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Research Paper

Alteration of the Pharmacokinetics of COL-3, a Matrix Metalloproteinase Inhibitor, Due to Acute Gastrointestinal Toxicity of Doxorubicin

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Purpose. Combination of COL-3, a matrix metalloproteinase inhibitor, and doxorubicin (DOX) might be a promising anticancer regimen. The present study was to examine the potential pharmacokinetic interactions and toxicity profile following their coadministration in rats.
Methods. Normal rats were treated with single agent or different combinations with oral or intravenous COL-3 and DOX, and the bile-duct cannulated (BDC) rats received oral COL-3 plus DOX. In a separate disposition study, the effects of DOX on the biliary, urinary, and fecal excretion of COL-3 were examined. In addition, the effects of DOX on *in vitro* protein binding, metabolism, and transport of COL-3 across Caco-2 monolayers were investigated.
Results. COL-3 did not affect the pharmacokinetics of DOX in rats. However, treatment with DOX significantly decreased the oral absorption, and prolonged the elimination, of COL-3 in the normal rats, but not in the BDC rats. DOX did not alter the biliary and urinary excretion of COL-3, but significantly decreased the fecal excretion of COL-3. DOX significantly enhanced the basolateral to apical flux of COL-3 across Caco-2 monolayers, but had no apparent effects on the protein binding and metabolism of COL-3. The combination of DOX with oral COL-3 did not significantly ($p > 0.05$) increase the acute diarrhea score and intestinal damage compared to rats receiving DOX alone.
Conclusions. These results indicated that DOX altered the oral absorption and elimination of COL-3, largely resulting from gastrointestinal toxicity caused by biliary excretion of DOX. Further studies are required to explore the efficacy and optimized dosage regimen of this promising combination.

KEY WORDS: COL-3; doxorubicin; gastrointestinal toxicity; matrix metalloproteinase inhibitor; pharmacokinetic interaction.

INTRODUCTION

6-Deoxy-6-demethyl-4-dedimethylamino-tetracycline (COL-3), an oral matrix metalloproteinase (MMP) inhibitor, has undergone phase I clinical trials in patients with refractory metastatic cancer (1) and AIDS-related Kaposi's sarcoma (2) and is currently being assessed in phase II trial to treat Kaposi's sarcoma and advanced brain tumors. COL-3 potently inhibits MMP-2, MMP-9, and MT1-MMP (3–6), which are overexpressed in a variety of tumors (e.g., breast, lung, and prostate cancers) and correlated with tumor metastasis and poor prognosis. The MMPs belong to a family of Zn^{2+}-dependent proteinases, which are known to regulate various cell behaviors relevant to cancer biology, including cancer cell growth, differentiation, apoptosis, migration, invasion, as well as tumor angiogenesis and immune surveillance (7).

Numerous MMP inhibitors (MMPIs) have been tested in cancer patients; however, results from phase II/III clinical trials are disappointing (8–10). One of the lessons from these trials is that MMPIs should be applied as cytostatic instead of cytotoxic agents. The cytostatic agent should, therefore, be used as an adjuvant to the conventional cytotoxic therapy to prevent regrowth of tumor between cycles of cytotoxic treatment, to delay the development of tumor resistance to cytotoxic therapy, and to ameliorate the dose-limiting toxicity of cytotoxic agents, thus allowing increased dosage of cytotoxic agents to maximize tumor killing effect (11,12). A number of MMPI–cytotoxic combinations, such as batimastat–cisplatin (13), AG3340–carboplatin (14), and AG3340–Taxol (15), were significantly more effective than either agent administered alone in prolonging survival or inhibiting tumor growth in animal studies, with the toxicity profiles improved or unchanged. Furthermore, phase I/II clinical studies combining a wide range of cytotoxic agents with MMPIs showed that such combinations, in general, were synergistic and well tolerated without indications of additional toxicity (16,17).

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Data are scanty concerning a potential pharmacokinetic and pharmacodynamic interaction between COL-3 and doxorubicin (DOX), an anthracycline antibiotic with multiple anticancer mechanisms including DNA intercalation, free radical formation, DOX-induced DNA breaks, chromosomal aberrations, and cell membrane alterations (18). Such information may be useful for developing a combination therapy strategy because the chance for treatment success can be compromised by the failure to anticipate the likely changes in plasma and unbound drug concentrations over time when the pharmacokinetics of a drug is significantly altered by concurrent drug administration, or the toxicity of either agent is greatly enhanced. The combination of COL-3 and DOX could be a useful strategy in the treatment of cancer. This prompted us to investigate whether there were potential pharmacokinetic interactions between COL-3 and DOX and whether there was a change in toxicity profile of either agent, which should be taken into account in the design of combination regimen.

MATERIALS AND METHODS

Chemicals and Reagents

COL-3 was a gift from CollaGenex Pharmaceuticals (Newtown, PA, USA). Carboxymethyl cellulose sodium (CMC), polyethylene glycol 400 (PEG-400), β-glucuronidase (Type B-3: from bovine liver, 4,000 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, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Minimum essential medium Eagle (with Earle's salts, L-glutamine, and nonessential amino acids, without sodium carbonate), penicillin-streptomycin, fetal bovine serum, 2.5% trypsin–ethylenediaminetetraacetic acid, and ^{14}C-mannitol (50 mCi/mmol) were bought from PerkinElmer Life Sciences (Boston, MA, USA). All other chemicals and reagents were commercially available and of analytical grade or high-performance liquid chromatography (HPLC) quality.

COL-3 suspension was freshly prepared in 2% CMC at COL-3 concentration of 5 mg/mL prior to its oral administration to the rats. COL-3 solution for intravenous injection was also freshly prepared, prior to its administration, in polyethylene glycol 400 (PEG-400): pH 7.6 phosphate buffer (4:6, v/v) at COL-3 concentration of 2.5 mg/mL. Doxorubicin, supplied as doxorubicin hydrochloride 50 mg dry substance for injection (Pharmacia & Upjohn, Bentley, WA, Australia), was dissolved with sterile water to obtain a final drug concentration of 2 mg/mL for intravenous injection.

Cell Culture

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained by serial passage in T-75 plastic culture flasks (Life Technologies, Paisley, Scotland, UK). The cells were cultured in complete Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% nonessential amino acids, and 100 U/mL penicillin and gentamicin (all from Life Technologies). The cells were grown in an atmosphere of 5% CO_2/95% air at 37°C and given fresh medium every 3 or 4 days. Viable cells were counted using the trypan blue exclusion method. The transmembrane specific resistance, expressed in $\Omega\ cm^2$, was measured using a Millicell-ERS apparatus (purchased from Millipore Corporation, Bedford, MA, USA) at room temperature. The integrity of Caco-2 monolayers was confirmed when the transepithelial electrical resistance exceeded 300 $\Omega\ cm^2$, and the paracellular flux of ^{14}C-mannitol was <1% per hour.

Animals

Male Sprague–Dawley (SD) rats (200–250 g) were obtained from Laboratory Animal Center (National University of Singapore, Singapore) and housed in the temperature-controlled room (25 ± 1°C) with a 12-h light–dark cycle. One day before the experiment, animals used for intravenous injection of COL-3 and/or DOX had the right femoral vein cannulated with polyethylene cannula (PE 10, I.D. 0.28 mm, O.D. 0.61 mm) under anesthesia with a mixture of midazolam/hypnorm/water (1:1:2) (intraperitoneal, 2.5 mL/kg). 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 experiment, the rats were euthanized using diethyl ester. The research adhered to the principles of laboratory animal care (NIH publication #85-23, revised 1985).

Pharmacokinetic Studies

Potential pharmacokinetic interaction between COL-3 and DOX was examined in three separate experiments: I, II, and III. In experiment I, oral administration of COL-3 was combined with intravenous injection of DOX. The rats were randomly divided into three groups, each with six to eight rats. Group 1 was given DOX by intravenous (i.v.) injection (10 mg/kg). Group 2 was given COL-3 suspension (5 mg/mL in 2% CMC) by gastric gavage (30 mg/kg). Group 3 received both drugs at the same dose as their respective groups 1 and 2, in which DOX was given by i.v. injection about 1 min after oral administration of COL-3. In experiment II, intravenous injection of COL-3 was combined with intravenous injection of DOX. The rats were randomly assigned into two groups, each with six to eight rats. One group was administered COL-3 solution by i.v. injection (10 mg/kg) as the control group, whereas the other was given both COL-3 and DOX, in which DOX (10 mg/kg) was intravenously injected about 1 min after the intravenous injection of COL-3 solution (10 mg/kg). In experiment III, pharmacokinetic studies were performed in the bile-duct cannulated (BDC) rats. Bile-duct cannulated rats were prepared as described previously (19). The rats were given COL-3 suspension by gastric gavage (30 mg/kg), either alone or in combination with DOX (i.v., 10 mg/kg). In all three experiments, the femoral venous cannula was flushed by 0.3 mL of 0.9% saline solution after each intravenous dosing. When one of the drugs was administered alone, the corresponding volume of 0.9% saline solution was given in place of the other drug.

For all the experimental rats, blood samples (about 400 μL) were collected, using orbital bleeding technique, prior to and at 0.5, 1, 3, 5, 7, 9, 11, 24, 30, 35, and 48 h after drug administration. A corresponding volume of 0.9% saline solution was injected through the femoral vein cannula to maintain a constant blood volume. The serum fraction was separated by centrifugation at 1,100 × *g* at 10°C for 10 min and stored at −20°C until analyzed. For the rats in experiment I given DOX (i.v., 10 mg/kg) alone or in combination with COL-3, tissue samples (heart, liver, and kidney) were collected at the end of experiment (48 h) to determine DOX tissue levels. The tissue was washed free of blood with 0.9% saline, blotted dry, weighted, and stored at −20°C until analyzed. For the BDC rats in experiment III, total bile was collected at the predetermined interval over a period of 24-h postdosing. The bile samples were kept at −20°C until analyzed.

Pharmacokinetic Calculation

Pharmacokinetic analyses were performed using the WinNonlin iterative curve-fitting program (Standard edition, 1.1, Scientific Consulting Inc., Lexington, KY, USA) based on nonlinear regression analysis. The serum concentration-time profile of DOX following its intravenous injection was well described by a two-compartment disposition model.

The single-dose intravenous and oral pharmacokinetics of COL-3 was analyzed using model-independent noncompartmental analysis. The peak serum concentration(s) (C_{max1}, C_{max2}) and the time(s) of occurrence for peak concentration (T_{max1}, T_{max2}) after oral administration were obtained by visual inspection of the serum concentration-time curve. The initial concentration (C_0) after intravenous injection was determined by extrapolating the curve to $t = 0$ obtained through linear regression on the logarithmic transformation using the first several data points. The area under the serum concentration-time curve (AUC_{0-t}) as well as the area under the first moment curve ($AUMC_{0-t}$) from time zero to the last measurable time (t_z) point was calculated using the mixed linear (for prepeak area) and log-linear (for postpeak area) trapezoidal rule and the log-linear trapezoidal rule for the oral and intravenous data of COL-3, respectively. The total area under the serum concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) was calculated as the sum of AUC_{0-t} and the extrapolated area, which was estimated as the last measurable serum concentration (C_z) divided by the terminal rate constant (λ_z), where λ_z was estimated using the terminal log-linear phase of the serum concentration-time curve. The area under the first moment curve from time zero to infinity ($AUMC_{0-\infty}$) was calculated as the sum of $AUMC_{0-t}$ and the extrapolated area, which was estimated as ($t_z \times C_z/\lambda_z + C_z/\lambda_z^2$). Terminal serum half-life ($t_{1/2,\lambda z}$) was calculated as $0.693/\lambda_z$. The absolute bioavailability (F) was estimated as the ratio of oral to intravenous dose-normalized mean $AUC_{0-\infty}$. The serum clearance for intravenous dose (CL) or the apparent serum clearance for oral dose (CL/F) was calculated as dose/$AUC_{0-\infty}$. The mean residence time for intravenous dose (MRT_{iv}) and oral dose (MRT_{oral}) was determined as $AUMC_{0-\infty}/AUC_{0-\infty}$. The mean absorption time (MAT) was estimated as $MRT_{oral} - MRT_{iv}$. The volume of distribution during the terminal phase (V_z) was estimated as CL/λ_z. The volume of distribution at steady-state (V_{ss}) was estimated as CL × MRT.

Assessment of Acute Diarrhea Severity and Intestinal Damage

The severity of acute diarrhea in all experimental rats was assessed according to the rating scale as described previously (20,21): 0 (normal), normal stool or absent; 1 (slight), slightly wet and soft stool; 2 (moderate), wet and unformed stool with moderate perianal staining of the coat; and 3 (severe), watery stool with severe perianal staining of the coat. Scoring of acute diarrhea was conducted at 4 and 24 h after dosing. Moreover, the intestinal epithelial injuries 24 h following drug administration in rats were evaluated by examining the histological changes at macroscopic and microscopic levels as described previously (22). Normal tissues from healthy rats were also examined for the purpose of comparison.

Urinary and Fecal Excretion of COL-3

A separate experiment was performed to determine the urinary and fecal excretion of COL-3 in the absence or presence of DOX. The rats were given intravenous (10 mg/kg) or oral (30 mg/kg) dose of COL-3 when given alone or in combination with DOX (i.v., 10 mg/kg). After dosing, the rats were individually housed in metabolic cages. Urine and feces were collected at the interval of 12 h over a period of 48 h and stored at −20°C until analyzed.

Rat Plasma Protein Binding Assay

The binding of COL-3 to rat plasma proteins was determined by ultrafiltration using Microcon® YM-3 centrifugal filter device with a molecular weight cutoff of 3,000 (Millipore), as described previously (23). Briefly, experiment was performed by spiking 0.5 mL of rat blank plasma with the drug(s) to obtain final COL-3 concentrations of 5, 10, 20, and 50 μg/mL, in the absence or presence of 2 μg/mL of DOX. Each concentration was run 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 at 3,000 × *g* at 37°C for 25 min. About 150 μL of ultrafiltrate was obtained, and an aliquot of 20 μL was subject to a validated HPLC method (24) to determine the unbound drug concentration.

In Vitro Metabolic Inhibition Assay

In vitro metabolism of COL-3 was studied with postmitochondria supernatant of rat liver, as described previously (23). Briefly, 1 mL of postmitochondria supernatant (containing 30 mg protein) was spiked with COL-3 (1.5 μmol) in the absence or presence of DOX (1 μmol). The reaction was initiated by adding 1 mL of NADPH-generating solution and conducted at 37°C in a shaking water bath for 1 h. Two negative control incubations, one without drug and the

NADPH-generating solution and the other with drug but without NADPH-generating solution, were concurrently run. After 1-h incubation, reactions were terminated by cooling on ice. An aliquot (200 μL) of acetonitrile–methanol–0.5 M oxalic acid (70:20:10, v/v) was added into an aliquot of 100 μL of incubation mixture, vortex mixed (30 s), and centrifuged at 5,000 × *g* at 4°C for 10 min. The supernatant was subject to the HPLC with SPD-M10Avp UV/VIS photodiode array (PDA) detector (Shimadzu) to detect COL-3 and its possible metabolites (24). The purity of COL-3 peak was confirmed by its UV spectrum on-line scanned with PDA detector (24).

Transport of COL-3 Across Caco-2 Monolayers

The inclusion of this model in the present study is based on the fact that there are negligible differences in the transport kinetics of substrates between Caco-2 monolayers and rat intestinal epithelia (25). The permeability of COL-3 across Caco-2 monolayers was determined, as described previously (23). Caco-2 cells were seeded at a density of 10^5 cells/cm^2 on the Transwell cell culture chamber insert (12-mm diameter and 0.4-μm pore size polycarbonate membranes) (Costar, Bedford, MA, USA). Transport studies were performed on 21–25 days postseeding. Briefly, the inserts were washed 3 × 10 min with warm transport buffer (HBSS containing 25 mM of HEPES, pH 7.4) and equilibrated for 30 min in transport buffer, at 37°C. COL-3 at 50 μM in transport buffer, 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 receiver chamber contained the corresponding volume of drug-free transport buffer. COL-3 at 50 μM demonstrated negligible cytotoxicity to Caco-2 cells when incubated for 24 h (23). At predetermined time points (0.5, 1, 2, and 3 h), 50 μL of sample was withdrawn from both the receiver and donor chambers simultaneously, and the corresponding volume of fresh transport buffer was replaced. The apparent permeability coefficient (P_{app}) expressed in cm/s was determined as $P_{app} = \frac{dCV}{dtAC_0}$, 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 receiver chamber (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). $V dC/dt$ represents the rate of appearance of COL-3 in the receiver chamber.

To examine the effect of DOX on the transport of COL-3 across Caco-2 monolayers, the cells were incubated with DOX (3 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 DOX (3 μM). Incubation of DOX at 3 μM for 1 h exhibited little inhibitory effect on Caco-2 cells. The permeability of COL-3 in both directions (AP to BL and BL to AP) was determined as described above.

Determination of COL-3 and Doxorubicin by High-Performance Liquid Chromatography

The concentrations of COL-3 in rat serum, bile, urine, and feces were determined by validated HPLC methods as described previously (24). The concentrations of DOX and one of its metabolites (doxorubicin aglycone) in rat serum and tissues were determined by validated HPLC methods with fluorescence detection, as described previously (26) with modifications. An aliquot of serum (100 μL) was deproteinized by adding 200 μL of acetonitrile–methanol–0.5 M oxalic acid (70:20:10, v/v) and centrifuged at 5,000 × g, 4°C for 10 min. An aliquot of 20 μL was injected into the HPLC. The chromatography separation was conducted on an MOS Hypersil column (200 × 4.6 mm I.D., particle size 5 μm) (Hewlett Packard, Wilmington, DE, USA). The excitation and emission wavelengths were 480 and 540 nm, respectively. The mobile phase consisted of acetonitrile and 0.05 M phosphate buffer (containing 0.05% triethylamine, pH 4.00; 33:67, v/v), delivered at a flow rate of 1.0 mL/min.

To determine concentrations of DOX and doxorubicin aglycone in tissues (heart, liver, and kidney), the tissue specimen was homogenized in five volumes of the ice-cold 0.9% saline with DIAX 900 homogenizer (Heidolph, Schwabach, Germany). An aliquot of 100 μL of the homogenate was first pretreated with 20 μL of silver nitrate (33%, w/v) at 4°C for 10 min to release DOX bound to DNA by intercalation and to precipitate proteins (27,28) prior to adding 180 μL of acetonitrile–methanol–0.5 M oxalic acid (70:20:10, v/v). The mixture was centrifuged at 5,000 × g for 10 min at 4°C, and supernatant was collected. An aliquot of 20 μL was injected into the HPLC with fluorescence detection as described above.

Statistical Analyses

Statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. Comparisons of means for the pharmacokinetic parameters were made using the two independent-samples *t* test. Comparisons of average diarrhea scores among the seven experimental groups were performed using the Kruskal–Wallis test, a nonparametric one-way analysis of variance, with *post hoc* multiple comparisons (29). A *p*-value of less than 0.05 was adopted as statistically significant.

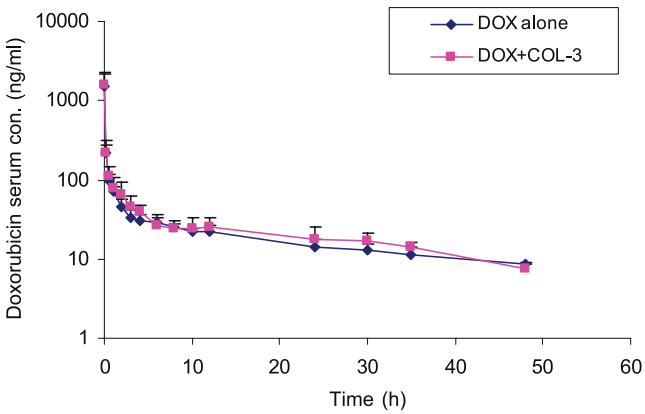

Fig. 1. Mean serum concentration-time profiles of doxorubicin in rats following its single intravenous dose (10 mg/kg) when given alone and in combination with COL-3. COL-3 was given orally at the dose of 30 mg/kg. Data represent the mean ± SD at each time point, $n = 7$.

Table I. Serum Pharmacokinetic Parameters of Doxorubicin (DOX) in Rats Following Its Single Dose Intravenous Injection (10 mg/kg) when Given Alone and in Combination with COL-3 (30 mg/kg, p.o.)

Parameters[a]	DOX (n = 7)	DOX + COL-3 (n = 6)	t test[b]
V_c (L)	3.130 ± 1.770	2.718 ± 1.471	NS
K_{10} (h^{-1})	3.550 ± 2.215	3.155 ± 1.005	NS
K_{12} (h^{-1})	8.526 ± 1.758	8.658 ± 2.173	NS
K_{21} (h^{-1})	0.1999 ± 0.0598	0.1988 ± 0.0749	NS
α (h^{-1})	12.22 ± 3.84	11.96 ± 2.67	NS
β (h^{-1})	0.05 ± 0.01	0.05 ± 0.02	NS
$t_{1/2,\alpha}$ (h)	0.06 ± 0.02	0.06 ± 0.02	NS
$t_{1/2,\beta}$ (h)	14.20 ± 3.25	14.90 ± 4.16	NS
C_{max} (ng/mL)	4781 ± 3863	4569 ± 2341	NS
CL (L/h/kg)	8.124 ± 1.477	7.473 ± 1.492	NS
$AUC_{0-\infty}$ (ng h/mL)	1266 ± 232	1385 ± 291	NS
MRT (h)	15.11 ± 3.86	15.95 ± 4.66	NS
V_{ss} (L)	121.9 ± 31.6	118.4 ± 42.0	NS

Results are given as the mean ± SD.
[a] Pharmacokinetic parameters (microconstants: V_c, k_{10}, k_{12}, and k_{21} and macroconstant slopes: α and β) are obtained from two-compartment model with first-order elimination.
[b] NS, No statistically significant differences (independent-samples t test, $p > 0.05$).

RESULTS

Effect of COL-3 on Doxorubicin Pharmacokinetics

The pharmacokinetics of DOX was characterized by a very short distribution phase with half-life $(t_{1/2,\alpha})$ of 3.6 min followed by a relatively long elimination phase with half-life $(t_{1/2,\beta})$ of 14.9 h, after intravenous injection. Co-administration of COL-3 did not alter DOX serum concentration-time profile (Fig. 1) and pharmacokinetic parameters (Table I). In addition, the concentration-time profile of doxorubicin aglycone (Fig. 2) and tissue (liver, kidney, and heart) distribution of DOX (Table II) was not altered by COL-3 ($p > 0.05$).

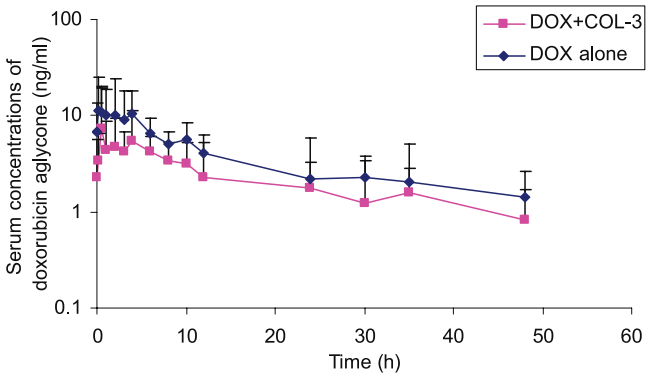

Fig. 2. Mean serum concentration-time profiles of doxorubicin aglycone in rats following single intravenous dose of doxorubicin (10 mg/kg) when given alone and in combination with COL-3. COL-3 was given orally at the dose of 30 mg/kg. Data represent the mean ± SD at each time point, n = 7.

Table II. Concentrations of Doxorubicin (DOX) and Doxorubicin Aglycone in Rat Heart, Liver, and Kidney at 48 h After Intravenous Injection of DOX (10 mg/kg) when Given Alone and in Combination with COL-3 (30 mg/kg, p.o.)

	DOX alone (n = 7)	DOX + COL-3 (n = 6)	t test[a]
DOX (ng/g)			
Heart	5212 ± 1089	5028 ± 1253	NS
Liver	1733 ± 300.0	1838 ± 255.3	NS
Kidney	8875 ± 1217	9040 ± 1319	NS
Doxorubicin aglycone (ng/g)			
Heart	41.7 ± 13.3	46.9 ± 26.2	NS
Liver	56.8 ± 27.9	64.5 ± 19.4	NS
Kidney	326.6 ± 67.6	289.8 ± 95.2	NS

Values are expressed as the mean ± SD.
[a] NS, No statistically significant differences (independent-samples t test, $p > 0.05$).

Effect of Doxorubicin on COL-3 Pharmacokinetics

Doxorubicin significantly altered both oral and intravenous pharmacokinetic profile of COL-3 in the normal (bile-duct intact) rats (Fig. 3). Doxorubicin markedly

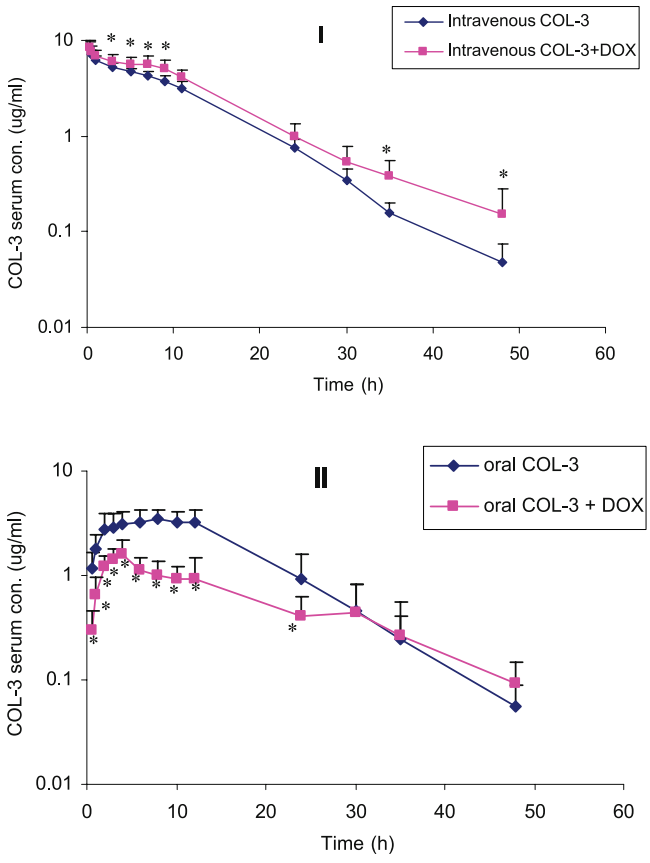

Fig. 3. Mean serum concentration-time profiles of COL-3 in rats following its single intravenous dose (10 mg/kg) (I) and oral dose (30 mg/kg) (II) with and without coadministration of doxorubicin (DOX). DOX was intravenously injected at the dose of 10 mg/kg. Data represent the mean ± SD at each time point (*COL-3 serum concentrations are significant different between single agent andcombination groups, $p < 0.05$).

reduced the extent of oral absorption of COL-3, as demonstrated by >50% reduction in the mean values of C_{max1}, C_{max2}, and $AUC_{0-\infty}$ of COL-3 when coadministered with DOX ($p < 0.05$) (Table III). Meanwhile, DOX significantly prolonged the terminal half-life and MRT of both oral and intravenous COL-3 ($p < 0.05$) (Tables III and IV). The mean CL of COL-3 was decreased by 21% in the combination group ($p < 0.05$), whereas no significant differences were found in C_0, V_{ss}, and V_z between the control (COL-3 alone) and COL-3/DOX combination groups (Table IV). Furthermore, DOX had no apparent effect on the pharmacokinetics of COL-3 in the BDC rats. As shown in Fig. 4, there were no significant differences in the serum concentrations of COL-3 at all sampling time points between the two groups ($p > 0.05$).

Assessment of Acute Diarrhea and Intestinal Damage

Acute diarrhea was evaluated at 4 and 24 h after dosing in all experimental rats (Table V). No or slight diarrhea was observed in the rats receiving COL-3 alone, whereas moderate to severe diarrhea was developed in the rats receiving DOX alone. The combination of DOX with oral COL-3 did not significantly increase the diarrhea score compared to the rats receiving DOX alone. Moreover, no or slight diarrhea was observed in the BDC rats coadministered with DOX and COL-3. Consistently, no or little macroscopic and microscopic intestinal tissue damages were observed for rats receiving COL-3, whereas DOX treatment resulted in typical macroscopic injuries such as wall thickening, hyperemia, hemorrhage, ulceration, and adhesion and microscopic damages including destruction of normal mucosal architecture, infiltration, edema, and degeneration of crypts. The extent of intestinal damages in rats receiving oral COL-3 in combination with DOX was comparable to that in rats given DOX alone.

Table III. Pharmacokinetic Parameters of COL-3 Following Its Single Oral Dose (30 mg/kg) when Given Alone and in Combination with Doxorubicin (DOX) (i.v., 10 mg/kg) in Rats

PK parameters[a]	COL-3 ($n = 7$)	COL-3 + DOX ($n = 6$)	p Value[b]
C_{max1} (μg/mL)	3.391 ± 1.035	1.623 ± 0.576[c]	0.008
C_{max2} (μg/mL)	3.773 ± 0.905 ($n = 6$)[d]	1.204 ± 0.389[c] ($n = 5$)[d]	0.000
T_{max1} (h)	3.33 ± 1.50	3.60 ± 0.55	0.718
T_{max2} (h)	10.00 ± 1.63 ($n = 6$)[d]	16.00 ± 9.38 ($n = 5$)[d]	0.291
AUC_{0-24} (μg h/mL)	60.22 ± 10.99	20.29 ± 7.32[c]	0.000
AUC_{24-48} (μg h/mL)	7.45 ± 4.27	6.34 ± 5.24	0.694
AUC_{0-48} (μg h/mL)	67.67 ± 14.08	26.63 ± 9.33[c]	0.002
$AUC_{0-\infty}$ (μg h/mL)	68.54 ± 14.67	27.75 ± 9.43[c]	0.002
λ_z (h^{-1})	0.1162 ± 0.0296	0.0776 ± 0.0178[c]	0.027
$t_{1/2,\lambda z}$ (h)	6.30 ± 1.54	9.31 ± 2.13[c]	0.017
MRT (h)	13.23 ± 1.53	17.19 ± 3.73[c]	0.028
CL/F (L/h/kg)	0.497 ± 0.145	1.202 ± 0.451[c]	0.023
V_{ss}/F (L/kg)	6.616 ± 2.365	20.39 ± 7.69[c]	0.014
V_z/F (L/kg)	4.730 ± 2.354	17.22 ± 10.01[c]	0.048
F (%)[e]	28	9	
CL (L/h/kg)[e]	0.139	0.108	
V_{ss} (L/kg)[e]	1.852	1.835	
V_z (L/kg)[e]	1.324	1.550	
MAT (h)[f]	3.36	5.74	

[a] Values are given as the mean ± SD.
[b] Independent-samples t test.
[c] Statistically different from the control (COL-3 given alone).
[d] The number of rats with the occurrence of the second peak.
[e] Calculated from the mean value.
[f] Estimated as (mean MRT_{oral} − mean MRT_{iv}).

Table IV. Pharmacokinetic Parameters of COL-3 Following Its Single Dose Intravenous Injection (10 mg/kg) when Given Alone and in Combination with Doxorubicin (DOX) (i.v., 10 mg/kg) in Rats

Parameters[a]	COL-3 ($n = 7$)	COL-3 + DOX ($n = 8$)	p Value[b]
C_0 (μg/mL)	8.300 ± 0.763	8.964 ± 1.462	0.300
AUC_{0-48} (μg h/mL)	80.62 ± 9.97	102.17 ± 18.48[c]	0.017
$AUC_{0-\infty}$ (μg h/mL)	81.43 ± 10.20	103.80 ± 18.74[c]	0.015
λ_z (h^{-1})	0.117 ± 0.019	0.095 ± 0.009[c]	0.012
$t_{1/2}$ (h)	6.04 ± 0.82	7.36 ± 0.77[c]	0.007
MRT (h)	9.87 ± 0.52	11.45 ± 0.90[c]	0.001
CL (L/h/kg)	0.125 ± 0.017	0.099 ± 0.016[c]	0.01
V_{ss} (L/kg)	1.225 ± 0.122	1.125 ± 0.159	0.201
V_z (L/kg)	1.090 ± 0.239	1.050 ± 0.211	0.733

[a] Values are given as the mean ± SD.
[b] Independent-samples t test.
[c] Statistically different from the control (COL-3 given alone).

Effect of Doxorubicin on COL-3 Disposition *in Vivo*

The renal route of elimination was negligible in the overall excretion of COL-3. There were 0.14 ± 0.08 and 0.92 ± 0.46% of COL-3 excreted in rat urine as the unchanged drug

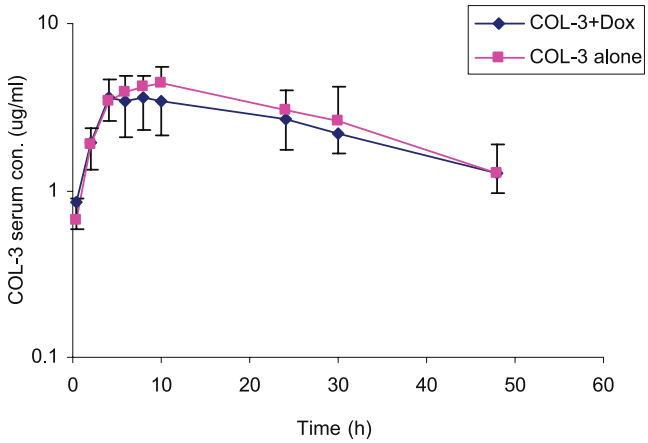

Fig. 4. Mean serum concentration-time profiles of COL-3 in bile-duct cannulated (BDC) rats following its single dose (30 mg/kg) oral administration when given alone and in combination with doxorubicin (DOX). DOX was intravenously injected at the dose of 10 mg/kg. Data represent the mean ± SD at each time point, $n = 5$.

Table V. Incidence of Acute Diarrhea After Administration of COL-3, Doxorubicin (DOX), or Their Combination in the Normal (Bile-Duct Intact) and Bile-Duct Cannulated (BDC) Rats

		Diarrhea score									
		4 h					24 h				
Experimental group	*n*	0	1	2	3	Mean rank[a]	0	1	2	3	Mean rank[a]
COL-3, p.o. (A)	12	12	0	0	0	12, C, D, E[b]	12	0	0	0	9, C, D, E[b]
COL-3, i.v. (B)	12	2	10	0	0	27.4, E[b]	0	12	0	0	26.5, E[b]
DOX, i.v. (C)	7	0	2	5	0	41.2, A, F[b]	0	1	6	0	41.5, A, F[b]
COL-3, p.o. + DOX, i.v. (D)	11	0	0	11	0	45.5, A, F, G[b]	0	0	11	0	44, A, F[b]
COL-3, i.v. + DOX, i.v. (E)	13	0	0	0	13	60, A, B, F, G[b]	0	0	0	13	59, A, B, F, G[b]
COL-3, p.o. (BDC rats) (F)	5	5	0	0	0	12, C, D, E[b]	5	0	0	0	9, C, D, E[b]
COL-3, p.o. + DOX, i.v. (BDC rats) (G)	5	3	2	0	0	19.4, D, E[b]	0	5	0	0	26.5, E[b]

Severity of diarrhea scores was assessed according to the rating scale with score: 0, normal; 1, slight; 2, moderate; and 3, severe. Values are given as the number of counts with each score.
[a] Kruskal–Wallis test ($p < 0.001$).
[b] Statistically different from groups at an experiment-wise error rate of 0.25 for *post hoc* multiple comparisons.

and the total of unchanged drug and its glucuronide conjugate, respectively, over 0–48 h following a single intravenous dose (Table VI). Doxorubicin had no apparent influence on COL-3 urinary excretion following intravenous administration of COL-3. However, unchanged COL-3 recovered in rat urine after its oral administration was significantly decreased in the combination group (34.6% of that in the control group), which was consistent with the decreased bioavailability of COL-3 in the combination group (32.1% of that in the control group; Table III).

COL-3 exhibited a significant fecal excretion, with 32.1 ± 9.9 and 38.8 ± 6.1% of unchanged drug recovered in rat feces over 0–48 h after a single intravenous and oral dose, respectively (Table VI). Doxorubicin seemed to significantly decrease the mean fecal excretion of COL-3 by 41% over 0–48 h after oral administration of COL-3 ($p < 0.05$) (Table VI). The amount of rat feces collected over 0–48 h was reduced by 74% ($p < 0.05$) in the combination group compared to the control group (Table VI).

COL-3 had a low biliary excretion. Within 24 h after a single oral administration of COL-3 (30 mg/kg), less than 3% (range, 0.37–2.13%; 1.36 ± 0.66%) and 5% (range, 1.92–4.14%; 2.97 ± 0.88%) of the dose were excreted in rat bile as the unchanged COL-3 and the total of COL-3 and its glucuronide conjugate, respectively. When rats were treated with coadministered DOX, the COL-3 biliary excretion and the total of COL-3 and its glucuronide conjugate as total dose over 24 h were 1.44 ± 0.96 and 2.31 ± 1.55%, respectively (Fig. 5). Doxorubicin had no significant influence on the biliary excretion of COL-3 and its glucuronide conjugate ($p > 0.05$).

Effect of Doxorubicin on COL-3 Plasma Protein Binding, Metabolism, and Transport in Caco-2 Monolayers *in Vitro*

No concentration dependence was observed with COL-3 over 5–50 μg/mL in both rat and human plasma. There were no significant changes ($p > 0.05$) in rat and human plasma

Table VI. Urinary and Fecal Recovery of COL-3 when It was Given Alone (i.v. 10 mg/kg or p.o. 30 mg/kg) and in Combination with Doxorubicin (DOX) (i.v. 10 mg/kg) in Rats, within 48 h Postdose

	Intravenous injection		Oral administration	
	COL-3	COL-3 + DOX	COL-3	COL-3 + DOX
Urine collected within 48 h (mL)	22.4 ± 6.1 (17.0–32.0)	25.8 ± 12.2 (15.0–51.0)	19.9 ± 6.8 (11.0–27.2)	21.6 ± 10.9 (9.6–34.5)
Urinary recovery of unchanged COL-3 (%dose)	0.139 ± 0.077 (0.062–0.228)	0.155 ± 0.092 (0.087–0.248)	0.026 ± 0.009 (0.015–0.037)	0.009 ± 0.008* (0.002–0.02)
Urinary recovery of the total of COL-3 and its glucuronide conjugate (%dose)	0.917 ± 0.464 (0.522–1.623)	1.67 ± 0.564 (0.582–1.856)	0.227 ± 0.078 (0.129–0.339)	0.289 ± 0.203 (0.137–0.588)
Feces collected within 48 h (g)	8.3 ± 2.2 (6.3–11.3)	ND	16.3 ± 2.3 (13.8–18)	4.3 ± 1.5 (2.3–5.5)*
Fecal recovery of unchanged COL-3 (%dose)	32.1 ± 9.9 (21.4–47.3)	ND	38.8 ± 6.1 (29.4–46.4)	22.9 ± 9.9 (12.3–31.9)*

Values are given as the mean ± SD (range) of five rats.
ND: Not determined as it was difficult to collect the feces because of the severe diarrhea that occurred in the rats receiving intravenous injection of both COL-3 and DOX concurrently.
*Statistically different from the COL-3 single agent group, $p < 0.05$.

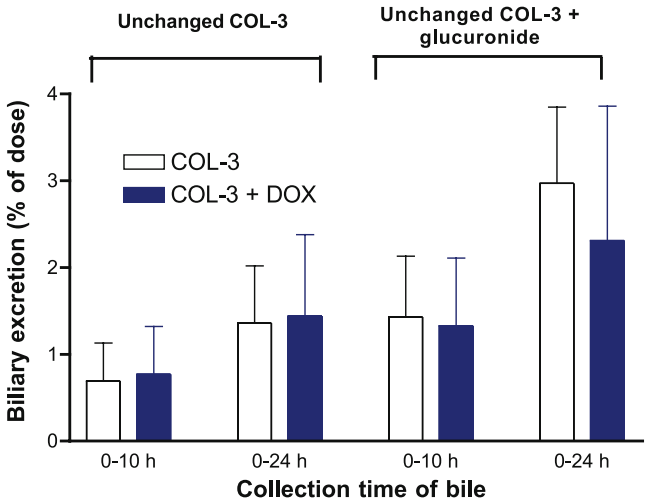

Fig. 5. Biliary excretion of COL-3 and its glucuronide conjugates following its oral administration (30 mg/kg), when given alone and in combination with DOX (i.v., 10 mg/kg) in BDC rats. Data are represented as mean ± SD of five rats.

protein binding of COL-3 over 5–50 μg/mL in the presence of 2 μg/mL DOX (Fig. 6). In addition, no metabolites were detected when COL-3 was incubated in the pooled rat liver postmitochondria supernatant in the absence or presence of DOX. The apparent permeability coefficients (P_{app}) of COL-3 across Caco-2 cell monolayers in both directions, AP to BL ($8.87 \pm 1.21 \times 10^{-6}$ cm/s) and BL to AP ($7.47 \pm 1.19 \times 10^{-6}$ cm/s), were similar ($p > 0.05$). Doxorubicin had no apparent effect on AP to BL ($8.58 \pm 1.36 \times 10^{-6}$ cm/s) permeability, but significantly increased the BL to AP ($9.01 \pm 0.09 \times 10^{-6}$ cm/s) transport of COL-3 ($p < 0.05$).

DISCUSSION

When choosing and optimizing the combinations of cytostatic agents such as MMP inhibitors with conventional cytotoxic agents, pharmacokinetic, pharmacodynamic, and toxicological properties of the combined agents must be considered with great caution. It is reasonable to propose the combination of low-toxicity COL-3 with a commonly used cytotoxic agent, such as DOX. Such combination regimens are expected to have a potentiated anticancer effect resulting from complementary mechanisms of action while the toxicity is reduced or remains unchanged. However, pharmacokinetic interactions because of altered absorption, distribution, metabolism, and excretion between the two compounds are highly likely. In particular, the oral absorption of COL-3 is highly likely to be interrupted because of acute GI toxicity induced by cytotoxic DOX. Such interactions may compromise the antitumor efficacy and safety profile, and dosage adjustment may be needed.

The present study indicated that acute DOX treatment significantly decreased the oral absorption of COL-3 (by ~60%, $p < 0.05$) and prolonged its oral or intravenous terminal half-life ($t_{1/2,\lambda z}$) and MRT ($p < 0.05$) in the bile-duct intact rats (Tables III and IV). However, DOX had no apparent effect on the serum concentration-time profile of COL-3 in the BDC rats with bile ducts exteriorized (Fig. 4). This observation provides a critical clue to identify the source of COL-3/DOX interactions in the bile-duct intact rats. On the contrary, acute COL-3 treatment did not change the pharmacokinetic and metabolic profiles of DOX as well as its tissue distribution (Figs. 1 and 2, and Tables I and II), suggesting that it does not necessarily adjust DOX dose when the two drugs are coadministered.

Like most cytostatic agents, oral COL-3 administration showed no or little gastrointestinal toxicity in rats, whereas DOX exhibited significant and typical gastrointestinal toxicity. Combination of oral COL-3 with DOX did not result in additional GI damage. These results indicate that COL-3 may be a suitable oral agent for chronic treatment of cancer, and predictable toxicity profile is expected when COL-3 is combined with DOX in cancer patients.

Numerous factors that are known to reduce the oral bioavailability of a solid drug include incomplete dissolution, metabolism in the gut lumen or by enzymes in the gut wall, substantial first-pass hepatic extraction, and P-glycoprotein (P-gp) mediated efflux (30). Increased metabolism in the gut lumen/gut wall and substantial hepatic first-pass effect were unlikely the mechanisms leading to the reduction of COL-3 oral absorption in the presence of DOX because COL-3 has an insignificant or no P-450-mediated metabolism, but undergoes phase II metabolism (i.e., glucuronidation) in a minor quantity, either in the absence (31,32) or presence of DOX (as demonstrated in the present disposition studies). Also, enhancement of P-gp mediated efflux was unlikely a source of drug interaction because COL-3 is not a P-gp substrate (23). It turned out that acute gastrointestinal (GI) toxicity induced by DOX biliary excretion directly altered the permeability of COL-3 across the intestinal mucosa and indirectly influenced COL-3 dissolution in the GI tract and COL-3 intestinal excretion via food effects.

It has been widely reported that substantial amount of DOX and its metabolites is rapidly excreted into the bile in both rats and patients (31,32). Within 12 h following intravenous injection of DOX (10 mg/kg), about 15, 5, and 5% of

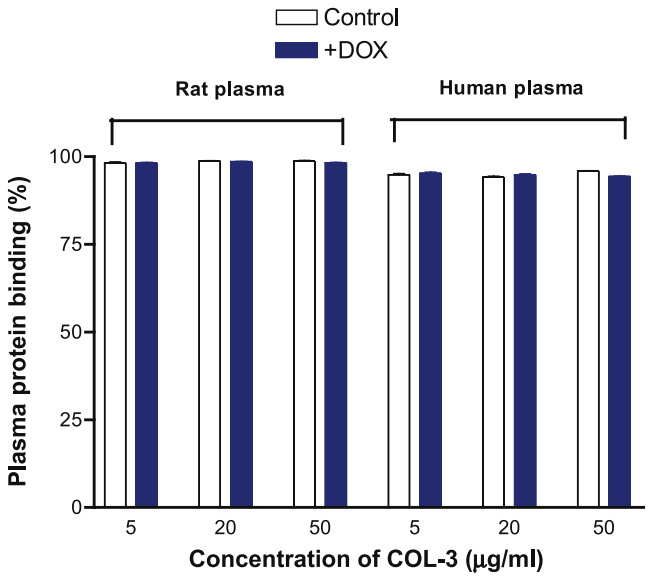

Fig. 6. *In vitro* protein binding of COL-3 in rat and human plasma, in the absence and presence of 2 μg/ml of DOX. Data represent the mean ± SD of five determinations.

the dose were recovered as the unchanged DOX, doxorubicinol, and its aglycones, respectively, in rat bile (31). In the present study, a reddish coloration of the bile was observed during the first hour to 24 h after DOX administration to BDC rats, indicating DOX and its metabolites excreted in bile. When bile flow was diverged out of the body in the BDC rats, no DOX and its metabolites were presented in the intestinal lumen. Lack of drug interaction between COL-3 and DOX in BDC rats suggested that the biliary excretion of DOX and its metabolites into the intestinal lumen was the source of drug interaction. It was likely that the presence of DOX in the GI tract was responsible for, in a large part, DOX's acute GI toxicity (i.e., mucositis), which has been widely reported in patients (33), and in the present study, severe diarrhea was noted in DOX-treated rats, especially those with bile-duct intact.

Similar to irinotecan hydrochloride (CPT-11), another cytotoxic agent, the cytotoxic activity of DOX and its active metabolite (i.e., doxorubicinol) can considerably damage GI mucosa cells both structurally and functionally (33). The acute diarrhea induced by DOX may stimulate intestinal contractility and disturb normal intestinal mucosa absorptive and secretory functions (33). The direct effect of DOX on the permeability of COL-3 across intestinal mucosa was demonstrated by the influence of DOX on the permeability of COL-3 across Caco-2 monolayers. COL-3 has been shown to permeate across the intestinal mucosa by bidirectional passive diffusion (23). In the absence of DOX, COL-3 AP to BL transport (absorption) with P_{app} of $(8.87 \pm 1.21) \times 10^{-6}$ cm/s was slightly higher than BL to AP transport (exsorption) with P_{app} of $(7.47 \pm 1.19) \times 10^{-6}$ cm/s. Doxorubicin had no apparent effect on the AP to BL transport, but significantly increased the BL to AP transport, of COL-3 ($p < 0.05$). Therefore, the net absorption of COL-3, demonstrated by the ratio of AP-to-BL P_{app} to BL-to-AP P_{app}, was decreased by 20% (from 1.19 to 0.95) in the presence of DOX. However, this direct effect was not able to account for all the 60% reduction of COL-3 oral bioavailability in DOX-cotreated rats, implying that other mechanisms for the reduction of COL-3 absorption might also be possible.

Significant decrease of food intake in DOX-cotreated rats, due to acute GI toxicity of DOX, could account for, in part, the reduction of COL-3 oral absorption. It was observed that DOX-cotreated rats barely ate. The reduction of food intake could be indirectly reflected by the significant reduction of feces excreted. The amount of feces collected within 48 h postdosing in DOX-cotreated rats was about 1/4 of that in COL-3 single drug-treated rats (Table VI). Lack of food in the GI tract is known to significantly hinder the dissolution, thereby decreasing the oral absorption, of COL-3 in rats (19).

Meanwhile, lack of food and digestive fluid in the GI tract could significantly influence the elimination of COL-3 because COL-3 is mainly eliminated by intestinal excretion, in which intestinal contents play a critical role (23). The intestinal contents draw COL-3 to diffuse from the circulation blood into the intestinal lumen and also form stable complex with COL-3 to prevent drug reabsorption during its passage down the intestine. At normal conditions, continuous entry of gastric contents and digestive fluids into the intestine provides a "sink" into which the blood-borne COL-3 is gradually eliminated with feces (23). However, because of the significant decrease of food intake in DOX-cotreated rats, no enough intestinal contents were available to bind COL-3, providing a strong "sink" condition and preventing drug reabsorption. It was observed that the overall fecal recovery of unchanged COL-3 in DOX-cotreated rats (22.9 ± 9.9% of oral dose) decreased significantly compared to that in single drug-treated rats (38.8 ± 6.1% of oral dose), within 48 h postdosing (Table VI). It was, therefore, not surprising that the elimination $t_{1/2}$ of oral or intravenous COL-3 was prolonged significantly in DOX-cotreated rats ($p < 0.05$) (Tables III and IV).

A biodistribution study of [^{3}H]-COL-3 in rats revealed that over 14 h after single oral dosing, 66 and 3% of [^{3}H]-COL-3 (including its metabolite, if any) were excreted in the feces (including the residual contents of the entire GI tract) and urine (including the residual urine from the bladder), respectively, whereas there were about 20.7% of [^{3}H]-COL-3 remaining in the tissues and visceral organs (mainly in muscle, 15.4%) and 5.8% of that in the GI tract (34). It is thus believed that there should be only a small percentage of unchanged COL-3 remaining in the body 48 h after its dosing in rats. The lower fecal recovery of COL-3 obtained in the present study was because of the fact that the recovery was assessed in terms of the drug excreted unchanged in feces only, without including the residual contents of the GI tract.

In addition, reabsorption of COL-3 in DOX-cotreated rats may further prolong the elimination $t_{1/2}$ of COL-3. As shown in Fig. 3(I), a minor second peak occurred between 5 and 9 h after intravenous injection of COL-3 in seven of eight DOX-cotreated rats, whereas these pronounced concentration fluctuations were not seen in the rats given COL-3 alone. Reabsorption was also observed after oral administration of COL-3 in the cotreated rats. As shown in Fig. 3(II), drug concentrations within 24 h postdose in the cotreated rats were significantly lower than those in the single drug-treated rats; however, after 24 h, there was no apparent difference in COL-3 concentrations between the two groups. Consistently, the AUC_{0-24} for the cotreated rats was 1/3 of that for the single-treated rats, whereas the AUC_{24-48} was almost the same for the two groups (Table II). Moreover, an apparent second peak was noted at 35 h postdose in the mean concentration-time curve of the cotreated rats [Fig. 3(II)]. These observations indicated that an apparent drug reabsorption occurred, probably after 24 h, following COL-3 oral administration in DOX-cotreated rats.

In summary, acute DOX treatment significantly decreased COL-3 oral bioavailability and prolonged COL-3 elimination, whereas acute COL-3 treatment did not modify the pharmacokinetic profile of DOX in rats. Acute GI toxicity caused, in a large part, by biliary excretion of DOX was likely the source of drug interaction between DOX and COL-3. Doxorubicin-induced GI mucosal damage might directly affect the permeability of COL-3 across intestinal wall and decrease food intake, resulting in decreased absorption of COL-3 because of hindered drug dissolution. Meanwhile, lack of food and digestive fluid in GI tract to bind COL-3 significantly reduced the fecal excretion of COL-3 and allowed drug reabsorption to occur, thus prolonging the elimination of COL-3. The combination of DOX with oral COL-3 did not generate additional toxicity compared to rats receiving DOX

alone. Further studies are required to explore the efficacy and optimized dosage regimen of this promising combination.

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