25 min, 37°C). About 150 μl of ultrafiltrate was obtained, and an aliquot of 20 μl was injected into HPLC for the determination of the unbound drug concentration.

Phase I Metabolism Studies

The post-mitochondria supernatant of rat liver was prepared according to the method previously described (12) with modifications. Rat livers were freshly collected from three adult male SD rats, pooled, and homogenized in 3 volumes of ice-cold 0.01 M potassium phosphate buffer (pH 7.6). The homogenate was centrifuged (12,500 × *g*, 20 min, 4°C), and the supernatant was collected. Aliquots (1.5 ml) of the supernatant were stored at −80°C until used. The protein concentration of the post-mitochondria supernatant was determined using a biuret-based total protein reagent according to manufacturer instructions (Sigma Diagnositics Inc., St Louis, MO, USA).

A NADPH-generating system was freshly prepared, as described previously (12). 10 ml of the NADPH-generating solution consisted of 8.0 ml of 0.1 M Tris buffer (pH 7.4), 1.0 ml of 0.15 M magnesium chloride solution, 1.0 ml of 0.5 M nicotinamide solution, glucose-6-phosphate (4 μmol/ml in final mixture), glucose-6-phosphate dehydrogenase (20 U/ml in final mixture), and 2 mM $NADP^+$. The solution was made up and $NADP^+$ was added immediately prior to use.

For drug metabolism reaction, COL-3 (1.5 μmol) was spiked with 1 ml of post-mitochondria supernatant (containing 30 mg protein). The reaction was initiated by adding 1 ml of the freshly prepared NADPH-generating solution; the incubation was conducted at 37°C in a shaking water bath for 1 h. Two negative control incubations, one without drug and NADPH-generating system, and the other with drug but without NADPH, were concurrently run. After 1-h incubation, reactions were terminated by cooling on ice, and 200 μl of acetonitrile-methanol-0.5 M oxalic acid (70:20:10, v/v) was added into an aliquot of 100 μl of the incubation mixture, vortex-mixed (30 s), and centrifuged (37,000 × *g*, 4°C, 10 min). The supernatant was subject to the HPLC with SPD-M10Avp UV-Vis photodiode array (PDA) detector (Shimadzu, Kyoto, Japan) to detect COL-3 and its possible metabolites. Mobile phase consisted of acetonitrile-methanol-oxalic acid (0.01 M, pH 2.0) at the ratio of 40:20:40 (v/v). Detections at the wavelengths of 350 and 260 nm were recorded. The purity of COL-3 peak was confirmed by its UV spectrum on-line scanned with PDA detector.

Transport Studies Across Caco-2 Monolayers

Cell Culture

The colon carcinoma derived Caco-2 cell line (at passage 18) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Caco-2 cells with passage number 40-47 were cultured in MEM (with Earle's salts and L-glutamine) (pH 7.4) supplemented with 10% FBS, 0.1 mM nonessential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO_2/95% air. For transport studies, Caco-2 cells were seeded at a density of 10^5 cells/cm^2 on the Transwell tissue culture chamber insert (12-mm diameter and 0.4-μm pore size polycarbonate membranes). The integrity of monolayers was assessed by measurement of transepithelial electrical resistance (TEER) and the permeability of a paracellular marker, ^{14}C-mannitol. Monolayers with TEER values more than 300 Ω cm^2 in culture medium were selected for transport experiment. The permeability of ^{14}C-mannitol was also evaluated in the presence of COL-3 and verapamil, and it was found that 50 μM of COL-3 and 50 μM of verapamil did not affect the integrity of the monolayers.

Transport Studies

The transport studies were conducted 21–25 days post-seeding. Prior to the experiment, the inserts were washed 3 × 10 min with warm transport buffer at 37°C (HBSS containing 25 mM of HEPES, pH 7.4). Studies were conducted after equilibrating the cells for 30 min in transport buffer. COL-3 (50 μM) in transport buffer (containing 0.5% DMSO), diluted from a 10 mM stock solution of COL-3 in DMSO, was added to either the apical (0.5 ml) or basolateral (1.5 ml) side of the inserts, whereas the receiving chamber contained the corresponding volume of drug-free transport buffer. At predetermined time points (0.5, 1, 2, 3 h), 50 μl of sample was withdrawn from both the receiver and donor chamber simultaneously, and the corresponding volume of fresh transport buffer was replaced. The TEER of the monolayers was checked at the beginning and at the end of each experiment.

To examine the effect of verapamil, a known P-glycoprotein (P-gp) inhibitor, on the transport of COL-3 across the Caco-2 monolayers, the cells were incubated with verapamil (50 μM) at both apical and basolateral sides at 37°C for 1 h, after which the medium at the apical and basolateral sides was replaced by HBSS or HBSS containing COL-3 (50 μM) in the presence or absence of verapamil (50 μM). The transports of

COL-3 in both directions (AP to BL, and BL to AP) were determined at the predetermined time points as described above.

The permeability of COL-3 in both directions was also determined with 4% BSA in the BL side of the monolayer, mimicing the plasma protein in the systemic circulation. To examine the effect of drug-protein interactions on the transport of COL-3, both disappearance from the donor and appearance in the receiver were determined when various concentrations of BSA (0–4% w/v) were included in either the basolateral (BL) or apical (AP) sides of the monolayers. Because the pH of the intestinal lumen has been reported to be around 6.5, the permeability of COL-3 across Caco-2 was also determined when apical side was adjusted to pH 6.5.

Efflux Studies

Caco-2 monolayers were washed three times with prewarmed HBSS containing 25 mM of HEPES (pH 7.4), and balanced at 37°C for 30 min. The cells were incubated with 50 μM COL-3 in both AP and BA sides for 1 h at 37°C. At the end of the incubation, the solution containing COL-3 was removed from AP and BA sides, the cells were rinsed with HBSS three times, and 0.5 and 1.5 ml of the fresh buffer with or without BSA were added to the AP and BA sides, respectively. At the predetermined time points (0.5, 1, 2, 3 h), 50 μl sample was withdrawn from the AP and BA sides concurrently, and an equal volume was replaced with buffer/BSA. The efflux from the AP and BA sides was determined simultaneously.

Data Analyses

The percent plasma protein binding was calculated as $(1 - C_f / C_t) \times 100$, where C_f is the drug concentration in the ultrafiltrate of the plasma (i.e., the unbound drug concentration), and C_t is the total drug concentration spiked in the plasma.

The erythrocyte partitioning of the drug was measured by the ratio of concentration in blood cells (C_{bc}) to that unbound in plasma (C_u). The ratio was expressed as $\rho = (H - 1 + C_b/C_p)/(f_u \times H)$, where H is the hematocrit, C_b is the blood concentration of drug, C_p is the plasma concentration of drug, and f_u is the unbound fraction in plasma (i.e., $f_u = C_f/C_t$) (13).

The apparent permeability coefficient (P_{app}) expressed in cm/s was determined as

$$P_{app} = \frac{dC \cdot V}{dt \cdot A \cdot C_0}$$

where dC/dt is the change in concentration on the receiving side over time (μM/s), V is the volume of the solution in the receiving compartment (cm^3 or ml), A is the surface area of the membrane (1.13 cm^2), and C_0 is the initial concentration in the donor chamber (μM). The flux or efflux rate across the monolayer was calculated from the slope of the cumulative amount transported *vs.* time.

Statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. Independent-samples *t* test was used for comparisons of means between two experimental groups. A value of $p < 0.05$ was taken to indicate statistical significance.

RESULTS

Urinary, Fecal, and Biliary Excretion

Less than 0.2% and 0.03% of COL-3 were excreted unchanged in rat urine within 48 h following a single intravenous and oral doses of COL-3, respectively; while 32.1 ± 9.9% and 38.8 ± 6.1% of the intravenous and oral doses were recovered unchanged in rat feces within 48 postdosing, respectively (Table I). 1.36 ± 0.66% (range, 0.37 to 2.13%) and 2.97 ± 0.88% (range, 1.92 to 4.14%) of the oral dose were excreted in rat bile as the unchanged COL-3 and the total of COL-3 and its glucuronide conjugate, within 24 h after dosing.

Rat/Human Plasma Protein Binding and Erythrocyte Partitioning

About 98% and 95% of COL-3 were found to bind to rat and human plasma protein, respectively, as determined by *in vitro* ultrafiltration method. The protein binding of COL-3 was concentration-independent over the concentration range of 5–50 μg/ml examined.

Along with the binding to plasma protein, COL-3 was also found to bind to erythrocytes in blood. By simultaneous determination of the serum and blood concentrations of COL-3 after a single oral dose in the rats with their respective time profiles as shown in Fig. 1, the ratio of blood-to-plasma concentration was calculated as 0.873 ± 0.058, over the concentration range of 500 to 5000 ng/ml achieved *in vivo*. The affinity of blood cells for drug (i.e., the ρ value) estimated was about 35.9 for COL-3 in rat blood, as the mean rat blood hematocrit was 0.45 as determined previously (14), the mean unbound fraction of COL-3 in rat plasma was 0.02, and the mean C_b/C_p value of COL-3 was 0.87.

Taken the partitioning of COL-3 to plasma protein (fu, 0.02) and erythrocyte (ρ, 35.9) together, it was estimated that of COL-3 in rat blood as a whole, only 2.3% existed as the

Table I. Urinary and Fecal Recovery of COL-3 and Its Glucuronide Conjugates Within 48 h after Intravenous (i.v. 10 mg/kg) and Oral (p.o. 30 mg/kg) Administration to Rats

	Intravenous injection		Oral administration	
	Urinary recovery	Fecal recovery	Urine recovery	Fecal recovery
Unchanged COL-3 (%dose)	0.139 ± 0.077 (0.062–0.228)	32.1 ± 9.9 (21.4–47.3)	0.026 ± 0.009 (0.015–0.037)	38.8 ± 6.1 (29.4–46.4)
Total (COL-3 + glucuronide conjugate) (%dose)	0.917 ± 0.464 (0.522–1.623)	ND	0.227 ± 0.078 (0.129–0.339)	ND

Data are shown as mean ± SD (range), n = 5. ND, not determined.

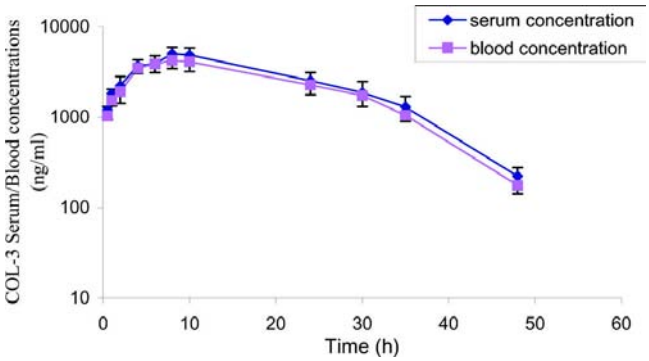

Fig. 1. Concentrations of COL-3 in rat serum and whole blood as function of time after oral administration (30 mg/kg) to rats. The vertical error bars represent the standard deviations of the mean (n = 3).

free drug, whereas there were 61.1 and 36.6% bound to plasma protein and erythrocyte, respectively.

Phase I/II Metabolism

In vitro phase I metabolism studies were conducted with two lots of pooled rat liver post-mitochondria supernatant (containing both microsomes and cytosol). After incubation with post-mitochondria supernatant under the various conditions, there was no apparent loss in parent drug concentration, and no peaks of metabolites were detected at the wavelength of 350 nm and 260 nm (chromatogram not shown here). The peak purity of COL-3 was confirmed by its UV spectrum on-line scanned with PDA detector, which was exactly the same as the spectrum of COL-3 methanol solution scanned with UV-Vis spectrophotometer (11), indicating no interference from possible metabolites. However, one of the phase II metabolites of COL-3, a glucuronide conjugate, was identified in a minor quantity in rat bile and urine.

Transport of COL-3 Across Caco-2 Cells

The apparent permeability coefficients (P_{app}) of COL-3 across Caco-2 cell monolayers in both directions, AP to BL (8.87×10^{-6} cm/s) and BL to AP (7.47×10^{-6} cm/s), were not statistically different ($p > 0.05$). In addition, verapamil, a P-gp inhibitor, had no apparent effect on the permeability of COL-3 across the monolayers, the P_{app} of COL-3 being 8.07×10^{-6} cm/s (AP to BL) and 6.83×10^{-6} cm/s (BL to AP) in the presence of verapamil. Therefore, COL-3 is unlikely a P-gp substrate. P-gp activity in the Caco-2 cells from the same source with identical passages was validated by performing the transport studies of digoxin, a known P-gp substrate, in another study (15).

However, protein binding and pH significantly affected COL-3 transport across Caco-2 monolayers. With 4% BSA included in the BL side of the monolayers (mimicing plasma protein in the systemic circulation), the permeability of COL-3 from AP to BL (absorption) was increased 2-fold ($p < 0.05$), while the permeability from BL to AP (exsorption) was decreased by >8-fold ($p < 0.01$) (Fig. 2). Because only unbound drug is thought to be capable of passing through biological membranes by passive diffusion, the protein binding in blood produced a "sink" condition to facilitate drug absorption, while to prevent its exsorption.

Additionally, pH in the AP side ("intestinal lumen") remarkably influenced the permeability of COL-3 across Caco-2 monolayers. When the pH of the AP side was adjusted from 7.4 to 6.5 (a normal pH in intestinal lumen), the transport of COL-3 from AP to BL (absorption) was increased 1.4-fold, while from BL to AP (exsorption) was decreased 2.1-fold (Fig. 2). With its pK_a values of 5.65 (pK_{a1}) and 8.35 (pK_{a2}) (13), the un-ionized form of COL-3 would be expected to increase to 12.2% from 1.57% as the pH was adjusted to 6.5 from 7.4. According to the pH partition hypothesis, only un-ionized nonpolar drug penetrates biological membranes. Hence, the change in transport of COL-3 from the AP to BL (increased absorption) or from BL to AP (decreased exsorption) was correspondingly with the increase of un-ionized drug when the pH in AP side was adjusted from 7.4 to 6.5.

The transport and efflux studies were also done when different concentration of BSA (mimicking "intestinal contents") was included in the AP side of Caco-2 monolayer to examine the role of "intestinal contents" on COL-3 intestinal excretion. The disappearance of COL-3 from the AP donor solution representing drug partitioning into the cell monolayer was rapid and exponential over time, while the concomitant appearance of COL-3 in the BL receiver solution representing drug absorption into system circulation was relatively

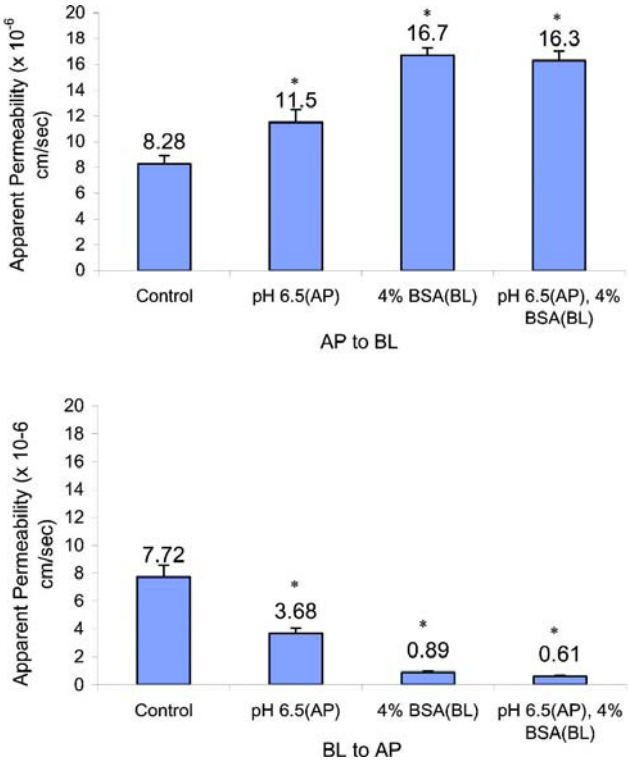

Fig. 2. Effect of 4% BSA in the BL side and/or pH 6.5 in the AP side on the apparent permeability of COL-3 across Caco-2 monolayers. COL-3 (50 μM) in HBSS was loaded either in the AP side for AP to BL transport study or in the BL side for BL to AP transport study. The control experiments were conducted with HBSS (pH 7.4) in both the AP and BL sides of the monolayers. Values are means of triplicate with the error bar representing one standard deviation from the mean. *Significantly different from the control, $p < 0.05$.

slow and linear over time (Fig. 3). With 0%, 0.5%, 1%, and 4% of BSA in the AP side, approximately 75%, 27%, 18%, and 11% of COL-3 partitioned into the monolayers, respectively, and concurrently approximately 20%, 14%, 10%, and 5% of COL-3 appeared in the BL receiver solution, respectively, within 3 h experimental time (Fig. 3).

In addition, inclusion of BSA in the AP side significantly affects the efflux (to AP) and flux (to BL) of COL-3. With 0%, 1%, and 4% of BSA in the AP side, the efflux rates (to AP) were 86 ± 6, 636 ± 94, and 1031 ± 142 ng/h, respectively; while the flux rates (to BL) were 209 ± 10, 135 ± 5, and 58 ± 7 ng/h, respectively (Fig. 4).

DISCUSSION

The large inter-individual variability in pharmacokinetics of COL-3 might be due to variations in absorption, distribution, metabolism, and elimination. A dissolution-rate limited absorption has been demonstrated to account for, for a large part, the irregular absorption profile of COL-3 in rats or humans (9,10). However, the occurrence of the second peak of COL-3 at 22–48 h (median, 25 h) (16) cannot be explained well by the dissolution rate-limited absorption, as the transit time of solids within the small intestine, the major absorption site for COL-3, is approximately 3 h in humans (13). Additionally, the long and variable terminal half-life (median, 56.7 h; range, 23.7–144.4 h) of COL-3 after oral administration in humans (6) is more likely due to some disposition sources rather than absorption, though a flip-flop phenomenon, that the terminal phase of the serum concentration-time curve reflects absorption whereas the initial portion of the curve reflects elimination, was proposed to explain, at least in part, the long and variable terminal half-life in cancer patients (9).

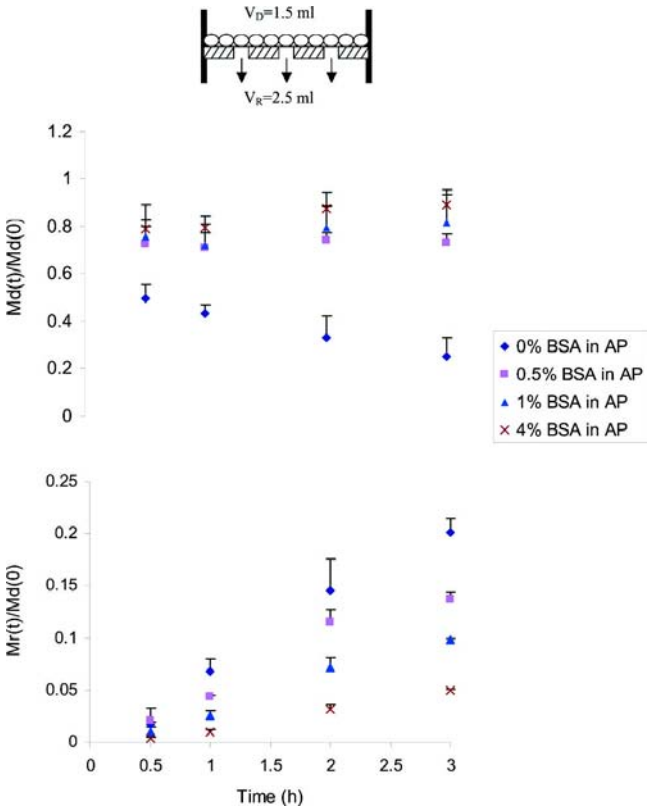

Fig. 3. Transport of COL-3 across the monolayers as a function of BSA concentration (w/v) in the AP donor chamber. COL-3 (50 μM) was loaded in the AP side. The mass of drug in the AP donor (Md) and BL receiver (Mr) at each time were calculated as a fraction of the mass in the AP donor at t = 0 [Md(0)]. The cumulative fraction is plotted as a function of time. Values are means of triplicate with the error bar representing one standard deviation from the mean.

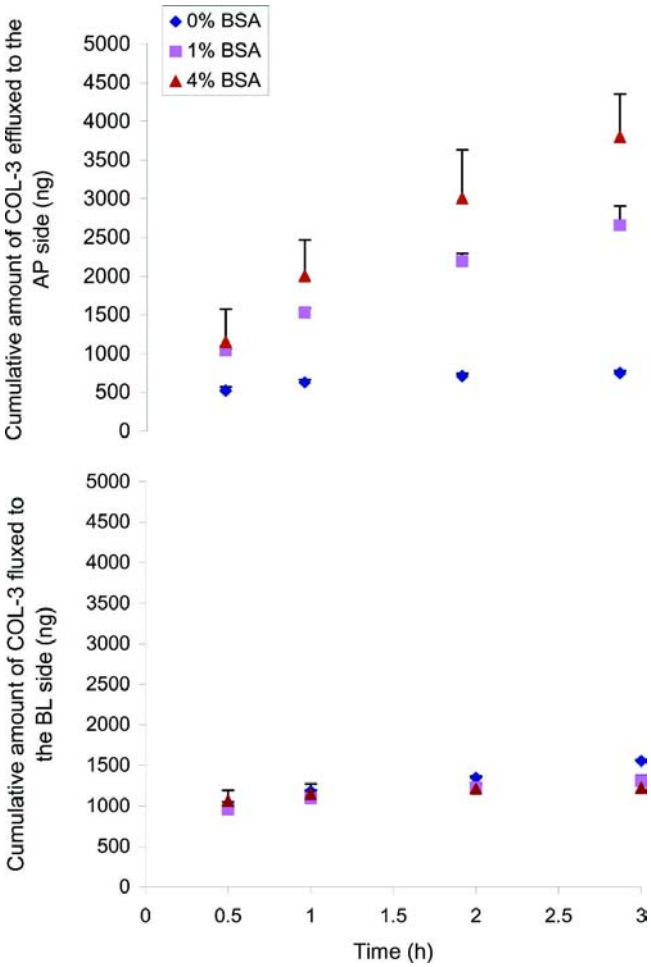

Fig. 4. Efflux to the AP side and flux to the BL side of COL-3 (50 μM) from the monolayers with 0%, 1%, and 4% of BSA in the AP side of the monolayers. Values are means of triplicate with the error bar representing one standard deviation from the mean.

Different from most tetracycline analogs, COL-3 showed a low biliary excretion and a negligible urinary excretion (Table I). The poor biliary excretion of COL-3 might be due to its relatively small molecular weight (371.35) and extreme lipophicility because for extensive biliary excretion to occur, molecules should possess a large molecular weight (MW >400 to 500) and a strong polar group (17). Also, the poor renal clearance of COL-3 is likely due to its extreme lipophilicity and high protein binding. It is well known that the excretion rate equals $(1 - F_R) \times$ (rate of filtration + rate of secretion), where F_R is the fraction reabsorbed from the lumen (13). The high plasma protein binding is unfavorable to COL-3 filtration and secretion; but the high lipophilicity is favorable to its reabsorption.

It is not surprising that cytochrome P450-mediated metabolism took place to an insignificant extent, or not at all, for

COL-3 in rats and humans (10), as COL-3 lacks dimethylamino functional group, the chemical site where other tetracycline analogs undergo metabolism (18,19). However, COL-3 might be the only tetracycline analogs documented *in vivo* (Table I) and *in vitro* glucuronidation. *In vitro* studies showed that four UGT isozymes (UGT1A1, 1A3, 1A9, and 2B7) were responsible for the glucuronidation of COL-3 (10). It is noted that COL-3 glucuronide can be converted back to the parent molecule, COL-3, by intestinal β-glucuronidase. Because COL-3 glucuronide undergoes a very low biliary excretion (<3% of total dose), the β-glucuronidase–mediated generation of COL-3 from its glucuronide cannot explain the 30–40% of fecal recovery.

Like doxycycline (18,19), another lipophilic tetracycline analogue, intestinal excretion turned out to be a major route of elimination for COL-3. Chen and colleagues (20) have shown that the gastrointestinal tract content and feces contained about 54% of the radioactivity by 14 h after oral administration of ^{3}H-COL-3 to rats. However, in their study, because no intravenous dose was given, a conclusion that COL-3 was mainly eliminated by intestinal excretion was not drawn. In the present study, a significant fecal recovery of COL-3 (Table I) was determined after COL-3 was given by intravenous injection in rats, suggesting that COL-3 is eliminated by excretion into the intestinal tract by a route different from biliary secretion. Transport studies across Caco-2 cell monolayers were further undertaken to understand the intestinal excretion of COL-3. The inclusion of this model in the current study is based on the fact that there are negligible differences in the transport kinetics of P-gp substrates between Caco-2 monolayers and rat epithelia (21).

P-glycoprotein (P-gp), an APT-binding cassette transmembrane transporter, acts as an energy-dependent efflux pump that mediates the intestinal excretion of a number of drugs such as digoxin (22), irinotecan and its metabolite SN-38 (23), docetaxel (24), and paclitaxel (25). However, COL-3 is unlikely a substrate of P-gp. The significant intestinal excretion of COL-3 is likely due to a mechanism different from intestinal P-gp–mediated efflux.

COL-3 is highly lipophilic. It might easily pass through the intestinal mucosa by passive diffusion, the natural tendency for molecules to move down a concentration gradient. The significant effects of protein binding and pH on the permeability of COL-3 across Caco-2 cell monolayers (as shown in Results) strongly support the passive diffusion of COL-3 across intestinal mucosa.

The bidirectional passive diffusion of COL-3 across Caco-2 cell monolayers provides an insight into its intestinal absorption and excretion. A significant correlation has been obtained between oral absorption fractions in humans and P_{app} values of drugs across Caco-2 monolayers. Drugs with P_{app} values of $>1 \times 10^{6}$ cm/s could be completely absorbed in humans given no dissolution problem exists (26). COL-3 had the P_{app} value of 8.40×10^{-6} cm/s (AP to BL). It is thus supposed to be absorbed completely once dissolved in the gastrointestinal tract; however, due to dissolution problem, the oral bioavailability of COL-3 was found less than 100% (9).

Meanwhile, a P_{app} value of 7.17×10^{-6} cm/s from BL to AP (exsorption) allows COL-3 to diffuse easily through the intestine wall from the systemic circulation into the intestinal lumen, where it could be bound to intestinal contents and ultimately excreted with feces. The intestinal contents may provide a "sink" condition to draw COL-3 to diffuse from the circulation blood into the intestinal lumen, and also prevent drug re-absorption by forming a stable complex with COL-3. The role of intestinal contents in the intestinal excretion of COL-3 was demonstrated by examining the transport of COL-3 across Caco-2 monolayers when various concentrations of BSA (mimicking intestinal contents) were included in the AP side ("intestinal lumen") of the Caco-2 cell monolayer.

With 1% and 4% of BSA in the AP side, the mean drug efflux rate (to AP side) was increased 7.4- and 12.0-fold, respectively; while the mean flux rate (to BL side) was decreased to 64% and 28%, respectively, compared to that without BSA in the AP side (Fig. 4). The significant increase in efflux of COL-3 (exsorption) when BSA was included in the AP side suggests that "intestinal contents" (BSA in the AP side) bind the drug to facilitate its diffusion from the circulation (BL side) into the intestinal lumen (AP side).

In addition, the effect of BSA ("intestine contents") binding with drug on the "re-absorption" of COL-3 was determined by simultaneous measurement of drug disappearance from the AP donor solution (representing drug partitioning into intestinal cells) and its appearance in the BL receiver solution (representing drug absorption into system circulation). With the increase of BSA concentration in the AP side, both drug cellular accumulation and absorption were reduced remarkably ($p < 0.05$) (Fig. 3), suggesting that drug-intestinal contents complex could efficiently prevent reabsorption of COL-3 during its passage down the intestinal lumen.

In normal conditions, continuous entry of food and digestive fluid into intestinal lumen provides a "sink" into which the intestinal excreted COL-3 is gradually eliminated with feces. However, in some pathologic conditions (i.e., with cancer) when food intake and digestive fluid excretion is hampered, no enough intestinal contents exist in lumen to bind free drug; so the intestinal excretion of COL-3 may be decreased, and reabsorption may occur. Indeed, reabsorption of the blood borne COL-3 was observed in the rats when COL-3 and doxorubicin were coadministered, in which food intake was reduced significantly due to severe gastrointestinal toxicity of doxorubicin (manuscript in preparation). Similarly, because cancer patients are often anorectic because of tumor burden and/or drug toxicity, intestinal contents in these patients are often deficient and vary greatly. Therefore, the clinically observed long and variable elimination half-life of COL-3 could be due to the variable and slow elimination of COL-3 via intestinal excretion in cancer patients. In addition, drug reabsorption in intestinal lumen could explain, at least in part, the occurrence of COL-3 second peak at 22–48 h (median, 25 h) in cancer patients (16).

CONCLUSIONS

COL-3 is extensively bound to plasma protein in rat (98%) and human (95%) plasma. It has a low biliary excretion and negligible urinary excretion in rats. Cytochrome P450–mediated metabolism takes place to an insignificant extent, or not at all, in rats and humans; however, COL-3 undergoes phase II metabolism (i.e., glucuronidation) in a minor quantity. The similarities in the disposition properties of

COL-3 (i.e., protein binding, urinary excretion, and metabolism) between rats and humans suggest that a rat is a suitable animal model for studying the disposition of COL-3.

The current study clarified that the major route of elimination for COL-3 in rats, and most probably in humans, is the passive diffusion of the drug from the blood into the intestinal lumen, from where it is bound to intestinal contents and ultimately excreted with feces. The passive diffusion of the drug across intestinal mucosa and the effect of intestinal contents on COL-3 intestinal excretion are well demonstrated by transport studies of COL-3 across Caco-2 monolayers. The variable intestinal excretion of COL-3, due to extreme variable intestinal contents (food and digestive fluids) in cancer patients, is likely to be one source, besides the dissolution rate-limited absorption, of the variability in COL-3 pharmacokinetics *in vivo*.

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