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Bile Acid as an Effective Absorption Enhancer for Oral Delivery of Epidermal Growth Factor Receptor–Targeted Hybrid Peptide

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ABSTRACT

The aim of this study was to improve the oral absorption of epidermal growth factor receptor–targeted hybrid peptide using bile acid as an absorption enhancer. The oral formulation of this peptide was formed through electrostatic interactions between the cationic peptide and anionic bile acid. Comparative studies of *in vitro* cell permeability and *in vivo* antitumor effects of peptide and peptide/bile acid complex were performed in Caco-2 cells and in a xenograft mouse model of human gastric cancer. The *in vitro* permeability of peptide/bile acid complex across Caco-2 cell monolayers was significantly enhanced to about 5.0-fold over those of peptide alone. Furthermore, *in vivo* mouse xenograft model treated with peptide/bile acid complex showed a 1.6-fold reduction in the mean tumor volume as compared with the peptide alone. A preliminary safety evaluation of blood cells counts, liver enzyme levels, and histopathology of gastrointestinal tissues and main organs showed that the peptide/bile acid complex did not induce any acute toxicity. These results suggest that bile acid is an effective absorption enhancer for improving the oral bioavailability and bioactivity of epidermal growth factor receptor–targeted hybrid peptide.

Introduction

Oral delivery is the preferred route for therapeutic agents because of the ease of administration and patient acceptance. However, oral delivery of drugs faces many hurdles such as poor absorption, poor permeability, and rapid degradation in the gastrointestinal tract. Until now, various strategies have been investigated to improve the bioavailability of poorly absorbable drugs. These strategies involve chemical modification, formulation vehicles, and the use of enzyme inhibitors, absorption enhancers, and mucoadhesive polymers.[1,2] Among them, the use of absorption enhancers is the simplest approach to enhance the oral absorption of biological agents across the intestinal epithelial membrane.[3,4] The study of absorption enhancers began in the 1960s, when ethylenediamine tetraacetic acid was shown to increase the absorption of heparin in rats and dogs.[5] Absorption enhancers that enhance the intestinal absorption of insulin, such as fatty acids and bile salts, have been investigated for the oral delivery of insulin.[6,7] Moreover, it has been reported that the absorption of human calcitonin, which is a polypeptide hormone consisting of 32 amino acid residues, is significantly increased by co-administration with absorption enhancers in rats.[8] Bile salts are endogenous surfactants, which have been the widely used absorption enhancers to increase drug delivery because of their high biocompatibility and unique ability to facilitate and promote drug permeation through biological membranes.[9-11] Bile acid carrier–mediated protein and peptide delivery can enhance the oral bioavailability and the biological activity of therapeutics, such as salmon calcitonin and insulin.[12-14] Furthermore, Song et al.[12] investigated the enhancement of the intestinal absorption of salmon calcitonin from proliposomes containing different types of bile salts. The results indicated that the absorption-enhancing effect of sodium taurodeoxycholate (TDCA) is more potent than that of other bile salts. TDCA is a secondary bile acid derived from primary bile acids in the intestine via enzymatic action. It was known that the bile acids can be absorbed at any level of the gastrointestinal tract by passive diffusion and active transport mechanism. Passive diffusion is the major absorption process for most drugs and also important mechanism for the absorption of osmotically active compounds

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such as TDCA. Meanwhile, pH is one of the important factors affecting the stability of orally administered drugs. A recent study investigated the combined effects of absorption enhancers and pH-lowering agents on the oral delivery of peptide drugs.[15,16] The results showed that combinations of pH-lowering agents and absorption enhancers potently protect peptide from degradation and enhance its intestinal absorption. Among various organic acid—based pH-lowering agents, citric acid (CA) is often used in dipping solutions for its anti-oxidative and pH-lowering effects on food and drug stability. A technique based on the use of CA has also been tested for the oral delivery of salmon calcitonin.[17]

Recently, many pharmaceutical companies have focused on developing oral delivery of peptides and proteins, and several formulations of proteins such as interferon-α and human growth hormone are at the clinical development stage.[18,19] A novel approach based on an enteric-coated tablet has been developed by Enteris BioPharma, Inc. (Boonton Township, NJ) for the oral delivery of peptide drugs. It is known as Peptelligence® and consists of an organic acid enzyme inhibitor (CA in the form of coated beads) and an absorption enhancer (acyl carnitine), which it is claimed helps penetration through the mucus layer. It has been demonstrated to be effective for improving the oral delivery of peptide-based therapeutics. This technology was used to develop an oral formulation of salmon calcitonin for a phase 3 clinical trial in patients with osteoporosis.[17] It was additionally used to develop an oral formulation of parathyroid hormone for a phase 2 clinical trial.[20]

We previously reported a novel targeted “hybrid peptide”, in which epidermal growth factor receptor (EGFR)-binding peptide was conjugated with lytic peptide (in a total of 32 amino acid residues and a molecular weight of 3774 Da) and demonstrated its highly potent and selective pharmacological activity toward KRAS-mutated EGFR-positive cancer cells.[21-23] It was previously suggested that the EGFR-binding peptide fragment specifically binds to EGFR on the cell surface, and that, the lytic fragment penetrate to make a pore and disrupts the cell membrane, leading to cell death regarding the mechanism of action of this hybrid peptide to target cells.[21,22] Moreover, our subsequent studies have shown that intravenous injection of this hybrid peptide is a potential treatment option for patients with colorectal cancer metastases in the liver.[24]

Therefore, based on the previous nonclinical and clinical findings of oral delivery strategies and our previous studies on hybrid peptides, we have focused on the development of effective oral peptide formulations using absorption enhancers. The absorption enhancers used for this purpose must be capable of enhancing peptide absorption without damaging the intestinal membrane. In this study, we developed an oral formulation of a hybrid peptide that was conjugated with the absorption enhancer TDCA and evaluated its permeability and biological activity both *in vitro* and *in vivo*.

Materials and Methods

Materials

The EGFR2R-lytic hybrid peptide used in this study was synthesized by the American Peptide Company (Sunnyvale, CA) as described previously.[22-24] TDCA and CA were purchased from Nacalai Tesque (Kyoto, Japan). All agents were of reagent-grade quality.

Cell Culture

Caco-2 cells an intestinal cell line derived from a human colorectal carcinoma were cultured in Dulbecco's modified Eagle's medium supplemented with 1% nonessential amino acids (Nacalai Tesque), 10% fetal bovine serum, 1% penicillin/streptomycin (P/S) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Human glioma U251 cells and human gastric cancer MKN45 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% P/S.

Preparation of EGFR-Targeted Hybrid Peptide (peptide)/Bile Acid Complex

TDCA and CA were dissolved in demineralized water, and EGFR2R-lytic hybrid peptide was dissolved in phosphate-buffered saline (PBS) solution (pH 7.4). The TDCA and hybrid peptide were mixed in molar ratios of 1:10, 1:5, 1:2, 1:1, and 1:0.5, and CA was added to the mixture in the same molar ratios as those of TDCA. The reaction mixture was incubated with gentle shaking overnight at 4°C. The peptide content in the peptide/bile acid complex was calculated from absorbance at 280 nm based on the absorbance of tyrosine, and tryptophan residues of hybrid peptide (Nanodrop spectrophotometer; Thermo Fisher Scientific, Rochester, NY).

In Vitro *Permeability Study*

We used the human colon carcinoma cell line Caco-2 as a model to investigate the permeability of the peptides across the intestinal cell membranes, as described previously.[25-27] Briefly, Caco-2 cells were (1×10^5 cells/well) seeded on 24-well Millicell® Hanging Cell Culture Inserts (Millipore Corporation, Billerica, MA) and cultured for 2 weeks to reach confluence. The culture medium was placed into the apical (0.3 mL) and basolateral chambers (0.9 mL), changed every other day for a week and daily thereafter. The Caco-2 cell monolayers with a *trans*-epithelial electrical resistance (TEER) value were measured using a Millicell-ERS meter with a chop-stick electrode (Millipore Corporation) as described previously.[26,27] Caco-2 cell monolayers were washed twice with PBS and preincubated with prewarmed transport medium (pH 7.4), comprised of Hank's Balanced Salt Solution (HBSS) and 10-mM HEPES, for 30 min at 37°C in a 5% CO_2 incubator. The buffer in the respective apical side was then replaced with a solution containing fluorescein-X (FAM-X)-labeled peptide or FAM-X-labeled peptide/bile acid complex (0.3 mL), and HBSS (0.9 mL) was added to the basolateral side. As a control, fresh HBSS was applied to both sides of the Transwell. Labeling of peptide was carried out according to the manufacturer's standard protocol. The FAM-X-labeled peptide is stored at 4°C until use. For preparation of the FAM-X labeled peptide/bile acid complex, FAM-X labeled peptide was added to the bile acid and CA (molar ratio of 1:2:2), the reaction mixture was incubated for overnight with gentle shaking at 4°C and protected from light. The FAM-X-labeled peptide or FAM-X-labeled peptide/bile acid complex from the apical side to the basolateral side were taken at 3 h post loading and analyzed with a plate reader equipped with a fluorescence module (Ex = 495 nm, Em = 520 nm; GloMax®-Multi Detection System; Promega, Madison, WI). Cells between passages 55 and 65 were used for all experiments.

In Vitro *Dissolution Study*

Dissolution experiments were performed at pH values ranging from 1.5 to 9.0. Buffer solutions with pH from 1.5 to 5.0 were prepared by combining different proportions of glycine and hydrochloric acid in aqueous solution, pH = 7.4 and pH = 9.0 buffer were prepared from phosphate or Tris buffer solution according to the published procedures and further diluted with deionized water.[28] All buffer solutions were checked for conformance to the desired pH using a pH meter. One hundred microliters of peptide and peptide/bile acid complex were incubated in the same volume of buffer solutions with various pH values gentle shaking at room