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Article

Oral Coadministration of Zn-Insulin with D-Form Small Intestine-Permeable Cyclic Peptide Enhances Its Blood Glucose-Lowering Effect in Mice

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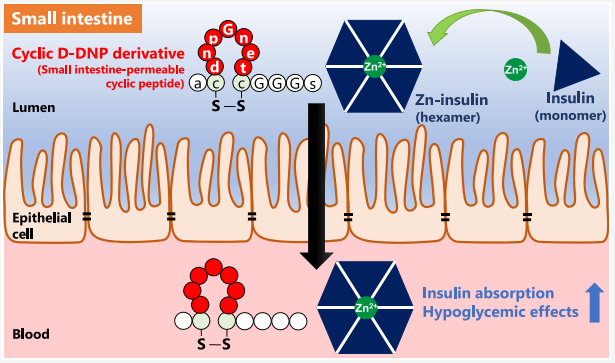
ABSTRACT: Oral delivery of insulin remains a challenge owing to its poor permeability across the small intestine and enzymatic digestion in the gastrointestinal tract. In a previous study, we identified a small intestine-permeable cyclic peptide, C-DNPGNET-C (C−C disulfide bond, cyclic DNP peptide), which facilitated the permeation of macromolecules. Here, we showed that intraintestinal and oral coadministration of insulin with the cyclic DNP derivative significantly reduced blood glucose levels by increasing the portal plasma insulin concentration following permeation across the small intestine of mice. We also found that protecting the cyclic DNP derivative from enzymatic digestion in the small intestine of mice using D-amino acids and by the cyclization of DNP peptide was essential to enhance cyclic DNP derivative-induced insulin absorption across the small intestine. Furthermore, intraintestinal and oral coadministration of insulin hexamer stabilized by zinc ions (Zn-insulin) with cyclic D-DNP derivative was more effective in facilitating insulin absorption and inducing hypoglycemic effects in mice than the coadministration of insulin with the cyclic D-DNP derivative. Moreover, Zn-insulin was more resistant to degradation in the small intestine of mice compared to insulin. Intraintestinal and oral coadministration of Zn-insulin with cyclic DNP derivative also reduced blood glucose levels in a streptozotocin-induced diabetes mellitus mouse model. A single intraintestinal administration of the cyclic D-DNP derivative did not induce any cytotoxicity, either locally in the small intestine or systemically. In summary, we demonstrated that coadministration of Zn-insulin with cyclic D-DNP derivative could enhance oral insulin absorption across the small intestine in mice.



KEYWORDS: *Cell-permeable peptide, Diabetes mellitus, Insulin, Insulin hexamer, Intestinal absorption*

INTRODUCTION

The oral route is an attractive noninvasive route of administration for insulin delivery, since oral insulin mimics the pharmacokinetics of endogenous insulin.[1] Moreover, oral delivery of insulin can overcome the problems related to the treatment of patients with type 1 diabetes mellitus and type 2 diabetes mellitus by insulin injection and insulin pump therapies.[2,3] However, oral delivery of insulin requires that the enzymatic barrier (that leads to rapid insulin degradation) and the permeability barrier (that limits the absorption of insulin in the small intestine) are both overcome. To achieve effective oral insulin delivery, several strategies have been suggested, including absorption enhancers,[4] enzyme inhibitors,[5] enteric-coated capsules,[6] and nanoparticles.[7] The efficacy of some oral insulin formulations has been investigated in clinical trials.[8−11] However, despite substantial research in this field, efficient oral delivery of insulin remains a challenge.

Cell-permeable peptides (CPPs) are short cationic and/or amphipathic peptides that facilitate the internalization of macromolecules by induction of macropinocytosis and/or other unidentified mechanisms.[12,13] CPPs have promising potential in improving insulin absorption in the small intestine as demonstrated in *in vivo* studies, where coadministration of CPPs, such as D-R8, and penetratin, enhanced insulin absorption from the small intestine of rodents.[14,15] However, cationic and amphipathic CPPs tend to accumulate in cells due to electrostatic interaction with intracellular organelles.[16,17] D-Penetratin was reported to show low permeability across Caco-

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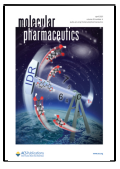

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2 cells, where it was retained in the intracellular compartment after internalization.[18] Thus, cationic and amphipathic CPPs could not facilitate insulin release from the small intestine epithelial cells to the blood.

In our previous study, we identified a novel small intestine-permeable cyclic peptide, C-DNPGNET-C (C−C disulfide bond, cyclic L-DNP peptide), which is negatively charged at physiological pH.[19] *In vitro* and *in vivo* permeability studies demonstrated that M13 phages displaying cyclic L-DNP peptide could permeate across Caco-2 cells (without disrupting the tight junctions) from the apical to the basal side within 3 min and across *in vivo* mouse small intestine from the lumen side to the portal vein within 10 min.[19] Transcellular transport of cyclic L-DNP derivative involves macropinocytosis initiated by a saturable interaction, possibly involving receptors.[19] Therefore, cyclic L-DNP peptide can be considered as a potential small intestine-permeable CPP candidate that can facilitate insulin absorption by enhancing both internalization and release of insulin in the small intestine.

Several studies related to CPP-mediated oral delivery of insulin have examined the absorption of insulin monomer in the small intestine.[14,15,20] Although insulin monomer is the active form of insulin, it has a tendency to be degraded by enzymes in small intestinal homogenates[5] and rat intestinal fluid,[14] and to form amyloid fibrils,[21,22] thus reducing bioactivity and stimulating immunogenicity.[23] Unlike insulin monomer, zinc ion (Zn^{2+}) stabilized insulin hexamer, Zn-insulin,[24] is more resistant to enzymatic degradation by chymotrypsin[25] and does not undergo fibrillation.[23] Zn-insulin can be converted to insulin monomer by Zn^{2+} dilution.[26] Insulin hexamer is available as a monomer in the portal vein within a few seconds after secretion from β cells of the pancreas.[27] In pharmaceutical formulations, insulin is generally present as hexamers in coordination with zinc.[28] Zinc is also used for the development of oral insulin delivery using nanoparticles.[7,29] Thus, Zn-insulin is a desirable pharmaceutical formulation of oral insulin (compared to insulin monomer). Based on these findings, it was hypothesized that cyclic DNP peptide could facilitate the permeation of insulin hexamer across the small intestine, and this may contribute to more insulin absorption from the small intestine.

In general, a coadministration strategy is easier to apply than a conjugation strategy. Herein, we investigated the effect of cyclic DNP peptide on the absorption of insulin and Zn-insulin from the small intestine of mice after intraintestinal and oral administration. We also examined changes in blood glucose levels in a mouse model of diabetes mellitus.

MATERIALS AND METHODS

Reagents. Cyclic DNP derivatives were synthesized by Scrum (Tokyo, Japan) and are shown in Table S1. Recombinant human insulin solution was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Humulin3/7 vial was obtained from Eli Lilly (Indianapolis, IN, USA). Streptozotocin (STZ) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were commercial products of analytical grade.

Animals. Institute of Cancer Research (ICR) mice (male; 7−10 weeks old, each weighing approximately 35−40 g) were purchased from Kyudo Co., Ltd. (Saga, Japan). The mice were housed in a room maintained at 18−24 °C and 40−70% relative humidity. They were subjected to a 12 h light/dark cycle and allowed free access to food and drinking water. All animal experiments were approved by the Institutional Animal Care and Use Committee at Kumamoto University and were performed in accordance with the regulations for animal experimentation at Kumamoto University (A2019−031).

Preparation of Insulin Solution. Human insulin solution was diluted with Hanks' Balanced Salt Solution (HBSS), containing 0.35 g/L sodium bicarbonate and 0.001% methylcellulose (which prevents the adsorption of insulin to the surface of the plastic vial). Immediately before preparing the mixed solution, the body weight of the mouse was measured. A mixture of insulin (10 or 60 IU/kg) and DNP derivatives (5 mM) in HBSS was incubated at 37 °C for 1 h. To form Zn-insulin, a mixture of insulin (10 or 60 IU/kg), DNP derivatives (5 mM), and 0.1 mM $ZnCl_2$ was incubated in HEPES buffer at 37 °C for 1 h. Each insulin/DNP derivative solution was clear after incubation.

***In Vivo* Pharmacological Study.** *In vivo* pharmacological and pharmacokinetic studies were performed on individual mice. In the intraintestinal administration study, intestinal closed loops were established in 6 h fasted mice as described previously.[19] The preincubated solutions (100 μL) containing insulin or Zn-insulin (10 or 60 IU/kg) plus DNP derivative (5 mM) was administered into the jejunum. In the subcutaneous (s.c.) administration study, 100 μL of insulin (1 IU/kg) was subcutaneously administered to mice that were fasted for 6 h. Blood samples (one drop) were collected from a small cut in the tail vein at 0 min (prior to dosing), 15, 30, 60, 90, and 120 min after administration, and blood glucose levels were measured using ACCU-CHEK Aviva Nano (Roche Diagnostics K.K., Tokyo, Japan). The blood glucose level was estimated as the percentage of blood glucose at 0 min. The area above the curve (AAC) showing a blood glucose level under 100% (from 0 to 120 min) and the area under the curve (AUC) showing a blood glucose level over 100% (from 0 to 120 min) were calculated using the trapezoidal method. AAC_{0-120} was then calculated from AAC−AUC. The pharmacological availability (PA) was calculated relative to the value obtained after s.c. injection, using the following equation (eq 1):

$$PA(\%) = \frac{[AAC_{0-120}]_{intraintestinal}/Dose_{intraintestinal}}{[AAC_{0-120}]_{s.c.}/Dose_{s.c.}} \times 100 \quad (1)$$

To obtain plasma from the portal vein, blood samples (800 μL) were collected from the portal vein at 0 min (before dosing), 5, 10, and 30 min after administration. The supernatants from blood samples were collected after centrifuging at 10,000 × g for 5 min at 4 °C. The plasma concentration of insulin in the portal vein was determined using a human insulin ELISA kit (Mercodia AB, Uppsala, Sweden), according to the manufacturer's protocol. Plasma insulin concentrations were calculated using a calibration curve. When insulin concentration in the plasma was below the lower limit of quantification (LLOQ), the insulin concentration in the sample was considered the LLOQ.

In the oral administration study, the preincubated solutions (100 μL) of insulin (60 IU/kg), Zn-insulin (60 IU/kg), or Humulin3/7 (60 IU/kg) plus DNP derivatives (5 mM) were administered using an oral gavage tube (i.d. 1.18 × length 50 mm, AS ONE Co., Osaka, Japan). Blood samples (one drop from each mouse) were collected from a small cut in the tail vein at 0 min (prior to dosing), 15, 30, 60, 90, and 120 min after oral administration, and then blood glucose levels were

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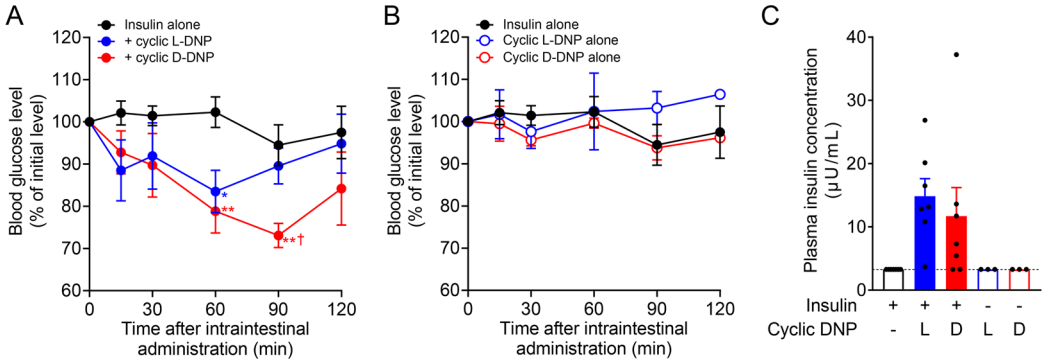

Figure 1. Effect of cyclic DNP derivatives on blood glucose levels and insulin absorption after coadministration of insulin into mouse jejunum. (A) Blood glucose levels after coadministration of insulin (10 IU/kg) and cyclic L-DNP derivative (5 mM) or cyclic D-DNP derivative (5 mM) into the mouse jejunum using the *in situ* closed-loop method. The glucose level in the blood from the tail vein was measured using ACCU-CHEK Aviva Nano at the indicated time points. Data represent the mean ± SEM (n = 6). *p < 0.05, **p < 0.01, significantly different from insulin alone at each time point. †p < 0.05, significantly different from cyclic L-DNP derivative at each time point (One-way ANOVA, Tukey's test). (B) Blood glucose levels after coadministration of cyclic L-DNP derivative (5 mM) or cyclic D-DNP derivative (5 mM) alone into the mouse jejunum using the *in situ* closed-loop method. The glucose level in the blood from the tail vein was measured at the indicated time points. Data represent the mean ± SEM (n = 3−6). (C) Plasma insulin in the portal vein after coadministration of insulin (60 IU/kg) and cyclic L-DNP derivative (5 mM) or cyclic D-DNP derivative (5 mM) into the jejunum of the mouse. The plasma insulin concentration was measured by ELISA. Data represent the mean ± SEM (n = 3−7). The dotted line shows the lower limit of quantification (3.27 μU/mL).

measured using ACCU-CHEK Aviva Nano. Blood glucose levels were estimated as the percentage of blood glucose at 0 min. To estimate AAC_{0-120}, the difference in blood glucose level between coadministration with or without DNP derivative was time-dependently plotted, and then AAC_{0-120} was calculated as described above.

Insulin Degradation Assay. The mice were administered 300 μL of insulin (10 IU/kg) or Zn-insulin (10 IU/kg) using the *in situ* closed-loop method as described above. Samples (200 μL) were collected from the closed-loop at 0, 10, 30, and 60 min after administration. The insulin concentration in the collected solution was measured by ELISA as described above. The data are shown relative to the value at time 0. The degradation rate constants were calculated from a semilog plot of the remaining percentage of insulin against time using the least-squares method with GraphPad Prism 7 (GraphPad Software, San Diego, USA). The half-life was calculated using the following equation (eq 2):

$$T_{1/2} = \ln(2)/K_{deg} \quad (2)$$

where K_{deg} is the degradation rate constant.

STZ-Induced Diabetic Mouse Model. After measuring baseline blood glucose levels (Day 0), mice were intraperitoneally injected with 300 μL of STZ (300 mg/kg body weight) in sodium chloride buffer (pH 7.0). After 5 days (Day 5), mice showing blood glucose levels >350 mg/dL were considered diabetic and used in this study. Preincubated Zn-insulin and the cyclic D-DNP derivative were administered using the *in situ* closed-loop method or orally, and the blood glucose level was measured using ACCU-CHEK Aviva Nano as described above.

Statistical Analysis. Unless otherwise indicated, data represent the mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism 7. An unpaired, two-tailed Student's t-test was used to determine the significance of differences between means of two groups. One-way analysis of variance (ANOVA), followed by Tukey's test, was used to assess the statistical significance of differences among means of more than two groups. Differences were considered statistically significant when p < 0.05.

RESULTS

Effect of Intraintestinal Coadministration of Insulin with Cyclic DNP Derivatives on Blood Glucose Levels in Mice. To examine whether the cyclic DNP derivative, consisting of cyclic heptapeptides with a short GGGS linker, enhances the absorption of insulin across the small intestine in mice, we examined the time-profile curve of blood glucose levels in mice using the *in situ* closed-loop method (Figure 1). The concentrations of insulin (10 IU/kg) and cyclic DNP derivative (5 mM) were selected based on an oral coadministration study of insulin and penetratin.[14] Intraintestinal administration of insulin alone did not decrease the blood glucose level at any time point during the 120 min (Figure 1A). In contrast, intraintestinal coadministration of insulin with cyclic L-DNP derivative decreased the blood glucose level by 18.4% until 60 min (p = 0.0306), compared to the administration of insulin alone. Subsequently, the blood glucose level returned to the initial level at 120 min (Figure 1A).

To investigate whether protection of cyclic DNP peptide against degradation in the small intestine could increase the insulin-induced hypoglycemic effect in mice, we examined the D-form of cyclic DNP peptide (D-DNP), which is composed of D-amino acids that are resistant to degradation. Results from the permeability studies demonstrated that 10 μM of FAM-labeled cyclic D-DNP derivative and cyclic L-DNP derivative could both permeate Caco-2 cells and the small intestine of the mouse; however, the permeability efficiency of FAM-labeled D-DNP derivative was lower than that of FAM-labeled cyclic L-DNP derivative by 34.0−38.4% (Figure S1).

Intraintestinal coadministration of insulin and cyclic D-DNP derivative decreased the blood glucose levels by 22.7% until 90 min (p = 0.0055), compared to insulin alone, and the blood glucose level did not return to the initial level within 120 min (Figure 1A). Compared to the intraintestinal coadministration of insulin with cyclic L-DNP derivative, intraintestinal coadministration of insulin with cyclic D-DNP derivative significantly decreased the blood glucose level within 90 min (p = 0.0300, Figure 1A). No significant reduction in the blood

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Table 1. Pharmacological Availability of Insulin after *In Situ* Coadministration with Cyclic DNP Derivatives[a]

	AAC_{0-120}		PA
	(% BGL·min)	fold change	(%)
Insulin alone (10 IU/kg)	85.4 ± 324	—	0.133
+ Cyclic L-DNP derivative (5 mM)	1247 ± 456	14.6	1.94
+ Cyclic D-DNP derivative (5 mM)	1865 ± 329**	21.8	2.90
Insulin alone (s.c.; 1 IU/kg)	6421 ± 481	—	—

[a]The AAC of blood glucose was estimated from 0 to 120 min, using the trapezoidal method. Fold change was calculated relative to insulin alone. The pharmacological availability (PA) was calculated using data obtained after s.c. administration of insulin solution. Data represent the mean ± SEM (n = 3−7). **p < 0.01, significantly different from insulin alone (One-way ANOVA, Tukey's test). Abbreviations: AAC, the area above the curve; BGL, blood glucose level; s.c., subcutaneous.

glucose levels was observed until 120 min after intraintestinal administration of cyclic L-DNP derivative or cyclic D-DNP derivative alone (Figure 1B). Furthermore, intraintestinal coadministration of insulin with linear L-DNP derivative or linear D-DNP derivative did not change the blood glucose level at any time point during the 120 min (Figure S2).

The PAs of insulin for intraintestinal coadministration of insulin and cyclic DNP derivatives were estimated relative to the subcutaneous (s.c.) injection of insulin (Figure S3, Table 1). The PAs for intraintestinal coadministration of insulin and cyclic L-DNP and cyclic D-DNP were 1.94% (p = 0.105) and 2.90% (p = 0.0113), respectively; a 14.6-fold and 21.8-fold increase (compared to insulin alone), respectively.

Considering the fact that approximately half of the amount of insulin released into the portal vein is removed by the liver during first-pass metabolism, we measured the plasma concentrations of insulin in the portal vein, using ELISA, to directly examine whether cyclic DNP derivatives could enhance insulin absorption (Figure 1C). Within 10 min after intraintestinal coadministration of insulin (60 IU/kg) with cyclic L-DNP derivative (5 mM) or cyclic D-DNP derivative (5 mM), the plasma concentration of insulin in the portal vein was 14.9 μU/mL (p = 0.0510) or 11.7 μU/mL (p = 0.235), respectively, but there was no statistical difference. After the administration of insulin alone, no insulin was detected in the plasma from the portal vein (LLOQ: 3.27 μU/mL). Thus, the cyclic DNP derivatives enhanced the absorption of insulin from the small intestine of the mouse.

Additive Effect of Zinc Chloride on the Intraintestinal Coadministration of Insulin with Cyclic DNP Derivative-Induced Blood Glucose Reduction in Mice. The hexametric form of insulin (Zn-insulin) is stabilized by zinc.[24] Since M13 phages displaying cyclic L-DNP peptide are internalized into Caco-2 cells by macropinocytosis,[19] we hypothesized that cyclic DNP derivatives would be able to enhance permeation of Zn-insulin (about 35 kDa) across the small intestine of mice. To examine whether the cyclic DNP derivative enhances the absorption of Zn-insulin across the mouse small intestine, we examined the time-profile curve of blood glucose levels in mice using the *in situ* closed-loop method (Figure 2). Zn-insulin was prepared by the addition of zinc(II) chloride (0.1 mM) to insulin solution. Intraintestinal coadministration of Zn-insulin did not decrease the blood glucose level at any time point during the 120 min (Figure 2A), which was not significantly different compared to insulin

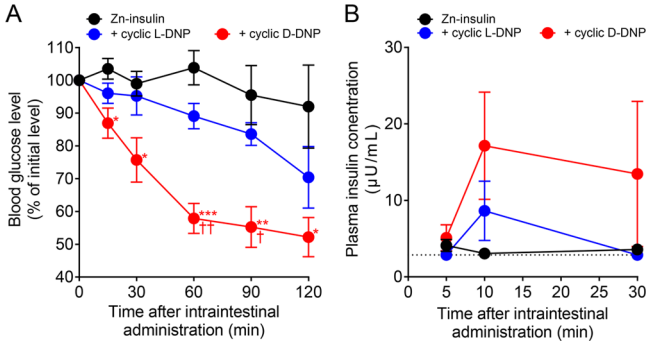

Figure 2. Effect of zinc chloride and cyclic DNP derivative on blood glucose levels and insulin absorption after coadministration of insulin into the mouse jejunum. (A) Time profile of blood glucose level after coadministration of insulin (10 IU/kg), zinc chloride (0.1 mM), and cyclic L-DNP derivative (5 mM) or cyclic D-DNP derivative (5 mM), into the mouse jejunum, using the *in situ* closed-loop method. The glucose level in the blood from the tail vein was measured at the indicated time points. Data represent the mean ± SEM (n = 5−6). *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from Zn-insulin at each time point. $^{\dagger}p$ < 0.05, $^{\dagger\dagger}p$ < 0.01, significantly different from cyclic L-DNP derivative at each time point (One-way ANOVA, Tukey's test). (B) Plasma concentrations of insulin in the portal vein after coadministration of insulin (60 IU/kg), zinc chloride (0.1 mM), and cyclic L-DNP derivative (5 mM) or cyclic D-DNP derivative (5 mM) into the mouse jejunum. The plasma insulin concentration was measured by ELISA. Data represent the mean ± SEM (n = 4). The dotted line shows the lower limit of quantification (2.86 μU/mL). Zn-insulin, addition of zinc chloride to insulin solution.

alone (Figure 1A). Intraintestinal coadministration of Zn-insulin and cyclic L-DNP derivative decreased the blood glucose levels by 23.4% until 120 min, compared to the intraintestinal administration of Zn-insulin (Figure 2A), but the reduction was not significant compared to Zn-insulin alone. In contrast, intraintestinal coadministration of Zn-insulin with cyclic D-DNP derivative also significantly decreased the blood glucose levels by 43.3% until 120 min (p = 0.0392), compared to the intraintestinal administration of Zn-insulin (Figure 2A). Compared to the coadministration of Zn-insulin with cyclic L-DNP derivative, intraintestinal coadministration of Zn-insulin with cyclic D-DNP derivative significantly decreased the blood glucose level at 60 min (p = 0.0015) and 90 min (p = 0.0034; Figure 2A).

The PA of insulin for intraintestinal coadministration of Zn-insulin and cyclic L-DNP derivative was 2.23% (Table 2), which was only 1.15-fold higher than that for intraintestinal coadministration of insulin and cyclic L-DNP derivative (1.94%, p = 0.287; Table 2). In contrast, the PA for intraintestinal coadministration of Zn-insulin and cyclic D-DNP derivative was 6.33% (p = 0.0008; Table 2), which was 2.18-fold higher than that for intraintestinal coadministration of insulin and cyclic D-DNP (2.90%; Table 1). The PA for intraintestinal coadministration of Zn-insulin with cyclic D-DNP derivative was significantly 2.84-fold higher than that of Zn-insulin with cyclic L-DNP derivative (p = 0.0197; Table 1).

To directly examine whether cyclic DNP derivatives enhanced Zn-insulin absorption from the small intestine of mice, the plasma concentration of insulin in the portal vein was measured using ELISA (Figure 2B). After intraintestinal coadministration of Zn-insulin (60 IU/kg) with cyclic L-DNP derivative (5 mM), plasma insulin in the portal vein was detected within only 10 min (7.92 μU/mL, Figure 2B), and

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Table 2. Area above the Curve and Pharmacological Availability Following *In Situ* Coadministration of Insulin and Cyclic DNP Derivatives with Zinc Chloride[a]

	AAC_{0-120}		PA
	(% BGL·min)	fold change	(%)
Zn-insulin [Insulin (10 IU/kg) + $ZnCl_2$ (0.1 mM)]	160 ± 668	—	0.249
+ Cyclic L-DNP derivative (5 mM)	1430 ± 365	8.94	2.23
+ Cyclic D-DNP derivative (5 mM)	4066 ± 581**,†	25.4	6.33
Insulin alone (s.c.; 1 IU/kg)	6421 ± 481	—	—

[a]AAC of blood glucose was estimated from 0 to 120 min using the trapezoidal method. Fold change was calculated relative to Zn-insulin. The pharmacological availability (PA) was calculated using data obtained after s.c. administration of insulin solution. Data represent the mean ± SEM (n = 3−6). ***p < 0.001, significantly different from insulin and zinc chloride. †p < 0.05, significantly different from cyclic L-DNP derivative (One-way ANOVA, Tukey's test). Abbreviations: AAC, the area above the curve; BGL, blood glucose level; s.c., subcutaneous; Zn-insulin, addition of zinc chloride to insulin solution.

was 46.8% lower than plasma insulin observed after intraintestinal coadministration of insulin with cyclic L-DNP derivative (14.9 μU/mL, Figure 1C). In contrast, after intraintestinal coadministration of Zn-insulin (60 IU/kg) with cyclic D-DNP derivative (5 mM), plasma insulin in the portal vein was detected in 5 min, increased until 10 min (17.2 μU/mL), and was stable until 30 min. Insulin concentration within 10 min after intraintestinal coadministration of Zn-insulin with cyclic D-DNP derivative was 2.17-fold higher than that with the L-DNP derivative (7.92 μU/mL, Figure 2B). However, there was no statistical difference in the plasma concentration of insulin in the portal vein at any time point.

Association between Insulin Levels in Portal Vein and Blood Glucose Levels after Intraintestinal Coadministration of Insulin with Cyclic DNP Derivative. To confirm that cyclic DNP peptide-induced insulin absorption was associated with the observed reduction in blood glucose level, a correlation analysis between insulin levels in the portal vein at 10 min (Figures 1C and 2B) and blood glucose levels at 15 min (Figures 1A and 2A) was performed. The plasma insulin levels in the portal vein at 10 min were associated with the blood glucose levels at 15 min (r = −0.971, Figure 3). This result suggests that insulin levels in the portal vein at 10 min were associated with the levels of initial insulin absorption from the small intestine in mice.

Additive Effect of Zinc Chloride on Insulin Degradation in Mouse Small Intestine. To examine whether the addition of zinc chloride affects insulin degradation in the small intestine of mice *in vivo*, the remaining percentage of insulin in the closed-loop was measured using ELISA (Figure 4). The percentage of insulin in the mouse jejunum was reduced by 84.4% within 10 min, and almost all insulin was degraded within 30 min (by 97.9%). However, after intraintestinal administration of Zn-insulin, the remaining percentage of insulin in the closed-loop was reduced by 50.1% within 10 min, by 69.7% in 30 min, and by 87.0% in 60 min. Degradation of insulin alone and of Zn-insulin alone both followed a one-phase decay until 30 and 60 min, respectively (r^2 = 0.9995, 0.9489). The degradation rate constant and half-life of insulin was estimated to be 0.0807 min^{-1} and 8.59 min, respectively (Table S2). In contrast, the degradation rate constant and half-life of Zn-insulin alone was estimated to be 0.0195 min^{-1} and 35.5 min, respectively (Table S2). The degradation rate constant of Zn-insulin was significantly reduced by 75.8% (p < 0.0001), and the half-life of Zn-insulin was 4.13-fold higher compared to insulin. These results suggest that the degradation of Zn-insulin in the small intestine of the mouse was slower than that of insulin alone.

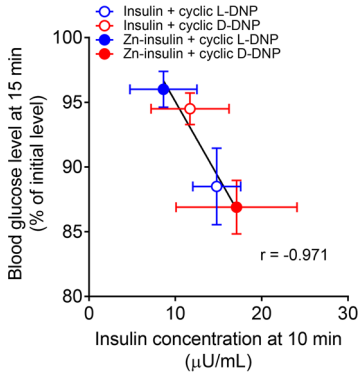

Figure 3. Association of insulin concentrations in the portal vein and blood glucose levels after intraintestinal coadministration of insulin or Zn-insulin and cyclic DNP derivatives. The correlation between the insulin concentration in the portal vein at 10 min and blood glucose level at 15 min was analyzed. The black line represents the linear regression line. Data represent the mean ± SEM (n = 4−7). Zn-insulin, addition of zinc chloride to insulin solution.

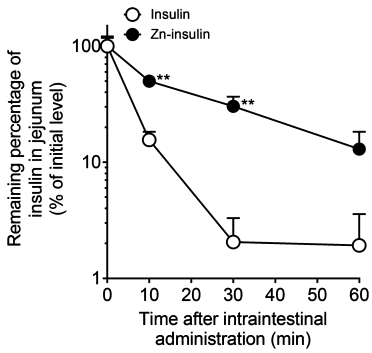

Figure 4. Degradation of insulin and Zn-insulin in mouse small intestine. Time profile of the remaining percentage of insulin after intraintestinal administration of insulin alone (10 IU/kg) or insulin (10 IU/kg) and zinc chloride (0.1 mM) into the mouse jejunum, using the *in situ* closed-loop method. Data represent the mean + SEM (n = 3−5). **p < 0.01, significantly different from insulin (Student's t-test). Zn-insulin, addition of zinc chloride to insulin solution.

Time Profile of Blood Glucose Levels after Oral Coadministration of Insulin or Zn-Insulin with Cyclic DNP Derivatives in Mice. Figure 5 shows the time profile of blood glucose levels after oral coadministration of insulin or Zn-insulin with cyclic DNP derivatives in mice. Because insulin is enzymatically degraded in the stomach, intestinal fluid, and the intestinal lumen, we administered 60 IU/kg insulin (instead of the 10 IU/kg insulin used in *in situ* closed-loop studies) in mice orally, in accordance with previous oral insulin reports.[30,31] Oral administration of insulin alone or Zn-insulin alone increased blood glucose levels until 30 min due to physical stress, and these then decreased until 120 min (Figure 5A and B). Figure 5A shows the time profile of blood glucose levels after oral coadministration of insulin with cyclic DNP derivatives. Compared to oral administration of insulin alone,

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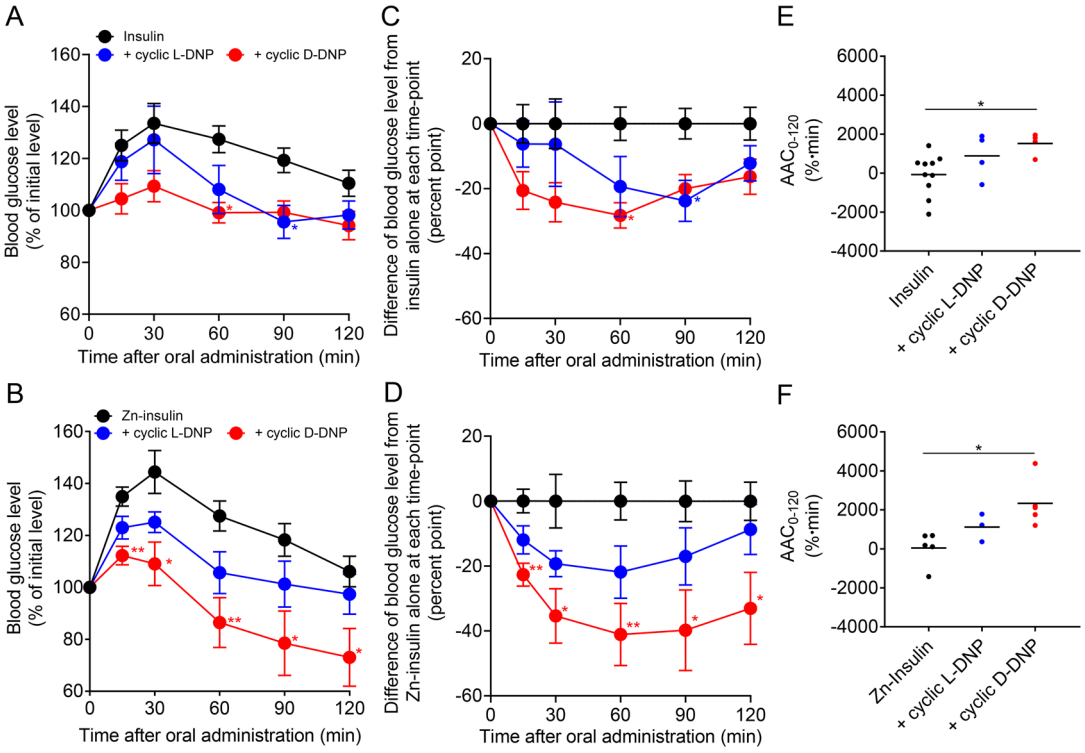

Figure 5. Effect of cyclic DNP derivatives and zinc chloride on blood glucose levels after oral coadministration of insulin and Zn-insulin in mice. (A,B) Time profiles of blood glucose level after oral coadministration of insulin (60 IU/kg) and cyclic DNP derivatives (5 mM) or cyclic D-DNP derivative (5 mM) (A, $n = 4-10$) and after oral coadministration of insulin (60 IU/kg), zinc chloride (0.1 mM), and cyclic L-DNP derivative (5 mM) or cyclic D-DNP derivative (5 mM) (B, $n = 3-5$). The glucose level in the blood from the tail vein was measured at the indicated time points. Data represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, significantly different from oral coadministration of insulin alone or Zn-insulin alone at each time point (one-way ANOVA, Tukey's test). (C,D) Time profiles of the differences in blood glucose levels after oral coadministration of insulin and cyclic DNP derivatives from insulin alone (C) or oral coadministration of Zn-insulin and cyclic DNP derivatives from Zn-insulin alone (D). The differences in blood glucose levels were estimated by subtracting the blood glucose level after insulin alone or Zn-insulin alone from the blood glucose level after coadministration of insulin or Zn-insulin with cyclic D-DNP derivative. Data represent the mean ± SEM ($n = 3-10$). $*p < 0.05$, $**p < 0.01$, significantly different from oral coadministration of insulin alone or Zn-insulin alone at each time point (one-way ANOVA, Tukey's test). (E,F) AAC_{0-120} values after oral coadministration of insulin and cyclic L-DNP derivative (E) or oral coadministration of Zn-insulin, and cyclic L-DNP derivative or cyclic D-DNP derivative from Zn-insulin alone (F). Data represent the mean ± SEM ($n = 3-10$). $*p < 0.05$, significantly different from oral coadministration of insulin alone or Zn-insulin alone at each time point (one-way ANOVA, Tukey's test). Zn-insulin, addition of zinc chloride to insulin solution.

oral coadministration of insulin with cyclic L-DNP derivative significantly reduced blood glucose levels at 90 min (by 19.9%, $p = 0.0242$), and oral coadministration of insulin with cyclic D-DNP derivative significantly reduced blood glucose levels at 60 min (by 22.2%, $p = 0.0195$) and 90 min (by 16.8%, $p = 0.0589$). No significant difference in blood glucose reduction was observed between coadministration of insulin with the L-form of cyclic DNP and coadministration of insulin with the D-form of cyclic DNP until 120 min. In contrast, oral administration of cyclic L-DNP derivative or cyclic D-DNP derivative alone did not reduce the blood glucose level until 120 min (Figure S4).

Figure 5B shows the time profile of blood glucose levels after oral coadministration of Zn-insulin with cyclic DNP derivatives. Oral coadministration of Zn-insulin with cyclic L-DNP derivative did not significantly reduce the blood glucose levels until 120 min. In contrast, oral coadministration of Zn-insulin with cyclic D-DNP derivative significantly reduced the blood glucose levels at 15 min (by 16.8%, $p = 0.0028$), 30 min (by 24.5%, $p = 0.0200$), 60 min (by 32.2%, p = 0.0088), 90 min (by 33.6%, $p = 0.0321$), and 120 min (by 31.1%, $p = 0.0470$). No significant difference in blood glucose reduction was observed between coadministration of insulin with the L-form of cyclic DNP and coadministration of insulin with the D-form of cyclic DNP until 120 min.

To assess the efficacy of blood glucose reduction after oral insulin administration, the differences in blood glucose levels between insulin alone and Zn-insulin alone at each time point were estimated (Figure 5C,D), and the corresponding AAC_{0-120} value was evaluated (Figure 5E,F). Oral coadministration of insulin with cyclic D-DNP derivative ($p = 0.0479$) or Zn-insulin with cyclic D-DNP derivative ($p = 0.0101$) significantly increased the AAC_{0-120} value; however, coadministration with cyclic L-DNP derivative did not significantly increase AAC_{0-120}. These results suggest that oral coadministration of Zn-insulin with cyclic D-DNP derivative showed a greater reduction effect on blood glucose levels than coadministration of insulin and cyclic L-DNP derivative (Figure 5).

Time Profile of Blood Glucose Levels after Oral Coadministration of Commercially Available Zinc-Formed Insulin Hexamer (Humulin3/7) with Cyclic DNP Derivatives in Mice. Humulin3/7 is a commercially available insulin injection that contains zinc-formed hexamer of human regular insulin. We examined whether cyclic D-DNP derivative can enhance oral absorption of commercially

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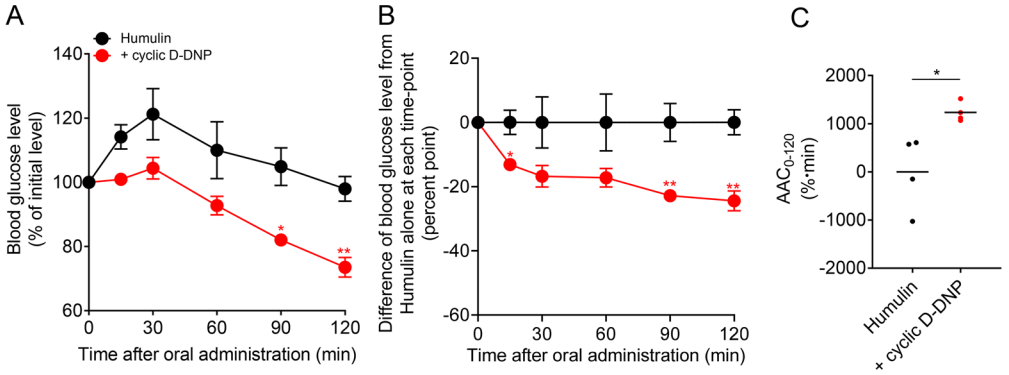

Figure 6. Effect of cyclic D-DNP derivatives on blood glucose levels after oral coadministration of commercially available zinc-stabilized insulin hexamer in mice. Humulin3/7 was used as a commercially available zinc-formed insulin hexamer. (A) Time profile of blood glucose levels after oral coadministration of Humulin3/7 (60 IU/kg) and cyclic D-DNP derivative (5 mM). The glucose levels in the blood from the tail vein were measured at the indicated time points. (B) Time profile of differences in blood glucose levels after oral coadministration of Humulin3/7 (60 IU/kg) and cyclic D-DNP derivative. The differences in blood glucose levels were estimated by subtracting the blood glucose level after Humulin3/7 alone from the blood glucose level after coadministration of Humulin3/7 with cyclic D-DNP derivative. (C) AAC_{0-120} value of oral administration of Humulin3/7 alone and oral coadministration of Humulin3/7 and cyclic D-DNP derivative. Data represent the mean ± SEM (n = 4). *p < 0.05, **p < 0.01, significantly different from oral coadministration of Humulin3/7 alone (Student's t-test).

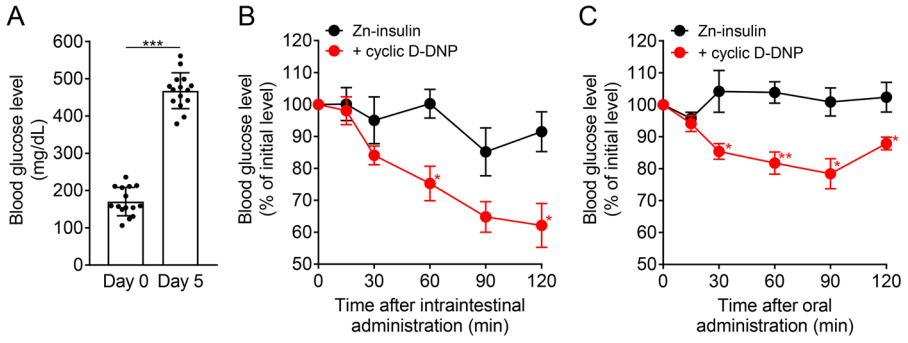

Figure 7. Effect of cyclic D-DNP derivatives on blood glucose levels after oral coadministration of Zn-insulin in the streptozotocin (STZ)-treated mice. (A) Blood glucose levels before (Day 0) and after (Day 5) treatment with STZ (300 mg/kg). Data represent the mean ± SEM (n = 15). ***p < 0.001, significantly different from Day 0. (B) Time profile of blood glucose levels after intraintestinal coadministration of Zn-insulin (10 IU/kg), and cyclic D-DNP derivative (5 mM). (C) Time profile of blood glucose levels after oral coadministration of Zn-insulin (60 IU/kg), and cyclic D-DNP derivative (5 mM). The glucose levels in the blood from the tail vein were measured at the indicated time points. Data represent the mean ± SEM (n = 3−5). *p < 0.05, **p < 0.01, significantly different from Zn-insulin at each time point (Student's t-test). Zn-insulin, addition of zinc chloride to insulin solution.

available insulin hexamer in mice (in addition to enhancing our prepared Zn-insulin hexamer). Oral administration of Humulin3/7 alone increased blood glucose levels until 30 min due to physical stress (Figure 6A), and levels then decreased until 120 min. Similar results were obtained following oral administration of Zn-insulin alone (Figure 5B). In contrast, oral coadministration of Humulin3/7 with cyclic D-DNP derivative significantly reduced blood glucose levels at 15 min (by 11.5%, p = 0.0138), 90 min (by 21.8%, p = 0.0099), and 120 min (by 24.9%, p = 0.0026) compared to Humulin3/7 alone (Figure 6A). The AAC_{0-120} for blood glucose levels after oral coadministration of Humulin3/7 and cyclic D-DNP derivative (p = 0.0210) was significantly higher compared with oral administration of Humulin3/7 alone (Figure 6B,C). The efficacy of blood glucose reduction after oral coadministration of cyclic D-DNP derivative and Humulin3/7 was not statistically different from that observed after oral coadministration of cyclic D-DNP derivative and Zn-insulin (Figure 5F). These results provide evidence that cyclic D-DNP derivative can enhance the absorption of both clinically approved Zn-insulin hexamer and our originally prepared Zn-insulin following coadministration.

Effect of Intraintestinal and Oral Coadministration of Zn-Insulin and Cyclic D-DNP Derivative on Blood Glucose Levels in Streptozotocin (STZ)-Treated Mice. To examine whether coadministration of Zn-insulin and cyclic D-DNP derivatives could reduce blood glucose levels in a diabetes mellitus mouse model, we examined the time profile of blood glucose levels after intraintestinal and oral coadministration of Zn-insulin and cyclic D-DNP derivatives in streptozotocin (STZ)-treated mice (Figure 7). Five days after intraperitoneal administration of STZ, the blood glucose levels increased from 171 mg/dL to 468 mg/dL (p < 0.0001, Figure 7A). Intraintestinal coadministration of Zn-insulin and cyclic D-DNP derivative reduced blood glucose levels at 30 min (through to 120 min) in STZ-treated mice compared to intraintestinal coadministration of Zn-insulin alone (Figure 7B); the blood glucose levels were reduced at 30 min (by 11.5%, p = 0.149), 60 min (by 24.9%, p = 0.0197), 90 min (by 23.9%, p = 0.0524), and 120 min (by 32.1%, p = 0.0282). In addition, oral coadministration of Zn-insulin and cyclic D-DNP derivative significantly reduced blood glucose levels at 30 min (by 18.1%, p = 0.0290), 60 min (by 21.3%, p = 0.0067),

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90 min (by 22.3%, p = 0.0202), and 120 min (by 14.2%, p = 0.0248), compared to Zn-insulin alone (Figure 7C).

Preliminary Cytotoxicity of Cyclic DNP Derivatives after a Single Intraintestinal Administration in Mice. *In vivo* cytotoxicity of cyclic DNP derivative was examined after intraintestinal administration. Lactate dehydrogenase (LDH) assays are used to examine damage to the intestines in rodents.[20] Intraintestinal administration of cyclic L-DNP derivative and cyclic D-DNP derivative did not increase the release of LDH from the small intestinal membrane of the mouse for 120 min, while sodium taurodeoxycholate significantly increased the release of LDH (Figure S5). Histological analysis showed no significant change in villi morphology in the mouse small intestine after coadministration of Zn-insulin and cyclic L- or D-DNP derivatives, while sodium taurodeoxycholate induced small changes within villi in the small intestine of treated animals (Figure S6). Because M13 phages displaying cyclic L-DNP peptide were predominantly detected in the liver and kidney after intravenous injection (Figure S7), cyclic DNP peptide is presumed to be predominantly distributed in the liver and kidney following permeation across the small intestine. However, neither cyclic L-DNP derivative nor cyclic D-DNP derivative had any effect on 23 serum biochemical parameters, including those related to liver and kidney dysfunction, over 120 min (Table S3).

DISCUSSION

In the present study, we have demonstrated that cyclic DNP derivatives improve insulin absorption from the small intestine in mice. Moreover, the D-form of cyclic DNP derivative was more effective in reducing blood glucose levels than the L-form after both intraintestinal and oral coadministration with insulin or Zn-insulin. Furthermore, oral coadministration of the cyclic D-DNP derivative enhanced the effect of Zn-insulin hexamers (Humulin3/7) in mice. The cyclic D-DNP derivative did not induce any cytotoxicity either locally in the small intestine or systemically after single-dose intraintestinal administration. These findings suggest that cyclic D-DNP derivative is a useful permeation enhancer for oral insulin.

Our pharmacological studies demonstrated that intraintestinal coadministration of cyclic D-DNP derivative and insulin or Zn-insulin reduced blood glucose levels in mice by about 1.5−2.8-fold (compared to coadministration with cyclic L-DNP derivative). Generally, peptides containing D-amino acids are more resistant to enzymatic degradation than peptides containing L-amino acids.[14,32−36] However, neither linear L-DNP derivative nor linear D-DNP derivative (which are not cyclized by a disulfide bond) could increase insulin absorption from the small intestine of the mouse. Therefore, protection of cyclic DNP derivative from enzymatic digestion in the small intestine (through the use of D-amino acids and by cyclization of the DNP peptide) is essential to enhance cyclic DNP derivative-induced insulin absorption across the small intestine.

The lumen of the upper gastrointestinal tract contains large amounts of pancreatic proteases that can degrade insulin, and hence protection of insulin from degradation by these enzymes is necessary to provide biological active insulin.[5,14,37,38] Insulin hexamer is resistant to physical and chemical degradation *in vitro*.[39,40] We demonstrate that the degradation rate of Zn-insulin in the small intestine of the mouse was slower by 75.7% (compared with insulin monomer). These findings demonstrate that coadministration of Zn-insulin and cyclic D-DNP derivative has a long-lasting effect in improving insulin absorption, and that this is a result of both an increase in insulin permeation and a reduction in insulin degradation in the small intestine.

In vitro and *in vivo* permeability studies demonstrated that the permeability of the cyclic D-DNP derivative was reduced by approximately 40% compared to the cyclic L-DNP derivative. This may be because of less efficient internalization into the small intestine epithelial cells and/or lower affinity to the target molecules caused by different chirality. However, there are discrepancies between this result and two other findings, the first concerning the relationship between the permeability of cyclic DNP derivative and plasma insulin concentration in the portal vein, and the second concerning the relationship between the permeability of cyclic DNP derivative and the hypoglycemic effect after coadministration of insulin or Zn-insulin with cyclic DNP derivative. Correlation analysis suggests that the permeability of the L-form of cyclic DNP derivative is likely to be reduced in the presence of Zn-insulin (compared to insulin), while the permeability of the D-form peptide appears to be increased.

The L-form peptide is generally more susceptible to degradation by enzymes than the D-form of the peptide.[14,32−36] Insulin is also degraded in the mouse small intestine with a half-life of 8.59 min, which is 4.13-fold lower than that of Zn-insulin (35.5 min). Based on these findings, we assume that cyclic L-DNP derivative and insulin are competitively degraded by digestive enzymes in the mouse small intestine. Thus, the cyclic L-DNP derivative remaining after intraintestinal coadministration of Zn-insulin is lower than after intraintestinal coadministration of insulin, and this may reduce the function of the cyclic L-DNP derivative as a permeability enhancer. In contrast, the stability of the cyclic D-DNP derivative is largely unaffected by the presence of insulin or Zn-insulin. Hence, the instability of cyclic L-DNP derivative (compared with cyclic D-DNP derivative) may partially explain the above discrepancies. Thus, the enhanced stabilities of cyclic D-DNP derivative and Zn-insulin in the small intestine may contribute to a greater hypoglycemic effect by sustainable absorption of cyclic D-DNP derivative-mediated insulin.

CPPs are used for oral insulin development in basic research. Compared to other CPPs, the advantage of the cyclic D-DNP derivative is a reduction in blood glucose levels from 15 min after oral coadministration with Zn-insulin (D-penetratin reduces blood glucose levels at least 60 min after oral coadministration of insulin).[14] Since several types of insulin such as fast-acting, intermediate-acting, and long-acting variants are used in patients with diabetes mellitus, several variants of oral insulin are also required. A long-acting oral insulin (I338) is currently in clinical trials.[11] Thus, oral insulin using cyclic D-DNP derivative may be suitable as a fast-acting type of oral insulin.

Reformulation of insulin injection to oral insulin is advantageous because the oral formulation may not be as effort intensive as the injection formulation. Injection formulations generally contain insulin as Zn-insulin hexamers. Results from our present study demonstrate that cyclic D-DNP derivative enhanced the permeation of human regular insulin in Humulin3/7 from the small intestine of mice. Based on our present findings, we believe that the addition of cyclic D-DNP derivative to an insulin injection solution could facilitate the development of oral insulin.

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The PA of oral coadministration of Zn-insulin with cyclic D-DNP derivative is assumed to be lower than the PA value after intraintestinal administration (6.33%) because there are several barriers in the gastrointestinal tract to prevent insulin absorption with cyclic D-DNP derivative after oral administration. Thus, the absorption efficacy of insulin after oral administration of cyclic D-DNP derivative is similar to that of D-penetratin (PA; 6.8%[14]). Furthermore, the bioavailability of insulin after oral coadministration of Zn-insulin with cyclic D-DNP derivative is estimated to be under 6%, because the PA value after intraintestinal coadministration of Zn-insulin with cyclic D-DNP derivative was 6.33%.

In the present study, we aimed to verify our hypothesis that coadministration of insulin with cyclic DNP derivative improves insulin absorption from the small intestine in mice. To improve the bioavailability of insulin using cyclic D-DNP derivative, further optimization studies are necessary. For example, Zn-insulin was resistant to degradation in the small intestine of mice, but Zn-insulin was still degraded with a half-life of 35.5 min. Soybean trypsin inhibitor may be effective in reducing insulin degradation in the gastrointestinal tract.[14] In some instances, oral insulin preparations used in clinical trials already contain protease inhibitors.[10] While 5 mM cyclic DNP derivative was used in the present study (in line with previous reports), 5 mM cyclic DNP derivative is a higher concentration than the estimated dissociation constant in Caco-2 cells (about 10 μM).[19] Although cyclic L-DNP peptide is thought to facilitate the permeation of conjugated compound across the mouse small intestine,[19] we have no evidence that the cyclic DNP derivative forms a complex with insulin. Therefore, with the above considerations taken into account, future developments should include (1) suppression of Zn-insulin degradation in the gastrointestinal tract; (2) a reduction in the concentration of cyclic D-DNP derivative to eliminate self-inhibition; and (3) conjugation of cyclic D-DNP derivative with Zn-insulin to improve insulin absorption by cyclic D-DNP derivative.

We verified whether coadministration of Zn-insulin and cyclic D-DNP derivative reduced blood glucose levels in a diabetes mellitus mouse model. Intraintestinal and oral coadministration of Zn-insulin and cyclic D-DNP derivative successfully reduced blood glucose levels in the STZ-treated mice, which mimics the type 1 diabetes mellitus phenotype in mice.[41] The time profile of blood glucose levels in STZ-treated mice was markedly different from that in control mice, after both intraintestinal and oral administration. In STZ-treated mice, the initial blood glucose reduction was delayed by 15 min compared with control mice, and the reduction in blood glucose levels in STZ-treated mice was lower than that in control mice. Under diabetic conditions, the rate of mucin secretion is increased in the intestine.[42] Mucus forms a selective barrier to particles and molecules, preventing their penetration to the epithelial surface of mucosal tissues. A difference in mucus thickness may explain the difference in the time profiles of blood glucose levels between STZ-treated mice and control mice. A pharmacokinetic study would be helpful to clarify the delay in initial blood glucose reduction after oral coadministration of Zn-insulin with cyclic DNP derivative in STZ-treated mice.

Patients with type 1 diabetes mellitus must be administered daily injections to manage their symptoms. In addition to therapeutic efficacy, safety is also important in the development of oral insulin. I338 is a possible long-acting type of oral insulin, and its therapeutic efficacy and safety have been demonstrated in humans and dogs.[11,43] Our *in vivo* experiments on LDH leakage and serum biochemical analysis demonstrated that cyclic D-DNP derivative did not exhibit cytotoxicity, damage peripheral tissues, or induce inflammation after a single administration. In addition, histological analysis suggests that intraintestinal coadministration of Zn-insulin with cyclic D-DNP derivative did not induce severe damage to the small intestinal villus (compared to Zn-insulin alone). We conclude that cyclic D-DNP derivative does not induce any cytotoxicity, either locally in the small intestine or systemically, after single-dose intraintestinal administration. However, our present study evaluated the therapeutic effect and preliminary cytotoxicity of only a single-oral administration of cyclic D-DNP derivative and Zn-insulin in mice. Therefore, future studies are required to determine the therapeutic efficacy and safety of repeated oral administration of cyclic D-DNP derivative and Zn-insulin during long-term clinical use.

CONCLUSIONS

In the present study, we provided evidence that cyclic D-DNP derivative increased the small intestinal permeability of both insulin and Zn-insulin. Together, cyclic D-DNP derivative and Zn-insulin effectively reduced blood glucose levels in the DM mouse model after oral absorption. Hence, cyclic D-DNP derivative may be useful in enhancing the oral absorption of insulin and Zn-insulin from the small intestine.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.0c01010.

Supplementary Materials and Methods with accompanying figures and tables: Permeation of cyclic DNP derivatives; Effect of linear DNP derivatives on blood glucose levels; Time profile of blood glucose levels after subcutaneous administration of insulin in mice and after oral administration of cyclic DNP derivatives in mice; Cytotoxicity of cyclic DNP derivatives in mouse small intestine; Histological analysis of mouse small intestine; Distribution of M13 phages; Amino acid sequences of the DNP derivatives; Summary of parameters for degradation of insulin and Zn-insulin in the mouse small intestine; Effect of cyclic DNP derivatives on serum biochemical parameters after administration into the mouse jejunum using the *in situ* closed-loop method (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AAC, area above the curve; BGL, blood glucose level; CPPs, cell-permeable peptides; DNP peptide, DNPGNET peptide; D-DNP, D-form of cyclic DNP peptide; HBSS, Hanks' Balanced Salt Solution; LDH, lactate dehydrogenase; LLOQ, lower limit of quantification; L-DNP, L-form of cyclic DNP peptide; PA, pharmacological availability; STZ, streptozotocin; s.c., subcutaneous; Zn-insulin, zinc containing insulin hexamer

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