Insulin is the most important therapeutic agent for diabetes, even though there are now many other oral antidiabetic medicines available. Therefore, many types of insulin formulations, including rapid- and long-acting insulin as well as mixed type insulin, have been developed to date.\(^1\)–\(^3\) Diabetes patients can appropriately choose injectable types of insulin formulations; however, more convenient forms of insulin that can be administered via noninvasive routes, such as oral administration, have not yet been developed because of poor absorption through mucosal regions.\(^4\)–\(^7\) To further improve the QOL and compliance of patients, we aimed to develop a noninvasive form of insulin by using pharmaceutical technologies and drug delivery systems. Moreover, the oral route of insulin absorption has another advantage as insulin absorbed from the intestine first passes through the liver, similarly to endogenous insulin secreted to the portal vein; therefore, oral insulin can circumvent the risk of hypoglycemia.

A major hurdle in the development of formulations for administration via noninvasive routes is the digestion and degradation in the harsh gastrointestinal environment. To protect biologics, such as insulin, from the acidic pH in the stomach and the actions of peptidases and proteases in the intestine, various nano- and micro-sized carriers composed of lipidic and polymeric moieties have been developed.\(^8\)–\(^10\) Another challenge is to permeate the barrier made by the epithelial cell layer, which limits the passage of hydrophilic macromolecules via the transcellular and paracellular routes. Some absorption enhancers can increase macromolecular drug absorption, including that of insulin, by disturbing the lipid membrane of epithelial cells and opening the paracellular tight junctions. However, we cannot exclude safety concerns, because such strategies can facilitate the invasion of exogenous pathogens.

Recently, in an in\(\text{vivo}\) study in mice, we found that cell-penetrating peptides (CPPs), particularly penetratin, can significantly increase the oral absorption of insulin.\(^11\) Furthermore, an in\(\text{silico}\) optimization study established that a partially modified penetratin analog, termed PenetraMax, has the greatest potential to enhance the intestinal absorption of insulin.\(^12\) Notably, our strategy using CPPs was easily achieved by the coadministration of insulin and CPPs as a physical mixture, which simplifies the complicated chemical decoration of insulin with CPPs and minimizes the loss of activity.\(^13\)\(^,\)\(^14\) We also demonstrated that intermolecular interaction between insulin and CPPs was a major factor governing the effectiveness of the absorption enhancement strategy, and intermolecular interactions can be measured using binding parameters, such as dissociation constants and concentrations of both insulin and CPPs.\(^15\)

This present study was aimed at determining the optimal oral insulin delivery conditions that would maximize the utility of cell-penetrating peptides (CPPs) by using a noncovalent strategy. We first compared the effectiveness of two potential CPPs, penetratin and its analog PenetraMax, as absorption enhancers for insulin. The combined effect was evaluated under in\(\text{vivo}\) oral administration conditions. Both \(\alpha\)-forms of CPPs were highly effective for increasing the oral absorption of insulin, and \(\alpha\)-PenetraMax showed a more rapid onset of absorption enhancement effects compared with those of \(\alpha\)-penetratin. However, synergistic absorption enhancement effects after combination treatment were not observed. Next, we tried a theoretical approach to establish optimized oral insulin delivery conditions. A surface plasmon resonance (SPR)-based analysis demonstrated that binding between insulin and penetratin (\(2\) \(\mu\)M) might be saturated at \(100–500\) \(\mu\)M penetratin, while the bound concentration of penetratin could increase in accordance with an increased concentration of mixed insulin. To test this hypothesis, we investigated the effectiveness of different insulin doses in the gastric pH-neutralized mice. The results showed that the dissociation of noncovalent complexes of insulin and CPPs at the low gastric pH was prevented in these mice. Our findings clearly suggested that a noncovalent strategy with CPPs represents an effective approach for the \(\alpha\)-form of CPP to increase the concentration of CPP-bound insulin to attain greater absorption of insulin, although this approach may not be appropriate for the \(\alpha\)-form of CPP. Our findings will contribute to the development of oral dosage forms of insulin for noncovalent strategies involving CPP.

**Key words** oral delivery; insulin; cell-penetrating peptide; penetratin; intermolecular interaction
MATERIALS AND METHODS

**Materials** Recombinant human insulin (27.5 IU/mg) and famotidine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The l- and d-forms of penetratin and PenetraMax (RQIKIWFQNRRMKWKK and KWFKIQMQIRRWKNKR, respectively) were synthesized by Sigma-Genosys, Life Science Division of Sigma-Aldrich Japan Co. (Hokkaido, Japan). Carboxymethyl dextran (CM5)-coated sensor chips were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). All other chemicals were of analytical grade and are commercially available.

**Preparation of Insulin/CPP Mixed Solutions** To prepare insulin solutions, specific amounts of recombinant human insulin were dissolved in 100 µL of 0.1 M HCl in a plastic vial. The insulin–HCl solution was diluted to 2.8 mL with phosphate-buffered saline (PBS, pH 6.0) containing 0.001% methylcellulose, which prevents the adsorption of insulin to the surface of the plastic vial, and the pH was then adjusted with 100 µL of 0.1 M NaOH. The insulin solution was prepared at 6, 15, or 30 IU/mL in PBS. Specific amounts of the l- and d-forms of CPPs (penetratin or PenetraMax) were weighed in a plastic vial and dissolved in PBS (pH 6.0) that contained 0.001% methylcellulose. Equal volumes of insulin and CPP solutions were gently mixed at room temperature. The final concentrations of insulin and CPP solutions were 3, 7.5, or 15 IU/mL (10, 25, or 50 IU/kg as insulin dose, respectively) and 1 or 2 mM, respectively. Each insulin/CPP solution was clear after mixing.

**Animals** In vivo experiments were performed at Kobe Gakuin University in compliance with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Seven-week-old male ddY mice weighing 35–40 g were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were housed under the following conditions: controlled temperature (23±1°C) with a 12:12-h light/dark cycle and relative humidity of 55±5%. They were provided free access to water and food during acclimatization. The mice were fasted for 24 h before experiments; however, they were allowed to drink water ad libitum.

**In Vivo Oral Administration Study** Protocols for the oral administration experiments are shown in Fig. 1. On the day before drug administration, the mice were first trained with oral administration tubes (i.d. 0.9 × length 50 mm, Natsu-me Seisakusho Co., Ltd., Tokyo, Japan) without insulin or CPP solution to acclimate them to oral gavage, which might induce an unexpected elevation in blood glucose levels due to stress. Furthermore, 60 min before actual drug administration, the mice were trained by tail cut for blood sampling as well as second oral gavage training. At 60 min after the tail cut and second oral gavage training, aliquots of blood were collected from the tail vein and blood glucose levels were measured using a glucometer (One Touch Ultra View®, Johnson & Johnson K.K., Tokyo, Japan). Considering the individual variation in blood glucose levels, the mice were appropriately divided into several groups, such as vehicle (PBS), insulin control, and insulin mixed with CPPs. In tests under normalized gastric pH conditions, 200 µL of famotidine solution (2.25 mg/mL in saline containing 5 mM HCl, equivalent to 15 mg/kg dose) was intraperitoneally injected 30 min before drug administration, as shown in Fig. 1B.16) In a preliminary experiment, the pH of gastric fluid was checked using universal pH indicator paper (Macherey–Nagel GmbH & Co., KG, Düren, Germany) before and after the oral dosing of PBS or the intraperitoneal (i.p.) injection of famotidine. Following group selection, the drug solution was orally administered, and blood was collected at 15, 30, 60, 120, 180, 240, 300, and 360 min after dosing. Blood glucose levels are shown as % of values at t₀, and the

![Fig. 1. Schemes for the in Vivo Oral Administration of Insulin and CPPs to Mice](image-url)
magnitude of the hypoglycemic response (pharmacological availability (PA)) was calculated based on the area above the curve (AAC) from 0 to 6 h by using a trapezoidal method. The PA was calculated relative to the subcutaneous (s.c.) injection data.

Surface Plasmon Resonance (SPR)-Based Binding Assay

The intermolecular interactions between insulin and L- or D-penetratin were analyzed by SPR (Biacore X-100, GE Healthcare, U.K.). To measure the binding of L- or D-penetratin to insulin, insulin was immobilized at the carboxymethyl dextran surface of a CM5 sensor chip by using amine coupling. For the immobilization procedure, insulin was diluted to a final concentration of 50 µg/mL using acetate buffer at pH 4.5, and immobilized on the chip surface in separate flow cells at 5 µL/min for 7 min. Reference surfaces were prepared by amine coupling activation followed by immediate deactivation. For binding measurements, different concentrations of L- or D-penetratin (2–100 µM) were injected for 90 s followed by an additional 90-s dissociation phase. At the end of each cycle, the surface was regenerated by a 30-s injection of 1 M NaCl. Measurements were carried out in Hanks’ balanced salt solution (HBSS) with different pH (3.0, 5.3, 6.0) or Japanese Pharmacopoeia 1st dissolution fluid (pH 1.2) at 20 µL/min and 25°C.

Each sensorgram was determined by subtracting nonspecific binding on the surface of the reference flow cell from total binding on the immobilized-insulin surface. First, the equilibrium binding of each cycle was calculated using BIAevaluation software, and then the dissociation constant (KD) and maximum amount (Rmax) were calculated using equilibrium amounts based on fitting with the MULTI program followed by Scatchard analysis. As observed in previous studies, Scatchard plot of the binding between insulin and penetratin shows two phase-binding patterns, and KD and Rmax for both phases were calculated and categorized as high- and low-affinity phases. The maximum binding capacity under transcellular transport assay conditions (Bmax) was calculated using Eq. 1:

\[ B_{\text{max}} = \frac{[\text{Ins}]}{[\text{Ins}]_{\text{immob}}} \times R_{\text{max}} \]

where [Ins], is the total ligand (insulin) concentration under the transcellular transport assay conditions, and [Ins]immob is the amount of immobilized ligand (insulin). The free ([C]f) and bound ([C]b) concentrations of penetratin in the transcellular transport assay conditions were calculated using Eqs. 2 and 3:

\[ [C]_f = \frac{[C]_b + B_{\text{max}} \times [C]_b}{(K_D + [C]_b)} \]

\[ [C]_b = [C]_f - [C]_f \]

where [C]f is the total penetratin concentration. The bound penetratin concentration under transcellular transport assay conditions [C]b was used as an index of the binding affinity between insulin and penetratin.

Statistical Analysis

All results are expressed as the mean±standard error of the mean (S.E.M.) of multiple determinations. The significance of the differences in mean values of multiple groups was evaluated using ANOVA followed by Dunnett’s test. Differences were considered significant when the p value was <0.05.

RESULTS AND DISCUSSION

Effect of Penetratin and PenetraMax on the in Vivo Oral Absorption of Insulin

Our recent work using mice demonstrated that oral absorption of insulin was enhanced by coadministration of penetratin, an amphipathic CPP.11) On the other hand, we also found that PenetraMax, which is a penetratin analog chosen based on our optimization analysis with self-organization maps and molecular orbital method, more strongly accelerated intestinal insulin absorption compared to that achieved with original penetratin in an in situ administration study involving the rat ileal loop.12) Therefore, we expected that PenetraMax may be the best choice as the bioenhancer for achieving the in vivo oral absorption of insulin, and thus examined the effect of coadministration with L- and D-forms of PenetraMax on the oral absorption of insulin in mice.

In this study, the strength of the absorption enhancement effect of CPPs was estimated by evaluating the hypoglycemic reaction after the oral administration of insulin with CPPs. Figure 2A shows the changes in the blood glucose levels after oral administration of the physical mixture of insulin...
studies). In this absorption study, the ability of L-form penetratin to enhance the oral absorption of insulin, as shown in Table 1. PA is an index of absorption enhancement efficiency. Therefore, we hypothesized that the combination of penetratin and PenetraMax, especially the D-form, strongly decreased the blood glucose level at only 240 min after administration. Although the coadministration with L-penetratin was partially effective on the blood glucose level at only 240 min after administration, its effect was weaker than that of D-penetratin; this finding was similar to that obtained in our previous study.11) PA is an index of absorption enhancement efficiency, as shown in Table 1.

Table 1  Pharmacological Availability of Orally Administered Insulin with CPPs

<table>
<thead>
<tr>
<th>Insulin-s.c. (1 IU/kg)</th>
<th>Vehicle (PBS)-p.o.</th>
<th>Insulin-p.o. (10 IU/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Penetratin (2 mM)</td>
<td>20.6±2.5</td>
<td>10.7±1.2</td>
</tr>
<tr>
<td>+Peptidase Max (2 mM)</td>
<td>42.0±10.7</td>
<td>2.19±0.55</td>
</tr>
<tr>
<td>+D-Penetratin (2 mM)</td>
<td>110.0±30.3</td>
<td>5.71±1.57</td>
</tr>
<tr>
<td>+L-Penetratin (2 mM)</td>
<td>67.2±19.7</td>
<td>3.49±1.02</td>
</tr>
<tr>
<td>+D-Penetratin (2 mM)</td>
<td>104.3±37.2</td>
<td>5.41±1.93</td>
</tr>
<tr>
<td>+D-Penetratin (1 mM)</td>
<td>68.8±19.1</td>
<td>3.57±0.99</td>
</tr>
<tr>
<td>+D-Penetratin (2 mM)</td>
<td>71.6±34.9</td>
<td>3.71±1.81</td>
</tr>
</tbody>
</table>

Data indicate mean±S.E.M. values (N=3–14). AAC, area above the curve.

We could confirm that blood glucose levels decreased most significantly after coadministration of insulin with D-penetratin (2 mM) in particular. The initial increase in blood glucose levels immediately after oral administration might be attributable to stress due to oral gavage and blood sampling, even though the mice were trained before the study. The hypoglycemic reaction after the oral administration of insulin with D-penetratin beyond the rapid initial elevation in blood glucose level reflected the strong potential of D-penetratin to enhance the oral absorption of insulin. Furthermore, the hypoglycemic effect of insulin coadministered with D-penetratin persisted at 30 min after their administration. Although the coadministration with L-penetratin was partially effective on the blood glucose level at only 240 min after administration, its effect was weaker than that of D-penetratin; this finding was similar to that obtained in our previous study.11) PA is an index of absorption enhancement efficiency, as shown in Table 1.

Figure 2B shows the results after the oral administration of insulin with L- or D-PenetraMax. Similar to penetratin, PenetraMax, especially the D-form, strongly decreased the blood glucose levels. In contrast to the effects of penetratin, the initial elevation in blood glucose levels after oral administration was only prevented by coadministration with D-PenetraMax (2 mM). This is not consistent with our previous results of D- and L-form PenetraMax, which showed almost the same degree of absorption enhancement effects on insulin intestinal absorption.17) This discrepancy could be a consequence of differences in absorption study methods (in situ versus in vivo studies). In this in vivo absorption study, the ability of L-form of PenetraMax was significantly diminished by the harsher environment of the entire gastrointestinal tract.

Obviously, penetratin and PenetraMax promoted the oral absorption of insulin in a different manner. D-Penetratin and D-PenetraMax showed relatively continuous enhancement effects and quite rapid onsets of effect, respectively. Therefore, we further hypothesized that the combination of these two CPPs would yield a synergistic action to gain more considerable oral absorption of insulin. Figure 2C shows the effect of the combined use of D-penetratin and D-PenetraMax on the hypoglycemic response following the oral administration of insulin. Indeed, after a combination of CPPs, the onset of the hypoglycemic effect was more rapid than when penetratin alone was added to insulin, in particular the initial rise in the blood glucose levels just after oral gavage was almost suppressed by combining with D-PenetraMax (2 mM). However, as shown in Table 1, the CPP combination strategy did not lead to an improvement in PA values. Additionally, PA did not change according to the concentrations of D-penetratin and D-PenetraMax (total 2 and 4 mM). In our previous study, we showed that excess concentrations of CPPs tended to result in the formation of insoluble aggregates in solution,13) and this trend was more clear for the D-form of CPPs. Therefore, the total concentration of D-penetratin and D-PenetraMax (4 mM) might form such precipitations in the intestine. To achieve a combined strategy using multiple CPPs, we need to further precisely investigate the eventual formation of complexes, the optimum effective doses, and the types of CPP to be combined.

Exploring Optimized Doses of Insulin Based on SPR Analysis and Effects on Oral Insulin Absorption  Our previous study showed that intermolecular interaction between peptide drugs and CPPs is a driving force for the stimulation of intestinal drug absorption via a noncovalent CPPs strategy.15,18) Furthermore, we showed that mixed CPPs are the key to improving interactions and the resulting absorption enhancement effect.15) In this step for exploring the optimized dosage condition, we tested the relationship among insulin concentrations, interactions with penetratin, and absorption enhancement efficiency.

Before conducting the animal study, we estimated the binding affinity and capacity of L- and D-penetratin to insulin by measuring the binding characteristics of penetratin to insulin by SPR analysis. Figures 3A and E show binding sensorgrams when various concentrations of L- and D-penetratin were applied to an insulin-modified surface at pH 6.0, which is equivalent to the pH of the administration solution used in the in vivo study. Based on the Scatchard analysis with response units detected for various concentrations of penetratin, we calculated the dissociation constants (K_D) and maximum binding capacity (R_max) of L- and D-penetratin to insulin (Table 2). Furthermore, Fig. 4 shows the relationship between total concentration of L- and D-penetratin mixed into various concentrations of insulin (18.8, 47.0, 93.9 µM) and the concentration of penetratin as the bound form. When insulin was administered at 101U/kg to mice, insulin solution was mixed at 3 IU/mL (18.8 µM) with L- and D-penetratin (2 mM), and ca. 50 µM of L- and D-penetratin could be estimated to be bound with insulin. The simulated curves of bound and total concentrations of penetratin in Fig. 4 and K_D values shown in Table 2 suggest that binding between insulin and L- or D-penetratin might be saturated at 100–500 µM, and therefore it may be difficult.
to facilitate interactions between insulin and penetratin by increasing the total concentration of penetratin. Alternatively, the binding curves simulated with higher concentrations of insulin (47.0, 93.9 \(\mu M\)) suggested that the bound concentration of penetratin may be elevated in accordance with the increase in the concentration of mixed insulin, so the absorption enhancement effect may also be improved. Therefore, we next examined the effect of D-penetratin on the oral absorption of insulin at several doses to confirm the importance of the bound concentration of D-penetratin under in vivo settings.

Figure 5 shows the hypoglycemic reaction after the oral administration of insulin at 10, 25, or 50 IU/kg (18.8, 47.0, 93.9 \(\mu M\), respectively) with or without D-penetratin (2 mM). For the group that received insulin with D-penetratin, variation in data tended to be small after increasing the dose of insulin, verifying the action of D-penetratin at this dose of insulin. However, contrary to our expectations, despite increasing the insulin concentration and the consequent increase in the bound form of D-penetratin, further improvement of the enhancement effects of D-penetratin on oral insulin absorption was not observed (Figs. 5B, C). Since the intermolecular interaction is drastically changed by pH (Fig. 3), insulin bound to CPP could be dissociated by the acidic environment of the

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Table 2. Binding Parameters Derived from Intermolecular Interactions between L- or D-Penetratin and Insulin at pH 6.0

<table>
<thead>
<tr>
<th></th>
<th>L-Penetratin</th>
<th>D-Penetratin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High affinity</td>
<td>Low affinity</td>
</tr>
<tr>
<td>Immobilized insulin amount (pmol/mm²)</td>
<td>0.214</td>
<td>0.214</td>
</tr>
<tr>
<td>(K_d) ((\mu M))</td>
<td>3.78</td>
<td>87.1</td>
</tr>
<tr>
<td>(R_{max}) (RU)</td>
<td>169</td>
<td>1292</td>
</tr>
<tr>
<td>(R_{max}) (pmol/mm²)</td>
<td>0.0751</td>
<td>0.575</td>
</tr>
<tr>
<td>Binding ratio (penetratin/insulin)</td>
<td>0.350</td>
<td>2.68</td>
</tr>
</tbody>
</table>

Parameters were calculated based on SPR binding data shown in Fig. 3 using Scatchard analysis.

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Fig. 4. Associations between Total Concentrations of L- or D-Penetratin Added to the Insulin Solution (18.8, 47.0, or 93.9 \(\mu M\)) and Concentrations of Penetratin Bound to Insulin Calculated Using Parameters Described in Table 1 and Eqs. (1), (2), and (3) Described in Materials and Methods. Solid and dotted curves indicate data obtained using L- and D-penetratin, respectively.
stomach. Therefore, it is thought that contributions of the increase in the bound form of D-penetratin might not occur.

**Evaluation of the Oral Delivery of Insulin under Neutralized Gastric pH Conditions**

As described above, facilitating intermolecular interactions between insulin and penetratin in the administered solution could not lead to further increases in the oral absorption of insulin. This might be because of the dissociation of complexes of insulin and penetratin by reduction of electrostatic forces in the low gastric pH after administration. Figure 6 shows that the gastric pH remained at 1 or 2, even after the oral administration of 100 µL PBS, although it slightly and temporally tended to be neutral (pH 4–7) just after its oral administration. As shown in Fig. 3, the SPR measurement at various pH values, including acidic conditions, confirmed that L- and D-penetratin can interact with insulin at weak acidic conditions (pH 5.3 or 6.0), whereas this interaction was abolished at strong acidic conditions (pH 1.2 or 3.0). As the isoelectric point of insulin is ca. 5.3, the net charge of insulin molecules reversed to positive at gastric low pH, and then it might dissociate from penetratin at pH <5.3. Therefore, it was difficult to keep the complex form of insulin and penetratin in the stomach after oral administration.

Therefore, to confirm our above-mentioned hypothesis, we tried to neutralize the pH in the stomach fluid. In this experiment, the histamine H2-receptor antagonist famotidine (15mg/kg) was injected intraperitoneally in mice 30 min before oral administration of insulin and penetratin. As shown in Fig. 6, we confirmed that the pH of the gastric fluid of mice receiving famotidine was neutralized and modified pH could be maintained for at least 30 min. We therefore examined the enhancement effect of L- and D-penetratin on oral insulin absorption using gastric pH-neutralized mice.

Figure 7 shows blood glucose levels after the oral administration of insulin (Fig. 7A (10IU/kg), 7B (50IU/kg)) with L- or D-penetratin (2mM) to gastric pH-neutralized mice. Theoretically, higher AAC was expected in the gastric pH-neutralized mice compared to that of the normal mice. However, with regard to D-penetratin, preventing molecular dissociation was not effective for improving oral insulin absorption (AAC; 113.1% gluc. reduc.·h (Fig. 5C, Table 3) vs. AAC; 73.3% gluc. reduc.·h (Fig. 7B, Table 3)). As the D-form of CPP is highly resistant to enzymatic degradation, maintaining the high concentration of D-penetratin-bound insulin might lead to aggregate formation, as we previously reported. Therefore, it is thought that this might have resulted in AAC reduction. These results suggest that a noncovalent strategy with CPP is not an effective approach for the D-form of CPP to increase the concentration of CPP-bound insulin to obtain more enhancing absorption of drug. Regarding L-penetratin, higher AAC values were observed in the gastric pH-neutralized mice compared to the normal mice administered insulin 10IU/kg (AAC: 42.0% vs. 54.1% glucreduc.·h (Table 3)). Furthermore, the AAC
extensively rose in gastric pH-neutralized mice (113.2% gluc. reduc.·h (Table 3)) at 50 IU/kg of insulin, which is higher than the value obtained in pH-unmodified normal mice (87.5% gluc. reduc.·h (Table 3, time profile of blood glucose level was not shown)), although it did not reach the predicted values. Theoretically, 5 times of AAC values obtained at 10 IU/kg administration could be obtained from the gastric pH-neutralized mice administered insulin 50 IU/kg. As the reason for this is that it required on the order of several tens of minutes until the mixed solution was completely discharged from the stomach, so there was a chance to drop pH value during that time. This might lead to dissociation of some CPP-insulin complexes. However, this situation could be overcome in the future by using a formulation approach, such as enteric-coated capsules. Taken together, these results suggest that at least compared to the D-form of CPP, a possible strategy to increase the amount of CPP-bound insulin would be effective for the L-form of CPP to further improve the effect of CPP in oral insulin delivery.

CONCLUSION

Our recent work shows that the noncovalent use of CPPs represents a strategy to greatly enhance the oral absorption of biopharmaceuticals, such as insulin. As a next step towards clinical development, our present study aimed to investigate optimized oral insulin delivery conditions that maximize the utility of CPPs in noncovalent strategy.

In vivo oral administration studies showed that both D-penetratin and D-PenmetaMax were highly effective for increasing the oral absorption of insulin, and D-PenmetaMax showed a more rapid onset of absorption enhancement effects than those of D-penetratin. On the other hand, combination of these CPPs did not show synergistic absorption enhancement effects in the in vivo oral insulin study. This finding suggested the combination effects were not obtained by combinations of similar types of CPPs. To achieve combined effects of multiple CPPs, we need to further precisely investigate the eventual formation of complexes, the optimum effective doses, and the types of CPP to be combined.

In this present study, we also tried a theoretical approach to establish an optimized oral insulin delivery condition to maximize the utility of CPPs using a noncovalent strategy. The SPR-based intermolecular binding analysis demonstrated that binding between insulin and penetratin (2 mM) might be saturated at 100–500 µM penetratin; however, the bound concentration of penetratin could be elevated in accordance with the increase in the concentration of mixed insulin. To test this hypothesis, we investigated oral insulin absorption profiles coadministered with CPP at different insulin doses in gastric pH-neutralized mice. The results showed that the dissociation of noncovalent complexes of insulin and CPPs at the low gastric pH was prevented in these mice. Our data clearly suggested that a noncovalent strategy with CPP represents an effective approach for the L-form of CPP to increase the concentration of CPP-bound insulin to attain more enhancing absorption effects.

Table 3. Areas above the Curves Were Calculated from Kinetic Profiles of Blood Glucose Levels after the Oral Administration of Insulin with CPPs to Gastric pH-Neutralized and Unmodified Mice

<table>
<thead>
<tr>
<th>Insulin dose (IU/kg)</th>
<th>pH-Neutralized</th>
<th>Normal (unmodified)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Vehicle (PBS)-p.o.</td>
<td>16.3±9.1</td>
<td>16.3±9.1</td>
</tr>
<tr>
<td>Insulin-p.o.</td>
<td>34.5</td>
<td>8.0±5.7</td>
</tr>
<tr>
<td>+L-Penetratin (2 mM)</td>
<td>54.1±17.6</td>
<td>113.2±13.3</td>
</tr>
<tr>
<td>+D-Penetratin (2 mM)</td>
<td>54.6±11.8</td>
<td>73.3±16.8</td>
</tr>
</tbody>
</table>

Data indicate mean±S.E.M. values (N=3–14), except for the group that received insulin (10 IU/kg; N=2).
absorption of insulin. However, this approach may not appropriate for the d-form of CPP. The findings in this present study will contribute to the development of oral dosage forms of insulin for a noncovalent strategy with CPP.

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Conflict of Interest The authors declare no conflict of interest.

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