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ORIGINAL ARTICLE

Physicochemical and preclinical pharmacokinetic and toxicological evaluation of LK-423—a new phthalimido-desmuramyl-dipeptide derivative with immunomodulating activity

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Abstract

Introduction: LK-423 is a new phthalimido-desmuramyl-dipeptide derivative with immunomodulating activity. As optimized delivery to the site of action appears crucial for further preclinical development of LK-423, the aim of this study was to perform a physicochemical and preclinical pharmacokinetic and toxicological evaluation. *Methods*: The solubility, partition coefficient, permeability, and stability profile were determined. Pharmacokinetics were evaluated in rats following intravenous and oral application of LK-423, and in dogs after intravenous administration and oral administration of microcapsules, designed for colon-specific delivery of LK-423 based on pH-, time-, and enzyme-controlled release mechanisms. Additionally, the acute and subchronic toxicity was examined. *Results and discussion*: LK-423 is hydrophilic, sparingly to slightly soluble, and poorly permeable. Stability profile in aqueous solution is pH dependent. A pharmacokinetic study following intravenous application to rats and dogs revealed that LK-423 is rapidly eliminated with a short terminal phase half-life, and high plasma clearance, as well as a limited distribution to the peripheral tissue. Oral bioavailability of LK-423 is low, presumably due to low permeability. Debris of insoluble microcapsule coating in feces and obtained plasma concentration profiles confirm that LK-423 microcapsules are a promising approach for local treatment of inflammatory diseases of the large intestine. Acute and a subchronic toxicity results indicate that LK-423 is a safe and nontoxic drug under the applied experimental conditions.

Key words: *Immunomodulating activity; LK-423; pharmacokinetics; physicochemical properties; toxicology*

Introduction

Inflammation is a physiological protective response to various stimuli, such as injury or infection, in which different cells of the immune system are activated to eliminate the causative stimulus. In the case of abnormal activity in the immune response, chronic inflammation may develop, resulting in detrimental tissue damage or even development of chronic inflammatory diseases, such as inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), multiple sclerosis, rheumatoid arthritis, osteoarthritis, atherosclerosis, psoriasis, and so on[1].

There is increasing evidence that disequilibrium in cytokine balance can lead to development of the aforementioned diseases, pointing out the pivotal role of cytokines in inflammation[2]. On the cytokine level, there are two possible approaches to diminish inflammation: inhibition of proinflammatory cytokine activity or promotion of anti-inflammatory cytokine activity. Considering the latter, much research work has been done on interleukin-10 (IL-10), which is known to have a fundamental

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role in immunoregulation. IL-10 is a pluripotent cytokine, but its main biological function appears to be limitation and termination of the immune response because of inhibition of proinflammatory cytokine production such as IL-1, IL-6, IL-8, IL-12, and tumor necrosis factor α (TNFα) and induction of synthesis of their inhibitors such as soluble TNFα receptor and IL-1 receptor antagonist[3–7]. Owing to this immunoregulatory activity, IL-10 appears to be a potent therapeutic candidate in treating chronic inflammatory and autoimmune diseases. This hypothesis was confirmed by many studies on animal models, as well as in clinical trials testing recombinant IL-10 (rIL-10)[3]. However, it should be borne in mind that rIL-10 is a protein molecule, which can induce the formation of neutralizing antibodies following repeated dosing. Additionally, there are many disadvantages owing to its pharmacokinetic properties and high costs of manufacture. Therefore, a nonprotein molecule able to induce the synthesis of IL-10 in vivo would be far more convenient[8].

LK-423 {*N*-[2-(2-phthalimidoethoxy)acetyl]-L-alanyl-D-glutamic acid; CAS 142489-47-2; Figure 1}, a new phthalimido-desmuramyl-peptide compound, could be a good candidate, as it has been reported that this drug is able to regulate cytokine production[8–10]. Furthermore, LK-423 was found to enhance IL-10 production and to suppress the synthesis of proinflammatory cytokines such as TNFα and interferon γ (IFNγ). In experiments on cyclophosphamide-treated mice, it was demonstrated that LK-423 significantly increased IL-10 production in the spleen cells at both the mRNA and the protein level[8]. Conversely, IFNγ synthesis was markedly reduced in LK-423-treated mice, suggesting that LK-423 could exert immunosuppressive and anti-inflammatory activity in vivo. This has been demonstrated in previous studies on animal models of various inflammatory diseases[8–10]. LK-423's exact mechanism of anti-inflammatory action has not been elucidated yet. However, it has been suggested that LK-423 acts on various types of cells with the capacity to produce and secrete IL-10 (Th_2-type, lymphoid, and nonlymphoid cells) and thus indirectly inhibits production of proinflammatory cytokine TNFα in other types of cells. In the pharmacological studies with LK-423 performed so far, LK-423 was administered locally, either as intraperitoneal injection of solution or intracolonal injection of suspension. Because no appreciable elevation in blood IL-10 occurred, it was speculated that the LK-423 effect was limited to local regions[10].

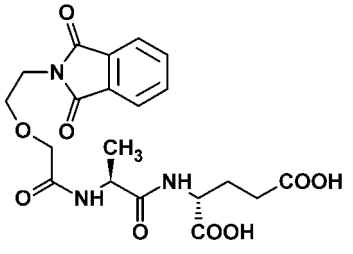

Figure 1. Chemical structure of LK-423.

As optimized delivery to the site of action appears crucial for further preclinical development of LK-423, the aim of this study was to perform a physicochemical and preclinical pharmacokinetic and toxicological evaluation. This is necessary to assess whether anti-inflammatory effect of the drug could be utilized therapeutically and for the design of pharmaceutical formulations for local or systemic drug delivery. For this purpose, the solubility, partition coefficient, permeability, and stability profile of LK-423 were determined. Furthermore, pharmacokinetic studies following intravenous and oral application of LK-423 to rats were performed. LK-423 pharmacokinetics was further evaluated in dogs after intravenous administration and oral administration of microcapsules for colon delivery. Additionally, the acute and subchronic toxicity of LK-423 was examined.

Materials and methods

Materials

LK-423 was synthesized as described by Urleb et al.[11] and supplied by Lek Pharmaceuticals (Ljubljana, Slovenia). Ketamine was purchased from Vetaquinol Biovet (Kosynierow Gdynskich, Poland). Xylazine was obtained from Chanelle Pharmaceuticals Manufacturing Ltd. (Loughrea, Ireland). Heparin was purchased from Krka (Novo mesto, Slovenia). Gelatin GE 0020 was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Peanut oil and fluorescein sodium salt were purchased from Sigma-Aldrich (Schnelldorf, Germany). Water for injections and sterile saline were provided by Lek Pharmaceuticals. Pectin (Genu® pectin type LM-104 AS-Z) was donated by Hercules Ltd. (Rijswijk, the Netherlands). Polyvinylpyrrolidone (PVP, Kollidon® 30) was purchased from BASF AG (Ludwigshafen, Germany). Eudragit® RS 30D, Eudragit® RL 30D, Eudragit® L 30D-55, and Eudragit® L 100-55 were supplied by Röhm GmbH, Chemische Fabrik (Darmstadt, Germany) and polycarbophil (PK, Noveon® AA1) by BFGoodrich Co. (Cleveland, OH, USA). Magnesium stearate, talc, and 3-ethyl citrate were supplied by Lek Pharmaceuticals. All other chemicals and solvents were of analytical grade.

LK-423 physicochemical characteristics

The aqueous solubility of LK-423 was determined in distilled water, aqueous solution of HCl (pH 3.3), and phosphate buffer (pH 6.0 and 7.5) at 25°C. A quantity of the drug exceeding the solubility was placed in 2 mL of

the corresponding media and stirred on a magnetic stirrer for 24 hours. At predefined time intervals, 0.2 mL aliquots were withdrawn and filtered. The concentration of the drug in the samples was determined using a high-performance liquid chromatography (HPLC)–UV method and the equilibrium solubility was calculated.

The partition coefficient P between octanol and 0.1 M HCl solution, and distribution coefficients D between octanol and distilled water, and octanol and phosphate buffer (pH 6.0 or 6.8), were determined using the shake flask method at 25°C and calculated as the partition ratio between octanol and aqueous phase. The concentration of LK-423 in the aqueous phase was determined using HPLC–UV.

LK-423 ionization constants K_{a1} and K_{a2} were determined using a potentiometric method at 25°C. The ionization constants were calculated according to the procedure for overlapping ionization processes as described by Albert and Serjeant[12].

Stability

The stability of LK-423 in five different aqueous media [phosphate buffers of pH 7.1 and 6.0, Ringer buffer (pH 7.4), and aqueous HCl solutions of pH 3.3 and 1.2] was determined. An appropriate amount of LK-423 was dissolved to give an initial concentration of 2000 ng/mL. Test solutions were incubated at 37°C, and LK-423 concentration was determined after 1, 2, 3, and 4 hours using HPLC–UV. Results were presented as the percentage of the drug remaining at a defined time.

Additionally, the metabolic stability of LK-423 was studied in vitro in Ringer buffer containing 10 mM D-glucose in the presence of rat jejunum tissue in EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, USA). Approximately 3-cm long tissue segments, opened along the mesenteric border, were mounted into inserts that were then placed between two cell chambers. The area of exposed tissue surface was 1 cm^2. Both chambers were filled with 2.5 mL of LK-423 solution in Ringer buffer (37°C) at a concentration of 2.36 or 118.50 mg/L and oxygenated by a stream of carbogen. At predefined times, 250 µL samples were withdrawn from both compartments and analyzed using HPLC–UV; the percentage of the drug remaining was then calculated.

Preparation and evaluation of LK-423 microcapsules

LK-423 microcapsules for delivering LK-423 to a rat (MCR) and dog (MCD) colon were prepared as described previously[13] with some modifications. First, LK-423 containing calcium pectinate cores were prepared by ionotropic gelation, by dropping dispersions of LK-423, pectin and additives, magnesium stearate for the cores of MCR[13] or PVP, and PK for the cores of MCD[14] into calcium chloride solution. After isolation and drying, the cores were coated with a retard coating using fluid bed technology (STREA-1; Niro-Aeromatic, Bubendorf, Switzerland). A 10% (w/w) of retard coating composed of a mixture of Eudragit® RS 30D and Eudragit® RL 30D at a ratio of 7:3 or 1:1 was applied to the cores for MCR or MCD, respectively. Following the 24-hour curing at 40°C, the MCR were coated with 30% (w/w) of enteric polymer Eudragit® L 30D-55 using the same coating technology. The coating was performed at inlet air temperature of 40°C and atomizing pressure of 2.0 and 1.6 bar for retard and enteric coating, respectively. The MCD were filled into gelatin capsules, which were then coated with enteric polymer Eudragit® L 100-55 by dipping the capsules into polymer dissolved in acetone. The microcapsules' size and LK-423 content was 1.6–2.0 mm and 16% for MCR and 2.0–2.4 mm and 38.8% for MCD. In vitro dissolution of LK-423 from the microcapsules was tested according to the USP XXX basket method (1 L of dissolution medium, 100 rpm, 37°C). To simulate pH changes along the rat and dog gastrointestinal tract (GIT), the following dissolution conditions were used: 4 hours in 1 mM HCl, 3 hours in phosphate buffer of pH 7.1, 1 hour in phosphate buffer of 7.6, and last 6 hours in phosphate buffer of pH 7.1 for rats; and 1 hour in simulated gastric fluid pH 1.2 and next 8 hours in phosphate buffer of pH 6.8 for dogs. At predefined intervals, 3 mL aliquots were withdrawn and the concentration of LK-423 in the samples was determined using HPLC.

Analytical methods

HPLC–MS method

Analysis of rat plasma samples was performed on an HPLC–atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) system composed of an LDC Constametric 4100 pump (TSP, Thermo Separation Products, Riviera Beach, CA, USA), a TSP (AS1000) autosampler with a fixed loop of 50 µL, an LCQ ion trap mass spectrometer (Finnigan, MAT, San Jose, CA, USA), and the Excalibur 1.1 TPS software program. The separation was carried out on a Luna C18(2) analytical column, 150 × 4.6 mm^2, 3 µm (Phenomenex®, Torrance, CA, USA) with a binary gradient (Table 1).

For detection, capillary temperature was set at 200°C, capillary voltage at −45 V, vaporizer temperature at 450°C, sheath gas flow (N_2) at 0.8 MPa, auxiliary gas flow (N_2) at 0.07 MPa, source voltage at 6.0 kV, and source current at 5 µA. An APCI (negative ionization) interface was used for online sample introduction into the mass detector (SIM: m/z = 447.5–448.5).

A 200 µL aliquot of plasma sample was denatured with 1.8 mL of acetic acid solution (pH 3.5). After 10 seconds

Table 1. HPLC–APCI–MS and HPLC–ESI–MS/MS method gradient.

HPLC–APCI–MS				HPLC–ESI–MS/MS			
Time (minutes)	% mobile phase A	% mobile phase B	Flow (mL/min)	Time (minutes)	% mobile phase A	% mobile phase B	Flow (mL/min)
0.0	100	0	1.0	0.5	100	0	0.3
2.0	100	0	1.0	1.5	0	100	0.3
2.1	50	50	1.0	2.0	0	100	0.3
7.0	20	80	1.0	3.0	100	0	0.3
7.1	20	80	0.5	5.0	100	0	0.3
9.0	0	100	0.5				
10.0	0	100	1.0				
10.1	100	0	1.0				
12	100	0	1.0				
Mobile phase A: 10 vol. % methanol in 10 mM ammonium acetate buffer, pH 8				Mobile phase A: 5 mM ammonium acetate in water			
Mobile phase B: 90% methanol in 10 mM ammonium acetate buffer, pH 8				Mobile phase B: 5 mM ammonium acetate in 99% acetonitrile			

of vigorous shaking on vortex and 5 minutes of centrifugation at 2600 × *g*, the supernatant was applied to a preconditioned [1 mL methanol and 3 mL 10 mM ammonium acetate buffer (pH 8)] solid phase extraction cartridge (Strata X cartridge 30 mg/mL; Phenomenex®). Cartridges were washed with 3 mL of 10 mM ammonium acetate buffer (pH 8.0) and 10% vol. of methanol in acetic acid solution (pH 3.5). The sample was eluted with 1 mL of methanol, evaporated to dryness under a stream of N_2 at 30°C, and reconstituted in 150 µL of formic acid solution (pH 4.0); then 50 µL was injected into the HPLC/MS system.

Calibration curves were obtained by plotting the LK-423 peak area versus the known spiked plasma concentration of the analyte using the $1/X$ weighted regression curve. The method was linear in the range of 10–1000 ng/mL. The analytical procedure was validated using analysis of quality control samples. The intra- and inter-day coefficients of variation (n = 3) for nominal concentrations of 50 and 500 ng/mL were below 10%, satisfying acceptance criteria for the method. Accuracy was 103.2% and 102.2% at 50 and 500 ng/mL, respectively. Short-term temperature stability and long-term storage stability of plasma samples were confirmed.

Analysis of dog plasma samples was performed on an HPLC–electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) system consisting of an LC pump (Series 1100; Agilent Technologies, Palo Alto, CA, USA), API 3000 triple quadrupole ESI–MS/MS detector (Applied Biosystems & MDS Sciex Foster City, CA, USA), and the Analyst 1.3.1 software program. The separation was carried out on a Luna C18(2) analytical column, 50 × 2.0 mm^2, 3 µm (Phenomenex®), and C18, 4.0 × 2.0 mm^2 guard column with a binary gradient (Table 1).

For MS detection, ESI interface (Turbospray) operating in positive mode was used. The source voltage was set at 5.5 kV, vaporizer temperature at 475°C; nebulizing gas flow (synthetic air) was 7 L/min and auxiliary gas flow (N_2) was 11 L/min. Both Q1 and Q3 were operated in unit mass resolution. For single reaction monitoring, the following mass transitions and collision energies were used: for LK-423 m/z 450.25 → m/z 303.15 at 17 V and for I.S. phenacetin m/z 180.23 → 110.22 at 29 V.

An 80 µL aliquot of plasma sample was denatured with 160 µL of methanol containing 200 ng/mL of phenacetin as internal standard. After 60 seconds of vigorous shaking on vortex, the sample was centrifuged at 36,000 × *g* for 5 minutes at 4°C. A 10 µL aliquot of supernatant was injected into the HPLC–ESI–MS/MS system.

The analytical procedure was validated using analysis of quality control samples. Calibration curves were obtained by plotting the LK-423/internal standard peak area versus the known spiked plasma concentration of the analyte using the $1/X$ weighted regression curve. Linearity was obtained in the range of 5–10,000 ng/mL. The intra- and inter-day coefficients of variation were below 10%. Accuracy of the method at nominal concentrations of 50, 500, and 5000 ng/mL was 97.0%, 104.8%, and 97.4%, respectively.

HPLC–UV method

For analysis of samples in physicochemical characterization, stability, dissolution, and in vitro GIT permeability experiments, an HPLC method with UV detection was applied. An Agilent 1100 Series HPLC system (Agilent Technologies) equipped with Hewlett-Packard 3D ChemStation software was used. The separation was achieved on a Hypersil ODS analytical column (5 µm, 250 × 4.6 mm^2; Thermo Hypersil-Keystone, Cheshire, UK) at room temperature with a mobile phase flow rate (acetonitrile:water:trifluoroacetic acid = 200:800:1) of 1.5 mL/min. LK-423 was detected by setting the UV diode array detector at 220 nm. The samples in media with pH above 3.3 were acidified to pH 1 before analysis to avoid degradation.

Fluorescein was detected using a fluorescence (λ_{EX} 485, λ_{EM} 535) microplate reader from GENious (Tecan, Salzburg, Austria).

Animals

Pharmacokinetic studies were performed on rats and dogs; for toxicological assessments, mice and rats were used. NMRI mice and male Wistar rats were provided by the Lek animal breeding unit (Lek Pharmaceuticals) and German shepherd dogs by Military Medical Academy (Belgrade, Serbia). Animals involved in pharmacokinetic studies were individually housed and fasted overnight before dosing. In all studies a 12-hour light/dark cycle and free animal access to food and water was applied. Experiments were conducted in accordance with the principles and procedures of the NIH Guide for Care and Use of Laboratory Animals. The study protocols for experiments on rats and mice were approved by the Veterinary Administration of the Republic of Slovenia, Ministry of Agriculture, Forestry and Food, under the number 323-02-236/2005/2. The study protocols for experiments on dogs were approved by the Ministry of Defense of the Republic of Serbia, Department for Military Medical Academy, Ethical Committee for Research, Care and Use of Laboratory Animals (number 27-104/15.03.2006).

In vitro intestinal transport studies

These experiments were performed analogously to those previously described[15]. The small intestine was excised and placed in an ice-cold solution of 10 mM D-glucose in Ringer buffer bubbled with carbogen (pH 7.4) immediately after the execution of the animal (male Wistar rats, deprived of food 18 hours before the experiment). The transport studies were carried out on jejunum (25 cm distally of the pyloric sphincter) and ileum (15 cm proximally of the cecum), excluding segments with Peyer's patches. The 3-cm long tissue segments were opened along the mesenteric border and mounted onto an insert with an exposed tissue area of 1 cm^2. In the next step, the inserts were placed between two EasyMount side-by-side diffusion chambers (Physiologic Instruments). The tissue was incubated with Ringer buffer containing 10 mM mannitol and 10 mM D-glucose on the mucosal and serosal side of the tissue, respectively, and gassed with carbogen at 37°C. After a 25-minute preincubation period, the buffer on the donor side (mucosal or serosal) was replaced with 2.5 mL of a solution containing 5 μM fluorescein, 250 μM LK-423, and 10 mM mannitol or D-glucose if studying mucosal-to-serosal (m-to-s) or serosal-to-mucosal (s-to-m) transport, respectively. Samples of 250 μL were withdrawn from the acceptor chamber at 25-minute intervals until the end of the experiment (175 minutes) and replaced with Ringer buffer containing 10 mM D-glucose or mannitol at the serosal (m-to-s transport) or mucosal (s-to-m transport) side, respectively.

Tissue viability and integrity during the experiment was controlled by monitoring the potential difference (PD), short circuit current (I_{SC}), and trans-epithelial electrical resistance (TEER, determined according to Ohm's law) using a multichannel voltage current clamp (model VCC MC6; Physiologic Instruments) every 25 minutes and by determining the permeability of fluorescein sodium salt, a hydrophilic transport marker. Additional confirmation of tissue viability was given by the increase of PD, I_{SC}, and TEER after the addition of D-glucose to the mucosal side of the tissue at the end of the experiment.

The apparent permeability coefficient (P_{app}) was calculated according to the following equation:

$$P_{app}(\text{cm/s}) = \frac{dQ}{dt}\frac{1}{AC_0},$$

where dQ/dt is the steady-state appearance rate on the acceptor side of the tissue, A is the exposed area of the tissue (1 cm^2), and C_0 is the initial concentration of the drug in the donor compartment.

Pharmacokinetic studies

Rats

To facilitate intravenous drug administration and blood sample collection in rats, chronic indwelling jugular catheters (inner diameter 0.5 mm, outer diameter 1 mm; Micro-Renathane Tubing; Braintree Scientific Inc., Braintree, MA, USA) were implanted under anesthesia with ketamine 60 mg/kg and xylazine 8 mg/kg into the jugular veins of rats[16].

For intravenous application (7.5 mg/kg) in rats (n = 10), LK-423 was dissolved in sterile phosphate buffer of pH 7.4 at a concentration of 7.5 mg/mL immediately before drug administration. For the oral route of application, 75 mg/kg of LK-423 was administered as suspension in 1% gelatin solution (n = 9) or in the form of LK-423 microcapsules (MCR, n = 9) by gavage. Blood samples (0.5 mL) were withdrawn into EDTA-containing tubes (BD Microtainer K2E, Franklin Lakes, NJ, USA) 0.5-hour predose and at 10, 20, 30, and 45 minutes, 1, 1.5, 2, 2.5, 4, 6, and 9 hours (intravenous injection); at 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 12 hours (oral suspension); and at 1, 2, 3, 4, 6, 8, 12, and 24 hours (oral MCR microcapsules). After each sampling, the volume of blood taken was replaced by the same volume of saline (NaCl 0.9%; Lek Pharmaceuticals).

The blood samples were immediately centrifuged (1000 × g, 10 minutes) and the plasma obtained was frozen in liquid nitrogen. The plasma samples were stored at −80°C until analysis.

Dogs

The dogs (25 ± 5 kg) were administered intravenously ($n = 3$) 5 mg/kg of LK-423, dissolved in sterile phosphate buffer (pH 7.4) at a concentration of 15 mg/mL immediately before application, through the cephalic vein. Orally 50 mg/kg of LK-423 in the form of microcapsules (MCD) were administered ($n = 5$). Blood samples (4 mL) were withdrawn from the cephalic vein 0.5-hour predose and at 10, 20, 30, and 45 minutes; at 1, 1.5, 2, 3, 4, 6, 9, 12, and 24 hours (intravenous injection); and at 1, 2, 3, 4, 6, 8, 10, 12, 14, 24, and 48 hours (oral MCD microcapsules).

Plasma samples were obtained immediately by centrifugation (1000 × *g*, 10 minutes) and stored at −80°C until analysis.

Pharmacokinetic analysis

Compartmental pharmacokinetic analysis was performed by a population pharmacokinetic approach using software NONMEM (version V, level 1.1; GloboMax LLC, Ellycott City, MD, USA) and Visual-NM (version V; R.D.P.P., Montpellier, France), a Windows-based interface for NONMEM. The first-order conditional method (FOCE) was used for parameter estimation. The structural model used was a two-compartment model with first-order absorption and absorption lag time as implemented in the ADVAN4/TRANS4 PREDPP subroutine. If possible, inter-animal variability in pharmacokinetic parameters was estimated by an exponential model. Residual variability (σ^2), comprising intra-animal variability and measurement error, was modeled by proportional, additive, and combination error models. The modeling strategy aimed at a final model with minimal structural and variability parameters needed to adequately describe the data. The model adequacy was evaluated by standard diagnostic plots of predicted versus observed concentration and weighted residuals versus observed concentration or time. Additional criteria were convergence of minimization, number of significant digits more than three, successful covariance step, and gradients in the final iteration in the range between 10^{-3} and 10^2. Alternative models were compared by the likelihood ratio test. The criterion for selection of a model was a change in the minimum value of objective function (ΔOBJ) of at least 3.84 per one additional parameter, corresponding to $P < 0.05$.

Toxicity testing

Acute toxicity screening

Acute toxicity screening was performed on male mice and male rats. Clinical observation following different modes of application of various doses of LK-423 was performed over a period of 14 days after the application. Each dose administered was tested on six animals.

Following intravenous application of 100 and 200 mg/kg and oral application of 25, 200, and 1000 mg/kg of LK-423, acute toxicity screening was performed on mice. For this purpose, LK-423 suspensions in water for injections at concentrations of 10 and 20 mg/mL were prepared and administered in the tail vein. For oral toxicity screening, LK-423 suspensions in peanut oil at concentrations of 2.5, 20, and 100 mg/mL were applied to mice by gavage.

Following intraperitoneal application of 25 and 1000 mg/kg of LK-423 (suspended in peanut oil at concentrations of 2.5 and 100 mg/mL), acute toxicity screening was performed on rats. The suspension was administered by slow injection.

Subchronic toxicity study

A subchronic toxicity study lasting 90 days with an additional 45-day recovery period was performed on rats. Each day the rats were dosed with 25 mg/kg of LK-423 (suspended in peanut oil at a concentration of 2.5 mg/mL) by intraperitoneal injection. In total, 40 rats were divided into four groups (10 rats per group), two for a 90-day, and two for a 135-day study. Two control groups received peanut oil, whereas the remaining groups received LK-423. Clinical observation, food consumption, and body weight were recorded. At the end of the experiment blood, biochemical and urine analyses, necropsy, and pathohistology were performed.

Statistical analysis

All results were expressed as the mean ± SD. Statistical analysis was performed using the SPSS software (version 12.0.1, SPSS Inc., Chicago, IL, USA, 2004). The one-way analysis of variance (ANOVA) test followed by the least significant difference test for post hoc comparison and independent samples *t*-test were applied for evaluation of the results. The significance level was set at 0.05.

Results

Physicochemical characteristics

LK-423 is a hydrophilic drug ($\log P = 0.065 \pm 0.015$) with two carboxylic groups; therefore two ionization constants were determined ($pK_{a1} = 3.37 \pm 0.06$ and $pK_{a2} = 4.94 \pm 0.01$). The distribution coefficient ($\log D$) increased with the pH of the medium from 0.53 ± 0.02 in bi-distilled water to 1.77 ± 0.08 in phosphate buffer (pH 6) and 2.10 ± 0.15 in phosphate buffer (pH 6.8). Regarding the solubility, it could be classified among sparingly soluble drugs in phosphate medium of pH 7.5 (20.05 ± 0.44 mg/mL) and among slightly soluble drugs in all

other media tested [2.48 ± 0.24 mg/mL in bi-distilled water, 2.41 ± 0.15 mg/mL in 1 mM HCl, 6.00 ± 0.05 mg/mL in phosphate buffer (pH 6)]. At least 80% of the equilibrium solubility of LK-423 determined after 24 hours was reached after 2 hours of dissolution. Despite the instability of LK-423 in media with pH higher than 6, no decrease in the concentration of LK-423 during the solubility determination occurred, because there was an excess amount of the drug that replaced the drug as it degraded.

Stability of LK-423

Figure 2 depicts LK-423 stability profiles in various media. It may be seen that the percentage of the remaining (undegraded) LK-423 was markedly dependent on the pH of the medium. In acidic media the drug was stable, because no degradation was observed within 4 hours and even in 24 hours (data not shown). On the contrary, LK-423 was found to be very unstable in buffer solutions of pH above 6. The lowest degradation was observed in phosphate buffer of pH 6, where only about 4% of the drug degraded in 4 hours. LK-423 appeared to be the most unstable in Ringer's buffer (pH 7.4) as less than 85% and 45% of LK-423 remained intact after 1 and 4 hours of incubation, respectively. Additionally, it was demonstrated that LK-423 degradation kinetics was not influenced by the presence of jejunum tissue (Figure 2) regardless of its concentration, suggesting that LK-423 was not subjected to metabolism by enterocytes.

LK-423 microcapsules

Dissolution profiles of LK-423 microcapsules used in rats and dogs are presented in Figure 3. To achieve colon-specific delivery of LK-423, the release characteristics of microcapsules were adjusted to the gastrointestinal conditions in the two animal species used in the experiments. In the stomach, LK-423 release was prevented by outer enteric coating. Premature release in the small intestine was prevented by inner retard coating that dissolved after a certain time, corresponding to the small intestine transit time of approximately 3 hours[17] in rats and 1–2 hours[18] in dogs. In the colon, LK-423 release was greatly enhanced because of colon-specific degradation of the pectin core by enzymes of colonic resident bacteria.

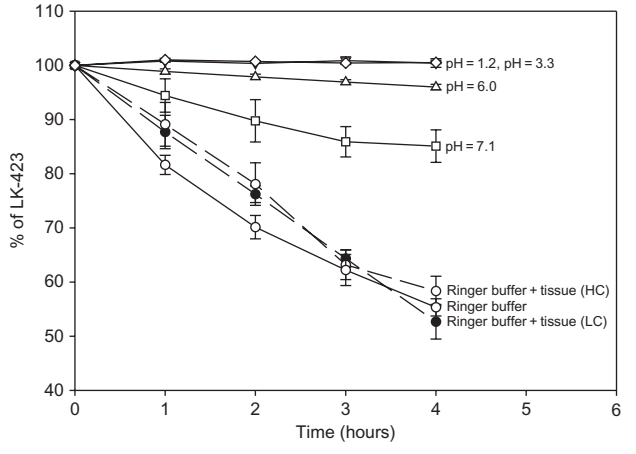

Figure 2. LK-423 stability in various media and in the presence of intestinal tissue at high (HC, 118.50 mg/L) and low (LC, 2.36 mg/L) LK-423 concentrations.

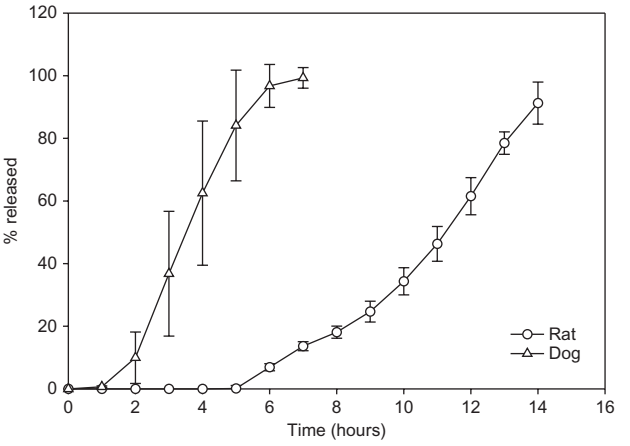

Figure 3. Release profiles of LK-423 from microcapsules for rats (MCR) and dogs (MCD). Dissolution methods mimicking conditions in the gastrointestinal tract were as follows: 4 hours in pH 3.3, 3 hours in phosphate buffer (pH 7.1), 1 hour in phosphate buffer (pH 7.6), and last 6 hours in phosphate buffer (pH 7.1) for rats; and 1 hour in simulated gastric fluid (pH 1.2) and next 8 hours in phosphate buffer (pH 6.8) for dogs.

In vitro intestinal transport studies

The apparent permeability coefficient of LK-423 across rat jejunum was determined using modified Sweetana-Grass diffusion cells. The concentration of LK-423 on the acceptor side increased slowly but linearly, regardless of the direction of transport. It was found that LK-423 is a poorly permeable compound with a P_{app} from m-to-s side of $(4.5 \pm 1.1) \times 10^{-6}$ cm/s. The P_{app} in the opposite direction (s-to-m side) was practically the same [$(4.8 \pm 1.4) \times 10^{-6}$ cm/s, $P > 0.05$]. Furthermore, the P_{app} from m-to-s side of the ileum was estimated at $(5.1 \pm 0.9) \times 10^{-6}$ cm/s and did not differ significantly from P_{app} across the jejunum ($P > 0.05$). For fluorescein, the P_{app} from m-to-s and s-to-m sides of the jejunum were estimated at $(4.7 \pm 0.9) \times 10^{-6}$ cm/s and $(5.5 \pm 1.5) \times 10^{-6}$ cm/s, respectively, and P_{app} from m-to-s of the ileum was estimated at $(5.0 \pm 0.7) \times 10^{-6}$ cm/s.

Pharmacokinetics of LK-423

LK-423 was quantified in the majority of plasma samples, except following oral application of microcapsules in rats, where the plasma concentration of LK-423 was below quantification limit in all samples taken, indicating negligible absorption. Consequently, the results of this experiment were excluded from further pharmacokinetic analysis. However, examination of feces revealed debris of microcapsule coatings, suggesting that drug containing pectin cores were disintegrated and the drug completely released.

A two-compartment model adequately described the plasma concentration profiles of LK-423 in rats and dogs. The model was simultaneously fitted to the observed concentration time data following bolus intravenous and oral administration. In each species, clearance (CL), central volume (V_1), peripheral volume (V_2), intercompartmental clearance (Q), bioavailable fraction after oral administration (F), absorption rate constant (K_a), and absorption lag time (T_{lag}) were estimated. Because of the limited amount of data, no variability of structural parameters in dogs could be estimated. However, in rats inter-animal variability of CL, V_2, and K_a was estimated based on an exponential model. A proportional error model was used to characterize the residual intra-animal variability in rats, whereas a combination error model comprising additive and proportional components most adequately described the residual variability in dogs. Parameters of the structural model were generally accurately estimated, with coefficients of variation between 0.4% and 34% in rats and 0.3% and 36% in dogs. Population clearance was estimated at 2.02 and 0.102 L/h/kg in rats and dogs, respectively. Bioavailable fraction in rats was 1.35% after oral administration of drug suspension and 1.17% in dogs after oral administration of microcapsules. Inter-animal variability of CL, V_2, K_a, and T_{lag} in rats was 11.8%, 64.3%, 72.2%, and 16.0%, respectively (Tables 2 and 3). Observed and model-predicted LK-423 concentration profiles in rats and dogs are presented in Figure 4. Apparently, the initial phase of plasma concentration profile after oral application of microcapsules in dogs is poorly predicted. This could be ascribed to high inter-animal variability of absorption lag time, which is governed by many physiological factors, such as gastric emptying and intestinal transit time. Nevertheless, the

Table 2. Population pharmacokinetic parameters of LK-423 in rats.

	Estimate	Inter-animal variability
Parameter	Mean (95% CI)	CV% (95% CI)
CL (L/h/kg)	2.02 (1.36, 2.68)	11.8 (3.3, 16.4)
V_1 (L/kg)	0.312 (0.162, 0.462)	—
V_2 (L/kg)	0.179 (0.136, 0.222)	64.3 (0.0, 93.9)
Q (L/h/kg)	0.335 (0.114, 0.556)	—
F (%)	1.35 (1.34, 1.36)	—
K_a (h^{-1})	0.309 (0.182, 0.436)	72.2 (37.3, 95.0)
T_{lag} (hours)	0.189 (0.159, 0.219)	16.0 (0.0, 25.1)
Residual error		
CV (%)	47.54 (38.90, 54.83)	

Table 3. Population pharmacokinetic parameters of LK-423 in dogs.

	Estimate
Parameter	Mean (95% CI)
CL (L/h/kg)	0.102 (0.0573, 0.147)
V_1 (L/kg)	0.0143 (0.00413, 0.0245)
V_2 (L/kg)	0.0822 (0.0363, 0.128)
Q (L/h/kg)	0.0397 (0.0156, 0.0638)
F (%)	1.17 (0.223, 2.12)
K_a (h^{-1})	0.0272 (0.0101, 0.0443)
T_{lag} (hours)	7.91 (7.86, 7.96)
Residual error	
CV (%)	38.73 (31.35, 44.92)
SD (ng/mL)	34.35 (22.62, 42.99)

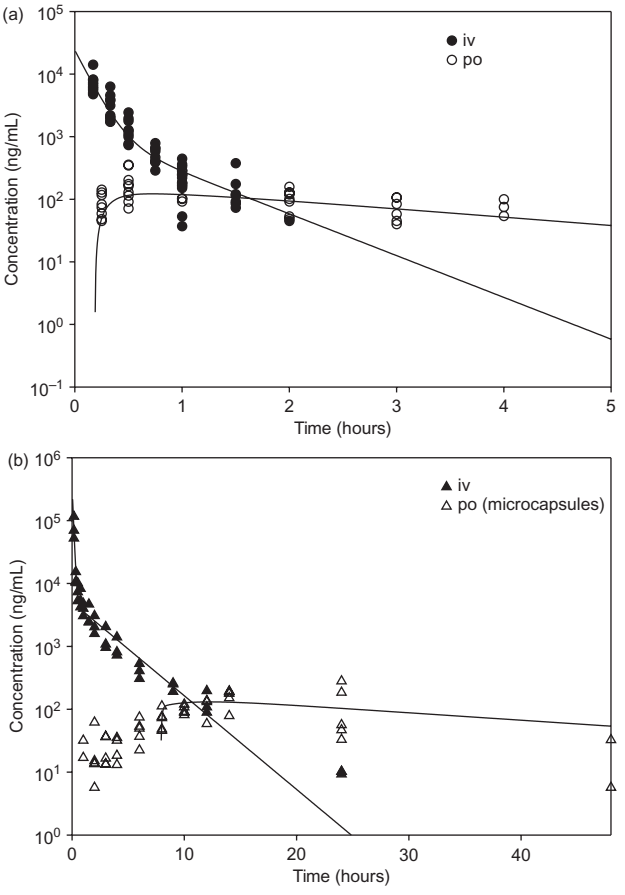

Figure 4. Individual observed and mean model-predicted plasma concentrations of LK-423 in (a) Wistar rats following intravenous (7.5 mg/kg) and oral (75 mg/kg) administration and in (b) dogs following intravenous (5.0 mg/kg) and oral (50 mg/kg, microcapsules MCD) administration.

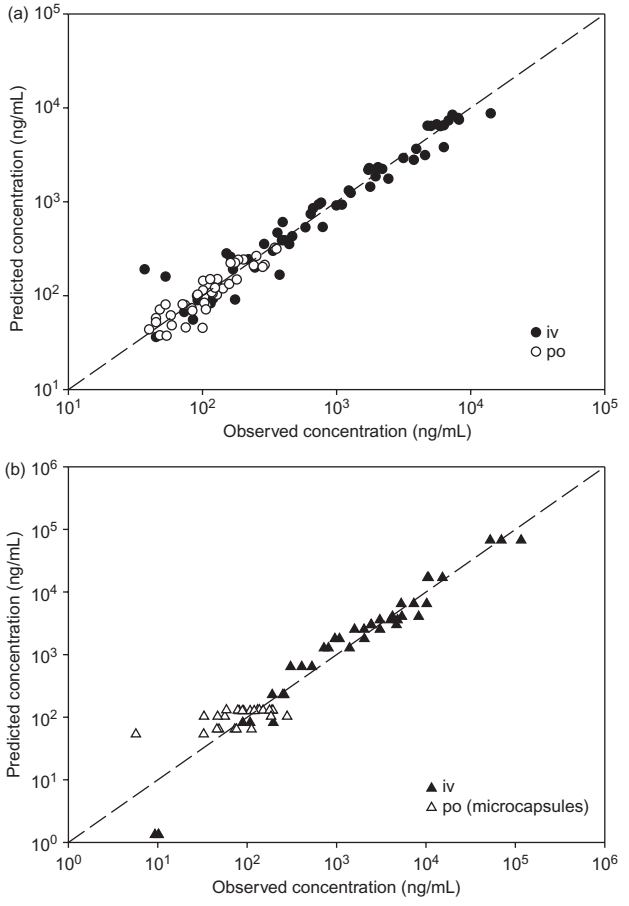

Figure 5. Individual model-predicted versus observed concentrations in (a) rats and (b) dogs, with line of identity.

mean estimate of T_{lag} (7.91 hours) indicates limited absorption in the upper part of the GIT, suggesting effective delivery of LK-423 to the colon. The plots of individual predicted versus observed concentrations demonstrated no bias (Figure 5).

Toxicity testing

All mice and rats survived single-dose application (acute toxicity screening). Symptoms of mild sedation were observed between 1.5 and 3 hours in mice receiving 200 mg/kg of LK-423 intravenously and in mice and rats receiving 1000 mg/kg orally and intraperitoneally, respectively, over a period between 2 and 6 hours. No LK-423 toxicity was observed in acute toxicity screening.

Similarly, in the subchronic toxicity study, all the rats survived 90 days of treatment with LK-423 at a daily dose of 25 mg/kg. No clinical symptomatology or deviations from normal behavior were observed. Moreover, there were no differences in food consumption, body weight, hematological parameters, urine analysis results, or organ weights between the control groups and the treated groups. Results of the biochemical blood analysis are summarized in Table 4.

Discussion

Biopharmaceutical, pharmacokinetic, and toxicological characteristics of LK-423 are of critical importance for the design of pharmaceutical formulations for local or systemic drug delivery. According to determined solubility and European Pharmacopoeia solubility classification, LK-423 is a sparingly to slightly soluble hydrophilic drug with pH-dependent solubility. In this respect low GIT permeability of LK-423 was expected, especially in the small intestine where the pH of intestinal fluid was about 7[19] and the drug was almost completely ionized. Limited membrane permeability was confirmed by determination of relatively low apparent permeability coefficients of LK-423 across rat jejunum and ileum. LK-423 contains a dipeptide moiety, which could be responsible for the active transport of LK-423 across GIT membranes through Pept1 transporters. The latter accounts for the high absorption fraction of many peptides and peptidomimetic drugs[20,21]. However, P_{app} in the opposite (s-to-m) direction was practically equivalent to that from the m-to-s side ($P > 0.05$), suggesting that LK-423 is absorbed passively, most probably through the paracellular route, which is a common way of absorbing small hydrophilic molecules.

Additionally, it was demonstrated that LK-423 is very unstable, especially when dissolved in media with pH above 6. As the pH of rat intestinal fluid ranges between 6.5 and 7.1[19], LK-423 might degrade to some extent in the lumen before being absorbed. Degradation of the drug in the lumen could also be promoted by resident bacterial or endogenous enzymes, leading to a lower fraction of the drug being available for absorption or delivery to the local site of action. Nevertheless, the results of the stability study in the presence of intestinal tissue indicate no influence of the tissue on LK-423 degradation rate.

The results of the pharmacokinetic study following intravenous bolus injection revealed that LK-423 is rapidly eliminated in rats with a terminal phase half-life of only 27 minutes, whereas in dogs a considerably longer terminal phase half-life of 127 minutes was determined. The extremely high total plasma clearance estimated in rats (2.02 L/h/kg) could be ascribed to drug degradation in plasma. However, the difference in clearance observed in rats and dogs is huge and cannot be explained by allometric scaling, which further implies that different enzymatic mechanisms are involved in LK-423 elimination in these two animal species.

Furthermore, low values of distribution volume of LK-423 in both species imply limited distribution to peripheral tissue. Based on parameters of the

Table 4. Biochemical blood analysis after 90-day and 90-day plus 45-day recovery period in the subchronic toxicity study in Wistar rats.

		90 days		90-day+45-day recovery	
	[a]Pretreatment	Control	LK-423	Control	LK-423
K^+ (mM/L)	6.3 ± 1.42	4.6 ± 0.28	4.6 ± 0.33	4.7 ± 0.2	4.6 ± 0.4
Na^+ (mM/L)	141.8 ± 4.1	143.8 ± 2.9	145.7 ± 1.3	140.4 ± 1.7	141.3 ± 1.8
Cl^- (mM/l)	105.8 ± 2.3	107.3 ± 2.3	110.9 ± 1.5*	107.9 ± 0.9	109.5 ± 1.1*
Urea (mM/L)	11.1 ± 1.84	6.9 ± 1.2	6.4 ± 0.8	7.5 ± 0.7	7.0 ± 0.9
Glucose (mM/L)	10.18 ± 1.33	6.1 ± 0.8	6.5 ± 0.7	6.2 ± 1.2	6.9 ± 0.9
Creatinine (μM/L)	49.3 ± 5.2	51.6 ± 5.0	48.7 ± 5.8	53.9 ± 5.0	50.7 ± 6.9
AST (U/L)	113.0 ± 16.1	81.6 ± 22.9	67.0 ± 14.1	79.1 ± 15.1	55.9 ± 8.7*
ALT (U/L)	20.6 ± 5.5	20.8 ± 17.0	12.3 ± 3.1	18.6 ± 5.3	14.8 ± 2.5
CK (U/L)	761.1 ± 231.4	398.2 ± 119.9	190.6 ± 101.1*	468.5 ± 155.9	134.8 ± 62.7*
LDH (U/L)	1111.6 ± 373.8	1534.6 ± 449.5	772.3 ± 489.8*	1761.1 ± 625.3	649.6 ± 363.0*
ALP (U/L)	268.1 ± 71.5	49.8 ± 7.4	45.6 ± 3.2	35.7 ± 5.8	31.1 ± 5.5
Total bilirubin (μM/l)	3.2 ± 0.5	3.1 ± 0.4	3.5 ± 0.3	2.7 ± 0.5	2.7 ± 1.2
Total protein (g/L)	54.0 ± 2.0	60.3 ± 3.7	58.2 ± 1.3	61.1 ± 2.5	59.6 ± 3.1
Cholesterol (mM/L)	1.0 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.3	0.7 ± 0.2

Values are mean ± SD; *$P < 0.05$ (independent samples t-test compared with control group). [a]Pooled results of all four groups are reported since their pretreatment values did not differ (ANOVA).

two-compartment model summarized in Tables 2 and 3, the distribution of LK-423 to peripheral tissues is very rapid with half-lives of 5 and 4 minutes in rats and dogs, respectively.

Following oral administration of LK-423 to rats as suspension in gelatin solution, maximum plasma concentration was reached in <1 hour. Additionally, the considerably longer terminal phase half-life observed after oral application compared with the half-life after intravenous application indicates a flip-flop phenomenon, with the absorption phase being the rate-limiting step of overall drug elimination. The very poor bioavailability of LK-423 (F = 1.35%) could be associated with several causes. With regard to the results of the stability study in the presence of intestinal tissue demonstrating that LK-423 is not subjected to metabolism in the enterocytes, it could be concluded that the LK-423 gut wall first-pass effect does not noticeably contribute to low systemic availability. Nevertheless, degradation of the drug in lumen by endogenous enzymes and resident bacteria cannot be excluded. Additionally, poor permeability characteristics could negatively affect bioavailability. However, there are several drugs with similar values of P_{app}—pravastatin $(2.47 \pm 0.70) \times 10^{-6}$, ranitidine $(3.97 \pm 1.00) \times 10^{-6}$, cimetidine $(2.15 \pm 0.37) \times 10^{-6}$, and atenolol $(3.74 \pm 0.96) \times 10^{-6}$ cm/s (unpublished data)—with relatively high absorption fractions in humans of 34%, 50%, 90%, and 50% for pravastatin[22], ranitidine, cimetidine, and atenolol[23], respectively, indicating that, besides limited permeability, there are other reasons contributing to the poor bioavailability of LK-423 in rats.

Following oral administration of LK-423 microcapsules to rats, systemic plasma concentrations were below the limit of quantification of the analytical method. These results indicate that LK-423 absorption was hindered. The colon-specific drug delivery using microcapsules is based on a combination of pH-, time-, and enzyme-controlled release mechanisms. In accordance with this design, the microcapsules are supposed to behave in vivo in GIT as follows: the outer enteric coating protects the drug delivery system in the stomach and the inner retard coating prevents drug release in the small intestine, whereas the polysaccharide core, which is degraded by enzymes of colonic resident bacteria, assures complete release of the drug in the large intestine. The effectiveness of microcapsules to deliver the drug in the colon was supported by two findings. First, the absence of undegraded microcapsules in feces (feces examination revealed only debris of insoluble microcapsule coating) confirms partial degradation of both coatings and complete degradation of the pectin cores, enabling complete release of LK-423. Secondly, nonappearance of LK-423 in systemic circulation suggests that LK-423 was released in lower parts of the GIT. In case of drug release in the upper parts of GIT, LK-423 would be absorbed as it was observed following oral administration of LK-423 suspension. Based on in vivo data and in vitro dissolution profiles, it may be assumed that microcapsules are an effective colon-specific drug delivery system that prevents drug release and thus also degradation before reaching the colon, enabling higher local concentration of the drug at the site of action.

Following oral administration of LK-423 microcapsules to dogs, the initial phase of plasma concentration profiles indicates limited absorption in the upper parts of GIT. Appearance of LK-423 in systemic circulation was highly variable, presumably because of variability in the gastric emptying rate. Again debris of microcapsule

insoluble coating and extremely swollen transparent cores were found in feces. LK-423 absorption was very slow, resulting in a flip-flop effect. Regarding all these facts and the plasma concentration profiles, it is possible that the drug is absorbed from distal parts of GIT. The bioavailability in dogs was low ($F = 1.17\%$), although markedly higher than following microcapsule administration in rats. Such species differences in availability were most probably associated with the higher permeability of dogs' intestinal wall because of larger pore size and higher frequency of pores[24].

Many studies on animal models and in humans revealed that systemic administration of rIL-10 was not as successful as expected, and according to the consideration of Herfarth and Schölmerich[25], one possible reason for the disappointing efficacy of systemically administered rIL-10 to patients suffering from CD could be the low concentration of the drug in the intestine. Therefore, it is thought that locally delivered IL-10 would be more effective and could even circumvent the systemic side effects related to high doses. This idea was realized by Steidler et al.[26], who observed significant improvement of colitis in two mouse models after intragastric application of genetically engineered IL-10 secreting *Lactobacilus lactis*. The same result was observed in the case of LK-423 when administered rectally to rats pretreated with trinitrobenzenesulfonic acid or dextransulfate sodium[9,10]. Therefore, LK-423 microcapsules for colon delivery seem to be a promising drug delivery system for local targeting of inflamed colonic tissue. However, the efficacy of LK-423 microcapsules for colon-specific delivery should be further evaluated in animal models of IBD.

In addition to pharmacokinetics, the toxicological characteristics of LK-423 were studied. As no animal died in acute toxicological screening, nor were any adverse effects observed, it can be concluded that the NOAEL (no observed adverse effect level) in mice following intravenous and oral application is above 200 and 1000 mg/kg, respectively. Similarly, NOAEL in rats following intraperitoneal application is higher than 1000 mg/kg. Biochemical blood analysis after 90 days of the subchronic toxicity study revealed a slight increase in the serum values of chloride and a decrease in urea concentration (Table 4). Additionally, the activity of creatinine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) was decreased. After the recovery period, the chloride, AST, and CK activities remained unchanged, whereas the activity of LDH further slightly decreased. These small deviations may be related to the daily administration of the vehicle (peanut oil) into the peritoneal cavity and tissue injuries during injection, as microgranulomas were found in some control and LK-423-treated rats during postmortem examination of the peritoneal cavity. Similarly, histopathological examination revealed different stages of peritonitis found as perihepatitis, perisplenitis, and serositis in both groups. In the organs examined (liver, spleen, kidneys, myocardium, lung, suprarenal gland, and testes) no toxic damage was found. Based on these results, it can be concluded that LK-423 at a daily dose of 25 mg/kg administered intraperitoneally for 90 days is nontoxic under the experimental conditions used.

In conclusion, this study demonstrates that LK-423 is a safe, nontoxic drug. Its pharmacokinetics is characterized by very rapid elimination, limited distribution, and poor bioavailability. Incorporated in microcapsules for colon-specific delivery, LK-423 seems to be appropriate for local treatment of inflammatory disease in the large intestine.

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