Safety of the Cell-Penetrating Peptide Penetratin as an Oral Absorption Enhancer

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Novel delivery technology using cell-penetrating peptides (CPPs) have recently shown their potential and are emerging as promising candidates for an oral protein and peptide delivery systems. As with for the development of any absorption enhancer that is meant to function across an epithelial layer covering a surface highly exposed to pathogens such as the intestines, concern arises about the safety of such enhancers. The purpose of this study was to investigate the effect of 7 d of consecutive oral administrations of CPPs and a typical enterotoxin, lipopolysaccharide (LPS) to mice to determine the degree, if any, of damage caused to the hepatic tissue. Following the 7-d dosing regimen, we could not detect significantly increased levels of the liver enzymes alanine aminotransferase and aspartate aminotransferase in plasma of mice treated with CPP and LPS compared to the controls, whereas heightened levels were observed in animals receiving the bile salt. In conclusion, the repeated use of CPPs as an oral absorption enhancer for macromolecules was found to be a safe strategy.

Key words oral delivery; cell-penetrating peptide; absorption enhancer; penetratin; lipopolysaccharide; toxicity

Conventionally, biodrugs such as protein and peptides are administered by subcutaneous or intravenous injections as a result of the poor pharmacokinetic and pharmacodynamics parameters following alternative routes of administration such as oral delivery—a fact owed mainly to the substantial barriers of the gastrointestinal tract. 1,2) Considering the greater convenience of oral administration of drugs in general, great effort is being invested in the development of oral formulations of biodrugs 3) and the growing number of diabetic patients worldwide further increases the potential benefits of an oral formulation of proteins such as insulin to help alleviate the inconveniences associated with subcutaneous injections. 4,5)

While progress within the field has been challenging, significant headway has been made by the use of cell-penetrating peptides (CPPs). This class of peptides is characterized by their innate ability to cross cellular membranes and in doing so enhance the absorption of proteins across mucosal surfaces such as the intestinal and nasal epithelium. 6) Penetratin is one such CPP which has been used for non-invasive delivery of proteins and our recent studies demonstrated the efficacy and safety of using penetratin for oral delivery 7) and chronic use in nasal delivery over a 30 d period. 8) There is concern, however, that chronic use of oral absorption enhancers such as CPPs may lead to systemic inflammation by allowing enterotoxins such as lipopolysaccharide (LPS) to penetrate the intestinal mucosa. Lipopolysaccharide is a component of the cell wall in Gram-negative bacteria and represents the major endotoxin released by the microenvironment in the gastrointestinal (GI) tract and chronic systemic exposure to LPS leads to peripheral damage such as hepatic necrosis. 7)

The aim of this study was to investigate if co-administration of CPPs and LPS would lead to hepatic injury by increasing the amount of LPS penetrating the intestinal epithelium and reaching circulation. By measuring the plasma concentration of two hepatic enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) released in hepatic necrosis, we could investigate if administration of CPPs over a 7d period lead to hepatic damage. 8)

MATERIALS AND METHODS

Preparation of Test Solutions Penetratin (α-form, RQIKIWFQNRRMKWKK, and α-form, rqikiwfqnrrmkwkk, synthesized by Sigma-Genosys, Life Science Division of Sigma-Aldrich Japan Co. (Hokkaido, Japan)), LPS (Escherichia coli O111:B4, Sigma-Aldrich Inc., St. Louis, MO, U.S.A.), sodium taurodeoxycholate (Sigma-Aldrich Inc.) and sodium caprate (C10) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were dissolved in sterile phosphate buffered saline (PBS) (pH 7.4).

Animal Studies All animal procedures were approved by the Kobe Gakuin University Animal Care and Use Committee, and were conducted in accordance with institutional guidelines that complied with “Basic Policies for the Conduct of Animal Experiments in Research Institutions under the Jurisdiction of the Ministry of Health, Labour, and Welfare of Japan” (2006). Animals were housed under controlled environmental conditions regarding humidity and temperature with a 12:12h light/dark cycle.

Analysis of AST/ALT Levels in Mice Plasma Seven weeks old male ddY mice (30–36g at start of experiment) (Japan SLC, Inc., Shizuoka, Japan) were dosed once per day for seven consecutive days by oral gavage with penetratin and LPS in order to simulate daily exposure to CPPs and thereby potentially increased levels of LPS reaching circulation. For oral administration, animals first received one dose of 100 µL LPS (5 mg/kg/d) followed 1 min later by 100 µL of penetratin (2 and 5 mm), PBS, taurodeoxycholate (96 mm) or

Note

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C10 (154 mM).

During the 7-d time course, animals had access to food and water ad libitum. On day 7, animals were dosed 4 h prior to a single blood sampling (300 µL) under anesthesia intraperitoneally (i.p.) injection of pentobarbital (50 mg/kg, SomnopentylR, Kyoritsu Seiyaku Corp., Tokyo, Japan) from the jugular vein using heparinized syringes. Blood samples were centrifuged at 13400 × g for 1 min at 4°C and plasma was then collected. Plasma AST and ALT levels were measured using a Wako Transaminase CII-test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Statistical Analysis** Significant differences in the mean values were evaluated by a one-way layout ANOVA followed by a Bonferroni’s multiple comparison test. A p-value of less than 0.05 was considered significant.

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**RESULTS**

As seen in Fig. 1, animals receiving no handling or administration (untreated group) gained weight steadily over the 7-d time course (115% at day 7). No significant difference in weight gain was observed for all the groups receiving an oral treatment (101% to 108%) suggesting that the handling of the animals or the administration of the 100/200 µL volume of test solution (regardless of content) was responsible for the lesser weight gain compared to the untreated group. The taurodeoxycholate treated group, however, did score the lowest weight gain (101%) suggesting a slight effect on the animals.

Figure 2 depicts plasma levels of the liver enzymes AST and ALT as measured on day 7. The observed values for AST were higher than ALT which is consistent with the fact that AST is found in other tissues such as skeletal muscle as well.

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**Fig. 1.** Changes in Body Weight Depicted as % Compared to Day 1 in Mice

- Untreated
- PBS
- LPS (5 mg/kg/d)
- +L-penetratin (2 mM)
- +D-penetratin (2 mM)
- +L-penetratin (5 mM)
- +C10 (154 mM)
- +taurodeoxycholate (96 mM)

Data points are depicted as mean ± S.E.M., n=4–5.

**Fig. 2.** Plasma Concentrations of Biomarkers for Hepatic Damage after 7 Consecutive Days of Oral or i.p. Administrations with LPS and Absorption Enhancer

A: Aspartate transaminase. B: Alanine transaminase. Data points are depicted as mean ± S.E.M., n=4–5, groups were analyzed using one way ANOVA followed by Dunnet’s post hoc test to test for individual group difference from untreated control. *: Statistical significant difference from untreated group, p<0.05.
DISCUSSION

Generally, the observed levels of ALT and AST in our controls and orally treated groups are lower than what is observed in other studies, but we ascribe the differences in absolute values to differences in the experimental procedures and mouse strains.

In the study by Sonaje et al., chitosan nanoparticles, which are hypothesized to open tight junctions, were used to deliver insulin orally without causing detectable damage to the mucosa nor an increase in serum biomarkers. C10, which is generally considered a safe absorption enhancer, did also not increase the values of AST and ALT significantly even at 100 mg/kg/d and was on par with the values for penetratin. Furthermore, penetratin has recently been investigated in a 30-d subchronic study with daily nasal co-administrations of insulin with which no apparent harmful effect or damage to the nasal mucosa was observed. The present study falls in line with these results and further underlines the mild nature of the absorption enhancing effect of penetratin. Rigorous studies, however, on the effect of absorption of therapeutically relevant molecules such as insulin and other protein and peptide based drugs and also a full-scale toxicity study are needed to conclude the usefulness and safety of penetratin as an oral absorption enhancer.

Penetratin is thought to enhance absorption of insulin in a transcellular manner as opposed to chitosans, fatty acid derivatives, bile salts and chelators whose mechanism of action is thought to be attributed to tight junction modulation. Absorption of LPS therefore requires passage through the cell and systemic exposure might be further limited due to this fact.

Preclinical development of absorption enhancers is faced with the difficult task of showing high efficiency in vitro while maintaining low toxicity in models that rarely resemble physiological relevant conditions, especially in terms of the ability of the tissue to regenerate such as the case is with intestinal tissue. However, on the basis of the weight gain/loss data and the hepatic biomarker levels in mice obtained in this study, we conclude that 7 consecutive days of oral administrations of solutions with a high concentration of penetratin and LPS does not lead to hepatic symptoms of systemic inflammation. These results are yet a step forward in the process of piecing together needed toxicological information regarding the use of CPPs prior to clinical development.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES