Effect of Solid Nanoparticle of Indomethacin on Therapy for Rheumatoid Arthritis in Adjuvant-Induced Arthritis Rat

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We designed new oral formulations containing indomethacin (IMC) solid nanoparticles, and investigate their usefulness by evaluating bioavailability and gastrointestinal lesions. The IMC solid nanoparticles were prepared using methylcellulose (MC), 2-hydroxypropyl- β -cyclodextrin (HP β CD), and the bead mill method, and high quality dispersions containing 1.0% IMC nanoparticles were prepared (IMC_{nano}, particle size: 76 ± 58 nm, means $\pm$ S.D.). The fate of serum IMC and the induction of paw edema in adjuvant-induced arthritis (AA) rats receiving low-doses IMC_{nano} (0.4 mg/kg) were similar to those following the administration of a therapeutic dose of conventional IMC prepared with MC and HP β CD (conventional IMC, 2 mg/kg), and the bioavailability in 0.4 mg/kg IMC_{nano} was 5.3-fold higher in comparison with that in 2 mg/kg conventional IMC. IMC-induced gastrointestinal lesions in AA rats administered IMC_{nano} (8 mg/kg), in consideration of bioavailability, were significantly less than for conventional IMC (40 mg/kg). On the other hand, the toxicity caused by conventional IMC and IMC_{nano} was similar in Caco-2 cells. It is possible that the oral administration of IMC solid nanoparticles will show increased effectiveness in treating RA without causing IMC-induced gastrointestinal lesions, since the bioavailability is higher than that of conventional IMC. An oral drug delivery system using drug nanoparticles may expand the usage of NSAIDs for therapy in the inflammatory field.

Key words nanoparticle; indomethacin; bioavailability; gastrointestinal lesion; adjuvant-induced arthritis

Arthritis is a chronic disease that affects several parts of the joints including the cartilage, synovium, tendons and muscles. Rheumatoid arthritis (RA) is a specific type of arthritis, and is a complex chronic inflammatory disease dependent on multiple interacting environmental and genetic factors, making it difficult to understand its pathogenesis and thereby to find effective therapies.¹⁾ In treatments for RA, the focus is on the reduction of pain, inflammation and joint damage. The principal pharmacological agents are nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs, glucocorticoids and specific inhibitors of the mediator response.²⁾ It is well known, however, that these drugs have significant side effects.^{3–8)} Gastroenteropathy is the most common side effect among patients taking NSAIDs, and RA patients taking NSAIDs are more susceptible to NSAIDs-induced gastrointestinal lesions in comparison with other patients.^{3–7,9)} NSAIDs-induced gastric lesions are believed to involve the depletion of endogenous prostaglandins (PG) *via* the inhibition of cyclooxygenase, and an overproduction of nitric oxide (NO) *via* inducible NO synthase (iNOS).^{3,6,10,11)} On the other hand, a number of elements, such as bacterial flora, neutrophils and iNOS, in addition to the depletion of endogenous PGs are involved in the pathogenesis of intestinal ulceration induced by NSAIDs,^{11–15)} and the presence of enterobacteria is essential for NSAID-induced ulceration to occur in the small intestine.^{11,14)} Kato *et al.* reported that the overproduction of NO plays a key pathogenic role in both the gastric and small intestinal ulcerogenic response to indomethacin (IMC) in RA model rats, and these gastrointestinal lesions increased in number with dose of oral administration.^{3,5–7)} Therefore, a decrease in amount administered orally by improving bioavailability may suppress the development of both of gastric and small intestinal ulcers in response to NSAIDs in RA patients.

Recently, strategies using micro/nanoparticles have been developed and investigated, and it was reported that these strategies increase bioavailability and usefulness of drug.^{16–18)} Implants fabricated using the biodegradable polymer PLGA [poly(DL-lactide-co-glycolide)] with mean particle diameters of 50–200 nm have been widely utilized as carriers for bioactive molecules and present a possible solution to limitations surrounding ocular drug penetration.^{19–22)} In addition, Sha *et al.*¹⁸⁾ developed a newly designed oral nanotherapeutic using redox nanoparticles (RNP⁰) with the potential to scavenge reactive oxygen species including nitroxide radicals for the treatment of inflammation in the gastrointestinal tract. RNP⁰ is a core-shell-type polymeric micelle prepared by the self-assembly of methoxy-poly(ethylene glycol)-*b*-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxymethyl-styrene]. It is expected that drug systems using nanoparticles may provide an alternative strategy for increasing drug bioavailability.^{19–22)} On the other hand, we have also developed methods to prepare tranilast solid nanoparticles by a bead mill method, and showed that these solid nanoparticles enhance drug bioavailability.^{23,24)} It is possible that decreasing the amount of orally administered NSAIDs by improving their bioavailability may lead to an expansion of their usage as therapy for RA patients.

Here, we have designed new oral formulations containing IMC solid nanoparticles, and investigated their usefulness by evaluating the drug bioavailability and gastrointestinal ulcerogenic responses.

MATERIALS AND METHODS

Animals and Materials Male Dark Agouti (DA) rats, aged 6 to 13 weeks, were housed under standard conditions (12 h/d fluorescent light (07:00–19:00), 25°C room temperature), and allowed free access to a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water. All procedures were

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performed in accordance with the Kinki University Faculty of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals. 2-Hydroxypropyl- β -cyclodextrin (HP β CD, average molar substitution, 0.6; average molecular weight (MW), 1380) was purchased from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). Low-substituted methylcellulose (MC, METOLOSE SM-4, average viscosity, 4Pa·s at 20°C) was provided from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Conventional IMC (solid, IMC microparticles, $17.2 \pm 12.8 \mu\text{m}$, means $\pm$ S.D.) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

Preparation of IMC Solid Nanoparticles IMC solid nanoparticles were prepared using zirconia beads and Bead Smash 12 (a bead mill, Wakenyaku Co., Ltd., Kyoto, Japan).^{23,24} Zirconia beads (diameter: 2mm) were added to the IMC microparticles with or without MC, and the mixtures were crushed with the Bead Smash 12 for 30s (3000rpm, 4°C). The mixtures were then dispersed in saline with or without 5% HP β CD, and crushed again with the Bead Smash 12 (5500rpm, 30s $\times$ 15 times, 4°C) in the presence of zirconia beads (diameter: 0.1mm). The compositions of the dispersions containing IMC are shown in Table 1. The pH was 6.5 for both dispersions containing IMC micro- or nanoparticles. The particle size was measured using a nanoparticle size analyzer SALD-7100 (Shimadzu Corp., Kyoto, Japan; refractive index 1.60–0.10i). The solubility of IMC in saline with and without 5% HP β CD was 0.003% and 0.012%, respectively (the inclusion complex by 5% HP β CD was 0.009%).

Stability of Drug Dispersions in IMC Formulations Three milliliters of dispersions containing IMC as described in Table 1 were incubated in 5mL test tubes in the dark at 20°C for 14d, after which 50 μL sample solutions were withdrawn from 5mm under the surface at the indicated time intervals (total height of liquid before withdrawn of samples was 4cm). The IMC concentrations in the samples were determined by the following HPLC method. Fifty microliters of samples was added to 100 μL methanol containing 0.1 μg propyl *p*-hydroxybenzoate (internal standard), and the mixture was filtered through a Chromatodisk 4A (pore size 0.45 μm , Kurabo Industries Ltd., Osaka, Japan). The filtered solution (10 μL) was injected into an Inertsil[®] ODS-3 (3 μm , column size: 2.1mm $\times$ 50mm) column (GL Science Co., Inc., Tokyo, Japan) on a Shimadzu LC-20AT system equipped with a column oven CTO-20A (Shimadzu Corp.). The mobile phase consisted of acetonitrile–50mM acetic acid (40:60, v/v) at a flow rate of 0.25mL/min; the column temperature was 35°C, and the wavelength for detection was 254nm.

Induction of Arthritis in DA Rats Arthritis was induced

by the injection of 50 μL of adjuvant, a suspension of 10mg/mL heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI, U.S.A.) in Bayol F oil, into the plantar region of the right hind foot and tail of DA rats. The control group received 50 μL of Bayol F oil. In this study, inflammation during the development of adjuvant-induced arthritis (AA) is assessed by measuring paw edema, which is one parameter of inflammation. The paw edema of arthritis was assessed by measuring the paw volume by plethysmometry, and was quantified by the following equation (Eq. 1):

$$\text{paw edema } (\Delta \text{ mL}) = \text{paw volume of arthritis rat} - \text{paw volume of normal rat} \quad (1)$$

The inflammatory scores are represented as AUC_{edema} (the area under the paw volume–time curve). The AUC_{edema} (AUC during 0–42d) was calculated from the following equation (Eq. 2):

$$AUC_{\text{edema}} = \int_0^{42 \text{ d}} V_{\text{edema}} dt \quad (2)$$

where V and t are the volume of paw edema and the days after adjuvant injection, respectively.

Administration of IMC_{micro} or IMC_{nano} IMC_{micro} or IMC_{nano} as described in Table 1 was administered orally to rats once a day starting on the day following adjuvant injection and continued for 42d. The doses administered were a low dose (0.4mg/kg) and a therapeutic dose (2mg/kg) in this study.

Assay of Serum IMC Concentrations On the day before IMC_{micro} or IMC_{nano} administration, a cannula filled with 30 μg /mL heparin (silicone tubing; i.d. 0.5mm, o.d. 1.0mm) was inserted into the right jugular vein of AA rats under isoflurane anesthesia. IMC_{micro} or IMC_{nano} (both of 0.4, 2mg/kg) was administered orally to the AA rats 14d after adjuvant injection (dose-volume 1mL/kg). Venous blood (200 μL) was collected at 0, 0.25, 0.5, 1, 2, 3, 4, 5 and 6h after the oral administration of IMC from the jugular vein through the cannula. The blood was centrifuged at 3000rpm for 20min at 4°C, and the serum obtained was stored at –80°C until IMC analysis by the HPLC method described above.

The IMC concentration in the serum after a single injection of 0.3mL of IMC solution (2mg/kg) into the femoral vein was analyzed by Eq. 3:

$$C_{\text{IMC}} = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad (3)$$

where t is time (0–6h) after IMC injection, and C_{IMC} is the concentration of IMC at the corresponding time. α

Table 1. Changes in IMC Particle Size in Formulations of Particle Dispersions Containing IMC 14d after Treatment with a Bead Mill

Formulation	Content (w/v%)			Treatment	Particle size (μm)	
	IMC microparticles	MC	HP β CD		Immediately	14 d after preparation
IMC _{micro}	1.0	0.5	5.0	—	17.2 $\pm$ 12.8	17.5 $\pm$ 12.9
Milled-IMC	1.0	—	—	Bead mill	—	—
Milled-IMC _{MC+}	1.0	0.5	—	Bead mill	0.19 $\pm$ 0.11	0.56 $\pm$ 0.28
IMC _{nano}	1.0	0.5	5.0	Bead mill	0.076 $\pm$ 0.058	0.083 $\pm$ 0.063

IMC particle sizes and standard deviations of dispersions containing IMC as described in Table 1 were determined by a nanoparticle size analyzer SALD-7100 (refractive index 1.60–0.10i). The data are presented as means $\pm$ S.D.

($2.24 \pm 0.27 \text{ h}^{-1}$) and β ($0.35 \pm 0.033 \text{ h}^{-1}$) show the elimination rate constants in the first and second-phases (mean $\pm$ S.E., $n=5$), and A ($8.40 \pm 2.14 \mu\text{g/mL}$) and B ($4.35 \pm 1.39 \mu\text{g/mL}$) are the IMC concentrations in the α - and β -phases, respectively.

The serum IMC concentration data after the oral administration of 0.3 mL of IMC_{micro} or IMC_{nano} (2 mg/kg) were analyzed by Eq. 4 (the each parameters were fixed with the value provided from the Eq. 3):

$$C_{\text{IMC}} = \frac{A \cdot k_a}{k_a - \alpha} e^{-\alpha(t-\tau)} + \frac{B \cdot k_a}{k_a - \beta} e^{-\beta(t-\tau)} - \left(\frac{A \cdot k_a}{k_a - \alpha} + \frac{B \cdot k_a}{k_a - \beta} \right) e^{-k_a(t-\tau)} \quad (4)$$

where C_{IMC} is the IMC concentration in the serum, k_a is the absorption rate constant. A nonlinear least-squares computer program (MULTI) was employed for the calculation.²⁵⁾

The area under the IMC concentration–time curve (AUC), area under the first moment curve ($AUMC$) mean residence time (MRT) and absolute bioavailability (BA) were calculated according to the following equations (Eqs. 5–8):

$$AUC = \int_0^{6 \text{ h}} C_{\text{IMC}} dt + \frac{C_{\text{IMC at 6 h}}}{\beta} \quad (5)$$

$$AUMC = \int_0^{\infty} C_{\text{IMC}} \cdot t dt \quad (6)$$

$$MRT = \frac{AUMC}{AUC} \quad (7)$$

$$BA = \frac{AUC_{p.o.} / \text{Dose}_{p.o.}}{AUC_{i.v.} / \text{Dose}_{i.v.}} \times 100 \quad (8)$$

Briefly, AUC was determined according to the trapezoidal rule up to 6 h, which was the time of the final IMC concentration measurement. The $AUC_{p.o.}$ and $AUC_{i.v.}$ are AUC after oral and intravenous administrations, respectively.

Assay of the IMC Absorption Using *in Situ* Loop Technique Fourteen days after adjuvant injection, AA were fasted overnight, and anesthetized with isoflurane. A small midline incision allowed the gentle exposure of a 4–5-cm target portion of intestine. This target small intestine, selected because of its suitable vasculature to collect venous blood, was washed gently and enough with saline solution. In addition, the bile duct was tied. An 8-cm length of silicon tubing (o.d. 2.0 mm, i.d.: 1.0 mm, TERUMO Corp., Tokyo, Japan) was inserted into one end of the intestine and tied securely with a surgical suture. The opposite end was tied and 1.5–2.0 mL (15–20 mg/rat) of IMC_{micro} or IMC_{nano} (1.0%) was injected into the intestine through the tube. Heparin (10 mg/kg) was injected in the femoral vein. The mesenteric vein was cannulated with an appropriate size of polyethylene tubing (Hibiki Co., Tokyo, Japan), and all venous blood was collected in a micro tube.^{26,27)} This blood was centrifuged at 10000 rpm for 30 min at 4°C, and the IMC concentration in the serum obtained was determined by the HPLC method described above.

Evaluation of Gastric Mucosal Lesions The AA rats were fasted for 18 h before experiments, but had free access to water. The rats were administered IMC_{micro} or IMC_{nano} (8 or 40 mg/kg) orally, and killed under deep ether anesthesia

6 h later.^{3,6,7)} The stomachs were excised, washed and fixed in 10% formalin solution, and the area of gastric glandular mucosal lesions was observed in digital photographs and quantified with Image J (NIH). Lesion area is expressed as a percentage of the total area of glandular stomach except the fundus.

Evaluation of Small Intestinal Ulcerogenic Lesions AA rats were administered IMC_{micro} or IMC_{nano} (8 or 40 mg/kg) orally, and killed under deep ether anesthesia 24 h later.^{3,5)} The small intestines were excised, washed and fixed in 10% formalin solution, and the area of the intestinal ulcerogenic lesions was observed in digital photographs and Image J. The lesion area is expressed as a percentage of the total area of small intestine.

RNA Preparation The gastric mucosa and small intestine (epithelium) were removed, after which the lenses and retinas were isolated, and the samples were snap-frozen in liquid nitrogen. Total RNA was prepared from each individual lens and retina by the acid guanidium thiocyanate–phenol–chloroform extraction method using Trizol reagent (Life Technologies Inc., Rockville, U.S.A.). The purity and concentrations of RNA were determined spectrophotometrically. The OD_{260}/OD_{280} values of all RNA used were greater than 1.8, which indicates low protein contamination and high purity RNA.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) The RT reaction was performed using an RNA PCR Kit (AMV Ver 3.0, TaKaRa Bio Inc., Shiga, Japan). One microgram of total RNA was mixed with 3 μL of 10 mM Tris–HCl buffer (pH 8.3) containing 5 mM MgCl_2 and 50 mM KCl. The following components were then added to give a final volume of 10 μL : 1 unit/ μL RNase inhibitor, 10 mM deoxynucleotide triphosphate, 2.5 units/ μL reverse transcriptase, and 0.125 μM oligo dT-adaptor primer. The RT reaction was performed at 42°C for 15 min, followed by 5 min at 95°C. PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I according to the manufacturer's instructions (Roche Diagnostics Applied Science, Mannheim, Germany). Briefly, 2 μL of cDNA was mixed with 2 μL of reaction mixture, LightCycler FastStart DNA Master SYBR Green I Reaction Mix, containing FastStart Taq DNA Polymerase, reaction buffer, MgCl_2 , SYBR Green I dye, and deoxynucleotide triphosphate mix. The following components were then added to give a final volume of 20 μL containing specific primers for iNOS or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 10 pmol each). The following primers were used: 5'-GGAGAGATTTT CACGAC ACCC-3' and 5'-CCA TGC ATA ATT TGG ACT TGCA-3' for iNOS (GenBank accession No. NM_012611); and 5'-ACG GCACAG TCA AGG CTG AGA-3' and 5'-CGC TCCT GGA AGAT GGTG AT-3' for GAPDH (GenBank accession No. NM_017008). The PCR conditions were 95°C for 10 min, 50 cycles of 95°C for 10 s (denaturing), 60°C for 10 s (annealing), and 72°C for 5 s (extension). The quantities of PCR products were measured fluorometrically in a real-time manner using a LightCycler DX 400 (Roche Diagnostics Applied Science, Mannheim, Germany). After completion of the PCR reactions, dissociation curves of the PCR products were generated using the LightCycler Software Version 4.0 program to detect nonspecific amplification, including primer-dimers, and to ascertain the quality of the amplification data. The differences in the threshold cycles for GAPDH and iNOS were used to calculate the levels

of mRNA expression in the rats.

Measurement of Protein Protein levels in the gastric mucosa and small intestine (epithelium) of rats were determined according to the method of Bradford²⁸⁾ using a Bio-Rad Protein Assay Kit (BIO-RAD, CA, U.S.A.) with bovine serum albumin as the standard.

Measurement of NO Levels The gastric mucosa and small intestine (epithelium) were excised from rats and stored at -80°C until use. Tissue samples were homogenized in saline on ice, and centrifuged at 10000rpm for 15 min at 4°C . The resultant supernatants were used for the measurement of NO levels. A concentric microdialysis probe (A-1-20-05, 5 mm in length; Eicom, Kyoto, Japan) was placed in the supernatant and perfused with Ringer's solution (140 mM NaCl, 4 mM KCl, 1.26 mM CaCl_2 , and 1.15 mM MgCl_2 , pH 7.4) at a constant flow rate of $2\mu\text{L}/\text{min}$ using a micro syringe pump (ESP-64, Eicom). NO_2^- and NO_3^- in the supernatant were separated on

a reverse-phase separation column packed with polystyrene polymer (NO-PAK, $4.6\times 50\text{mm}$, Eicom); NO_3^- was reduced to NO_2^- in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). NO_2^- was mixed with Griess reagent to form a purple azo dye in a reaction coil, and placed in a column oven set at 35°C . The absorbance of the color product dye at 540 nm was determined on a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase consisted of 10% methanol containing 0.15 M NaCl- NH_4Cl and 0.5 g/L $\text{Na}_4\text{-EDTA}$ delivered by a pump at a rate of 0.33 mL/min. The Griess reagent, 1.25% HCl containing 5 g/L sulfanilamide with 0.25 g/L *N*-naphthylethylenediamine, was delivered at a rate of 0.1 mL/min. In this paper, the amounts of NO reflect the level of the NO_2^- metabolite, which is produced from NO.⁷⁾

Measurement of IMC Concentration in the Mucosal Membrane of Stomach and Small Intestine Following Oral

Fig. 1. Cumulative Size Distribution and Frequency of IMC Dispersions with or without MC, HP β CD and Bead Mill Treatment

The compositions of the IMC dispersions are shown in Table 1. Particle size was determined using a nanoparticle size analyzer SALD-7100 (refractive index 1.60–0.10i). (A) Cumulative distribution and frequency of IMC microparticles, (B) cumulative distribution and frequency of Milled-IMC, (C) cumulative distribution and frequency of Milled-IMC_{MC+}, (D) cumulative distribution and frequency of IMC_{nano}.

Administration The AA rats were fasted for 18h before the experiments, but had free access to water. The rats were administered IMC_{micro} or IMC_{nano} (40mg/kg) orally, and killed under deep isoflurane anesthesia 3, 6 and 12h later. The stomachs, duodenum (lower 2cm of the stomach), jejunum (40% of the upper part of small intestine, 28.1±3.9cm), and ileum (60% of the lower part of small intestine, 40.1±7.2cm) were washed by saline, and these mucosal membrane were excised (mean±S.E., *n*=25). These samples were homogenized in methanol on ice, and centrifuged at 10000rpm for 15min at 4°C. IMC in the supernatant was analyzed by the HPLC method described above.

Stimulation Studies with Caco-2 Cells The Caco-2 cell line was used in this study. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Tokyo, Japan) containing 10% (v/v) heat-inactivated fetal bovine serum (GIBCO), 1% non-essential amino acid solution (GIBCO), 1% L-glutamine and 10μg/mL streptomycin and 1000IU/mL penicillin (GIBCO). Caco-2 cells (1.5×10⁴ cells) were seeded in 96-well microplates (IWAKI, Chiba, Japan). At 1d after seeding, the medium was changed to medium containing 0–1% IMC_{micro} or IMC_{nano}, and the cells were incubated for 24h. In this study, we showed the concentration range (0–0.16%) where cytotoxicity was seen (Fig. 6). Following stimulation, culture medium containing TetraColor One (SEIKAGAKU Co., Tokyo, Japan) was added, the absorbance at 490nm was measured, and cell viability was calculated according to the manufacturer's instructions as represented by Eq. 9:

$$\text{cell viability (\%)} = \text{Abs}_{\text{treatment}} / \text{Abs}_{\text{non-treatment}} \times 100 \quad (9)$$

Statistical Analysis All values are presented as mean±standard deviation (S.D.) or standard error of the mean (S.E.). Unpaired Student's *t*-test was used to evaluate statistical differences, and multiple groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparison. The *p* values less than 0.05 were considered significant.

RESULTS

Preparation of Dispersions Containing IMC Nanoparticles Figure 1 shows the particle size distribution of dispersions containing IMC as described in Table 1. The mean particle size using the IMC treated bead mill method was 15.8±14.3μm (Milled-IMC, means±S.D.), with the particle size being on the micrometer order (Fig. 1B). On the other hand, IMC particles obtained by the addition of MC and the bead mill method had a mean particle size of 190±110nm (Milled-IMC_{MC+}, means±S.D.). In addition, the mean particle size using the IMC treated bead mill method was 76±58nm by the addition of both MC and HPβCD (IMC_{nano}, means±S.D.), and the mean particle size of IMC_{nano} was lower than that of Milled-IMC_{MC+}. Figure 2 shows the stability of drug dispersions in 1.0% IMC formulation as described in Table 1. The IMC_{micro} preparation precipitated 4h after preparation, and the stability of drug dispersions in IMC formulation was increased by the combination of added MC and the bead mill method. Furthermore, the addition of HPβCD further enhanced the stability of drug dispersion in IMC_{nano}, and no precipitation was observed 14d after preparation. Although the particle size of Milled-IMC_{MC+} increased to 0.56±0.28μm

Fig. 2. Stability of Drug Dispersions in IMC Formulation with or without MC, HPβCD and Bead Mill Treatment

One percent IMC dispersions were kept in the dark at 20°C for 14d, and the amounts of remaining IMC were determined at the indicated time points. The compositions of the IMC dispersions are shown in Table 1. The data are presented as means±S.E. of 5 independent samples.

Fig. 3. Serum IMC Concentration Following the Oral Administration of IMC_{micro} or IMC_{nano} in AA Rats

Serum IMC concentration following the oral administration of IMC in AA rats. IMC, 0.4mg/kg or 2mg/kg, was administered to AA rats 14d after adjuvant injection. Solid lines present fitting curves calculated using Eq. 4 (see Materials and Methods). The data are presented as the means±S.E. of 6–7 experiments. **p*<0.05 vs. IMC_{micro}-administered rats.

from 0.19±0.11μm, there was no change in the particle size of IMC_{nano} between 0 and 14d after preparation (Table 1).

Pharmacokinetics in the Oral Administration of Dispersions Containing IMC Nanoparticles Figure 3 shows the serum IMC concentrations following the oral administration of IMC_{micro} and IMC_{nano} at a low dose (0.4mg/kg) or therapeutic dose (2mg/kg) in AA rats 14d after adjuvant injection, and Table 2 summarizes the pharmacokinetic parameters calculated from the *in vivo* intestinal penetration data. The serum IMC levels, absorption rate constant (*k_a*), *AUC* and *AUMC* in AA rats administered 2mg/kg IMC_{nano} were all significantly higher than those in rats administered 2mg/kg IMC_{micro}, and the *T_{max}* in the case of IMC_{nano} was faster than that in the case of IMC_{micro}. On the other hand, there were no significant differences in the pharmacokinetic parameters (*C_{max}*, *AUC*, *AUMC* and *MRT*) between the administration of low dose IMC_{nano} (0.4mg/kg) and therapeutic dose IMC_{micro} (2mg/kg). The bioavailability in the case of 0.4mg/kg IMC_{nano} was 5.3-fold higher than that in the case of 2mg/kg IMC_{micro}.

Gastric and Small Intestinal Ulcerogenic Response to IMC in AA Rats Figure 4 shows the changes in gastrointestinal lesions and NO in AA rats administered IMC_{micro} and IMC_{nano}. The oral administration of IMC_{micro} or IMC_{nano} (40mg/kg) caused hemorrhagic lesions in both the gastric

Table 2. Pharmacokinetic Parameters for Plasma IMC Concentration after Oral Administration of IMC_{micro} and IMC_{nano}

Rp.	k_a ($\times 10/h$)	T_{max} (h)	C_{max} (nmol/mL)	AUC (nmol/mL $\cdot$ h)	$AUMC$ (nmol $\cdot$ h ² /mL)	MRT (h)	BA (%)
IMC _{micro} (2 mg/kg)	0.38 $\pm$ 0.11	0.92 $\pm$ 0.09	5.29 $\pm$ 0.13	19.11 $\pm$ 1.93	40.08 $\pm$ 3.47	2.29 $\pm$ 0.08	14.8 $\pm$ 4.7
IMC _{nano} (0.4 mg/kg)	1.21 $\pm$ 0.49*	0.59 $\pm$ 0.19*	5.37 $\pm$ 0.49	21.67 $\pm$ 2.40	48.43 $\pm$ 4.96	2.34 $\pm$ 0.16	78.8 $\pm$ 9.3*
IMC _{nano} (2 mg/kg)	1.35 $\pm$ 0.56*	0.53 $\pm$ 0.31*	28.5 $\pm$ 4.60***	121.8 $\pm$ 24.3***	321.5 $\pm$ 63.8***	2.46 $\pm$ 0.24	83.4 $\pm$ 15.5*

Dispersions containing IMC as described in Table 1 were administered orally to rats. Parameters were calculated according to Eqs. 4–8 (see Materials and Methods). α 2.24 $\pm$ 0.27 h⁻¹, β 0.35 $\pm$ 0.033 h⁻¹, A 8.40 $\pm$ 2.14 μ g/mL, B 4.35 $\pm$ 1.39 μ g/mL. IMC_{micro}, IMC_{micro}-administered AA rats; IMC_{nano}, IMC_{nano}-administered AA rats. The data are presented as means $\pm$ S.E. of 6–7 independent rats. * p <0.05 vs. 2 mg/kg IMC_{micro}. *** p <0.05 vs. 0.4 mg/kg IMC_{nano}.

Fig. 4. Effect of Orally Administered IMC_{micro} or IMC_{nano} on Indomethacin-Induced Gastrointestinal Lesions in AA Rats

(A) Gastrointestinal images of the stomachs and small intestines of AA rats administered IMC_{micro} or IMC_{nano}. (B, D) Lesion scores (B) and NO levels (D) in the stomachs of AA rats administered IMC_{micro} or IMC_{nano}. AA rats 14d after adjuvant injection were fasted for 18h before the experiments. AA rats were orally administered IMC (8 or 40mg/kg), and killed 6h later. (C, E) Lesion scores (C) and NO levels (E) in the small intestines of AA rats administered IMC_{micro} or IMC_{nano}. AA rats 14d after adjuvant injection were orally administered IMC (8 or 40mg/kg), and killed 24h later. The data are presented as the means $\pm$ S.E. of 5 experiments. * p <0.05 vs. 40mg/kg IMC_{micro}-administered rats.

mucosa and small intestine of AA rats 14d after adjuvant injection, with the lesion score in AA rats administered 40mg/kg IMC_{nano} tending to be higher than that in AA rats administered 40mg/kg IMC_{micro}. Following the administration of 8mg/kg IMC_{nano}, a dose given in consideration of the increased bioavailability in the case of the IMC_{nano} preparation, the lesion areas were significantly less than in the case of 40mg/kg IMC_{micro}. In addition, the iNOS mRNA and NO levels in the gastric mucosa and small intestines of AA rats administered 8mg/kg IMC_{nano} were also clearly lower

than in the case of 40mg/kg IMC_{micro} administration (iNOS mRNA, gastric mucosa, 8mg/kg IMC_{nano} 0.46 $\pm$ 0.19, 40mg/kg IMC_{micro} 1.87 $\pm$ 0.28, small intestine, 8mg/kg IMC_{nano} 1.01 $\pm$ 0.29, 40mg/kg IMC_{micro} 4.94 $\pm$ 0.42, iNOS/GAPDH, means $\pm$ S.E., n =3–5). Figure 5 shows the amounts of IMC in the mucosal membrane of stomach and small intestine of AA rats following the oral administration of IMC_{micro} and IMC_{nano} 14d after adjuvant injection. IMC was detected in the mucosal membrane of stomach and small intestine following the administration of IMC_{micro} and IMC_{nano} for 12h,

Fig. 5. Changes in IMC Amount in Mucosal Membrane of Stomach (A), Duodenum (B), Jejunum (C) and Ileum (D) Following the Oral Administration of IMC_{micro} or IMC_{nano}

IMC_{micro}, IMC_{micro}-administered AA rats. IMC_{nano}, IMC_{nano}-administered AA rats. AA rats were fasted for 18h before the experiments, but had free access to water. The rats were administered IMC_{micro} or IMC_{nano} (40mg/kg) orally, and killed under deep ether anesthesia 3, 6 or 12h later. The data are presented as means±S.E. of 5 independent rats.

Fig. 6. Effects of IMC_{micro} and IMC_{nano} on the Viability of Caco-2 Cells

Caco-2 cells were incubated in medium containing IMC_{micro} or IMC_{nano} for 24h. Cell viability was calculated using TetraColor One according to Eq. 9 in Materials and Methods. The data are presented as means±S.E. of 5–10 experiments.

and the amounts in the mucosal membrane of stomach and small intestine were similar between IMC_{micro} and IMC_{nano} administration. The amount of IMC in mucosal membrane of the stomach reached a maximum 3h after administration, and then decreased with time. On the other hand, the amounts of IMC in the mucosal membrane of small intestine remained constant during the period 3–12h after administration. Figure 6 shows the changes in the viability of Caco-2 cells following treatment with IMC_{micro} and IMC_{nano}. The viability of Caco-2 cells decreased with increasing IMC concentration, with the viabilities of Caco-2 cells treated with IMC_{micro} and IMC_{nano} being similar. The viabilities of Caco-2 cells treated with 0.04% IMC_{micro} and IMC_{nano} were approximately 32.7% and 35.6%, respectively.

Preventive Effects of the Oral Administration of IMC_{micro} and IMC_{nano} on Paw Edema in AA Rats

Fig. 7. Changes in Paw Edema of the Right (A) and Left (B) Hind Feet of AA Rats Administered IMC_{micro} or IMC_{nano}

IMC_{micro} and IMC_{nano}, a low dose (0.4mg/kg) or therapeutic dose (2mg/kg), was administered orally to rats once a day starting on the day following adjuvant injection. Treatments were continued for 42d after adjuvant injection. The data are presented as means±S.E. of 5–10 independent rats. **p*<0.05 vs. vehicle-administered rats.

Table 3. Effects of IMC_{nano} on Paw Edema in AA Rats

	AUC_{edema} (mL·d)	
	Right	Left
Vehicle	47.87±1.77	31.56±1.13
IMC _{micro} (0.4 mg/kg)	43.99±1.83*	27.10±0.94*
IMC _{micro} (2 mg/kg)	22.13±1.81***	17.80±1.05***
IMC _{nano} (0.4 mg/kg)	21.62±1.76***	16.97±0.99***

Dispersions containing IMC as described in Table 1 were administered orally to AA rats once a day starting on the day following adjuvant injection. Parameters (AUC_{edema}) were calculated according to Eq. 2 (see Materials and Methods). IMC_{micro}, IMC_{micro}-administered AA rats; IMC_{nano}, IMC_{nano}-administered AA rats. The data are presented as means±S.E. of 5 independent rats. * $p<0.05$ vs. vehicle. ** $p<0.05$ vs. 0.4 mg/kg IMC_{micro}.

7 shows the changes in paw edema in the right (A) and left (B) hind feet of AA rats administered IMC_{micro} or IMC_{nano}. IMC_{micro} or IMC_{nano} at a low dose (0.4 mg/kg) or therapeutic dose (2 mg/kg) was administered orally to rats once a day starting on the day following adjuvant injection, and Table 3 shows the AUC_{edema} values. Paw edema in the right hind foot, into which adjuvant was injected, appeared on the day following injection, and reached a maximum 14 d after injection. On the other hand, paw edema in the left hind foot, which was not injected with adjuvant, was not observed during the first 7 d after adjuvant injection, but clearly increased from 10 d. Fourteen days after adjuvant injection, paw edema in the right and left hind feet of AA rats administered 0.4 mg/kg IMC_{micro} was significantly less than in AA rats administered vehicle, and paw edema in the right and left hind feet of AA rats administered 2 mg/kg IMC_{micro} was significantly less than in rats receiving 0.4 mg/kg IMC_{micro}. The AUC_{edema} values in the right and left hind feet of AA rats administered 2 mg/kg IMC_{micro} were 46.2% and 56.4% those of AA rats administered vehicle, respectively. Furthermore, paw edema in the right and left hind feet of AA rats administered 0.4 mg/kg IMC_{nano} was significantly less than in AA rats receiving vehicle; paw edema and AUC_{edema} values for AA rats receiving 0.4 mg/kg IMC_{nano} were similar to those for AA rats receiving 2 mg/kg IMC_{micro}.

DISCUSSION

IMC, [1-(4-chlorobenzoyl)-5-methoxy-2-methylIMC_{ol}-3-yl] acetic acid, molecular weight 357.8, pK_a 4.5, is practically insoluble in water, but has been used as a therapy for RA patients. Gastrointestinal lesions are the most common side effect in patients taking IMC, and RA patients taking IMC are more susceptible to IMC-induced gastric lesions in comparison with other patients.^{3-7,9} Therefore, the development of IMC formulations that do not cause gastrointestinal lesions is highly anticipated. In this study, we designed new oral formulations containing IMC solid nanoparticles, and investigated their usefulness by evaluating drug bioavailability and the toxicity (gastric and small intestinal ulcerogenic responses to IMC).

First, we attempted to mill IMC microparticles further by the bead mill method, but the mean particle size remained on the micro order (Milled-IMC, Fig. 1B); therefore, new innovations to regulate IMC particles were required, so we searched for useful additives to aid the performance of the bead mill. MC, a derivative of cellulose, is a water-soluble substance with a high degree of purity, uniformity and transparency. MC

molecules link to water molecules by intermolecular hydrogen bonds to form a cage-like structure, and such MC solutions are neutral, odorless and tasteless. MC solutions are also stable over the pH range,²⁹ and it is not necessary to remove the gel following the complete release of a drug.³⁰ MC is highly biocompatible³¹⁻³³ and is used to prepare drug formulations. The gel strength depends on the degree of substitution and the molecular weight.^{34,35} The particle size of IMC was decreased using a combination of MC addition and the bead mill method with the obtained IMC particles having a mean particle size of 190±110 nm (Milled-IMC_{MC+}, mean±S.D.).

Next, the stability of dispersions containing IMC was demonstrated. The IMC_{micro} preparation precipitated 4 h after preparation. The precipitation was related the big particle size (weight) of IMC_{micro}. The stability of drug dispersions in IMC formulation (Milled-IMC_{MC+}) was improved by the combination of MC addition and the bead mill method; however, precipitation was still observed 2 d after preparation (Fig. 2, Table 1). Mori *et al.*³⁶ reported that adsorption to the surface of cyclodextrin decreases the cohesion of nanoparticulate solids, and we previously reported that the addition of HPβCD to the mixture is suitable for the preparation of nanoparticles using mill methods.^{23,24} Therefore, we attempted to prepare an IMC dispersion containing 0.5% MC and 5% HPβCD using the bead mill method. HPβCD was found to enhance the stability of the IMC dispersion (IMC_{nano}), and no precipitation was observed 14 d after preparation (Table 1). In addition, there was no difference in the particle size of IMC_{nano} between 0 and 14 d after preparation, and the addition of HPβCD was also found to enhance the recovery percentage of IMC by the bead mill method (Milled-IMC_{MC+} 73.2±4.1%, IMC_{nano} 93.5±3.3%, means±S.E., $n=5$). The size of HPβCD (approximately 0.8 nm) is smaller than IMC_{nano}. Therefore, the HPβCD attach the surface of IMC nanoparticles, and may enhance the stability.

In this study, we investigated whether the IMC in IMC_{nano} and IMC_{nano} penetrated the membrane of small intestine by using the *in vivo* study (Fig. 3). The serum IMC level, absorption rate constant (k_a), AUC and $AUMC$ for AA rats administered 2 mg/kg IMC_{nano} (therapeutic dose) were significantly higher than those for rats administered 2 mg/kg IMC_{micro}, and the T_{max} for IMC_{nano} was faster than that for IMC_{micro} (Table 2). In addition, the AUC of serum IMC following the administration of low dose IMC_{nano} (0.4 mg/kg) was similar to that following the administration of therapeutic dose IMC_{micro} (2 mg/kg), and the bioavailability for 0.4 mg/kg IMC_{nano} was 5.3-fold higher than that of 2 mg/kg IMC_{micro} (Table 2). Furthermore, the particle size of IMC_{nano} was 85±67 nm (means±S.E.) in the pH 1.2 buffer based on Japanese Pharmacopoeia (JP) test protocols. These results show that the amount of IMC administered as RA therapy can be reduced by using IMC_{nano}. It is important to clarify the mechanism for the BA enhancement by nanoparticles. The solubility of IMC_{nano} may be increased in the *in vivo*, since the specific surface in nanoparticles is larger than that in microparticles. Moreover, we reported that the HPβCD was increased the absorption *via* membrane.³⁷ Taking these findings together, we hypothesize that high solubility of IMC_{nano} caused the enhancement of BA.

In studies to identify the mechanisms of NSAIDs-induced gastrointestinal lesions in RA, the selection of an experimental animal is very important. The AA rat is an animal model

in which arthritis is induced by the injection of an adjuvant. Inflammatory pain during the development of AA is assessed by measuring paw volume (paw edema).^{38,39} Paw edema in AA rats is known to involve two inflammatory stages, primary and secondary inflammation. The primary inflammation starts from the day following the injection of adjuvant into the right hind foot. Secondary inflammation is observed from 7 d after adjuvant injection, and inflammation reaches a maximum 14 d after adjuvant injection into the right and/or left hind foot.^{3,7} In addition, it has been reported that gastrointestinal lesions induced by conventional NSAIDs, such as indomethacin, naproxen and aspirin, are significantly aggravated in AA rats as compared with normal rats. It is noteworthy that changes in the biological characteristics of AA rats correspond to those that occur in human RA.^{3,5,6,38,39} Therefore, AA rats may provide a useful model for studies on the mechanisms of NSAIDs-induced gastrointestinal lesions in RA. It is known that the severity of arthritis in Wistar rats is moderate and occurs with an incidence of only 50–60%. On the other, the severity of arthritis in DA and Lewis rats is much higher than in Wistar rats, and occurs with an incidence of 100%.^{3,5–7} Therefore, in this study we investigated the effect of orally administered IMC solid nanoparticles on drug bioavailability and the gastric and small intestinal ulcerogenic responses to NSAIDs using IMC-administered DA rats injected with adjuvant.

The oral administration of IMC_{micro} or IMC_{nano} (40 mg/kg) resulted in the formation of hemorrhagic lesions in both the gastric mucosa and small intestine of AA rats 14 d after adjuvant injection, and the lesion score at AA rats administered 40 mg/kg IMC_{nano} tended to be higher than that of rats administered 40 mg/kg IMC_{micro}. When the difference in bioavailability was taken into account, and the dose of IMC_{nano} was reduced to 8 mg/kg, the lesion area in rats administered IMC_{nano} was significantly less in rats administered 40 mg/kg IMC_{micro} (Fig. 4), while the fates of serum IMC were similar (8 mg/kg IMC_{nano}: AUC, 453.0 ± 54.7 nmol/mL·h, mean ± S.E. n=5; 40 mg/kg IMC_{micro}: AUC, 441.9 ± 44.1 nmol/mL·h, mean ± S.E. n=5). In addition, the *in situ* loop experiment, the intestinal penetration in IMC_{nano} increased with time; however, there was little permeation in the case of IMC_{micro} found (the IMC levels 40 min after the injection of IMC_{micro} were only 1.3% those of the levels following the injection of IMC_{nano}, n=5). Also, the iNOS mRNA and NO levels in the gastric mucosa and small intestine of AA rats administered 8 mg/kg IMC_{nano} were clearly lower than in rats administered 40 mg/kg IMC_{micro}. It is known that the overproduction of NO plays a key pathogenic role in both the gastric and small intestinal ulcerogenic responses to IMC in RA patients.^{3,5–7} Furthermore, we showed that the amounts of IMC in the mucosal membrane of stomach and small intestine of AA rats following the oral administration of IMC_{micro} and IMC_{nano} 14 d after adjuvant injection was similar (Fig. 5), and the cell stimulation by IMC_{micro} and IMC_{nano} were similar in the *in vitro* study (Fig. 6). These results show that the high IMC concentration in blood and intensity of cell stimulation relate the gastrointestinal toxicity by the oral administration of IMC_{micro} and IMC_{nano}. We conclude that treatment with low doses of IMC_{nano} may enable RA therapy without gastric and small intestinal ulcerogenic responses to NSAIDs. Moreover, paw edema of the right hind feet of AA rats receiving oral administration of low dose IMC_{nano} (0.4 mg/kg) was signifi-

cantly less in comparison with AA rats who received vehicle, and the paw edema and AUC_{edema} values of AA rats receiving low doses IMC_{nano} (0.4 mg/kg) was similar to that of AA rats receiving therapeutic dose IMC_{micro} (2 mg/kg). These results show that the oral administration of IMC_{nano} for RA treatment can be kept at a low dose, since the drug bioavailability is higher than that of conventional IMC. In addition, the oral administration of IMC_{nano} provides therapy for RA with fewer IMC-induced gastrointestinal lesions.

Further studies are needed to elucidate the precise mechanism for the gastrointestinal toxicity of IMC_{nano} by hematoxylin–eosin (HE) staining. In addition, it is important to clarify the drug solubility of the IMC formulation and structure of the delivery system. Therefore, we are now investigating the structure of the delivery system for IMC nanoparticles by scanning electron microscopy.

In the present study, we attempted to establish a new method for the preparation of drug solid nanoparticles, and succeeded in preparing a high quality dispersion containing IMC nanoparticles (particle size, 76 ± 58 nm, mean ± S.D.). It is possible that the oral administration of IMC nanoparticles will provide increased effectiveness in treating RA without the toxicity (gastric and small intestinal ulcerogenic responses to NSAIDs), and that an oral drug delivery system using drug nanoparticles may expand the usage of NSAIDs for therapy in the inflammatory field.

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