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Buccal mucosal delivery of a potent peptide leads to therapeutically-relevant plasma concentrations for the treatment of autoimmune diseases

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ABSTRACT

Stichodactyla helianthus neurotoxin (ShK) is an immunomodulatory peptide currently under development for the treatment of autoimmune diseases, including multiple sclerosis and rheumatoid arthritis by parenteral administration. To overcome the low patient compliance of conventional self-injections, we have investigated the potential of the buccal mucosa as an alternative delivery route for ShK both in vitro and in vivo. After application of fluorescent 5-Fam-ShK to untreated porcine buccal mucosa, there was no detectable peptide in the receptor chamber using an in vitro Ussing chamber model. However, the addition of the surfactants sodium taurodeoxycholate hydrate or cetrimide, and formulation of ShK in a chitosan mucoadhesive gel, led to 0.05–0.13% and 1.1% of the applied dose, respectively, appearing in the receptor chamber over 5 h. Moreover, confocal microscopic studies demonstrated significantly enhanced buccal mucosal retention of the peptide (measured by mucosal fluorescence associated with 5-Fam-ShK) when enhancement strategies were employed. Administration of 5-Fam-ShK to mice (10 mg/kg in a mucoadhesive chitosan-based gel (3%, w/v) with or without cetrimide (5%, w/w)) resulted in average plasma concentrations of 2.6–16.2 nM between 2 and 6 h, which were substantially higher than the pM concentrations required for therapeutic activity. This study demonstrated that the buccal mucosa is a promising administration route for the systemic delivery of ShK for the treatment of autoimmune diseases.

1. Introduction

The voltage-gated potassium Kv1.3 channel is highly expressed by terminally-differentiated effector memory T (T_{EM}) lymphocytes [1], which are the major mediators in the pathogenesis of autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA) [2,3]. Due to their roles in modulating the activation of T_{EM} cells, Kv1.3 channels represent an important target for the treatment of autoimmune diseases, for which there are limited clinically available therapeutic options. *Stichodactyla helianthus* neurotoxin (ShK), isolated originally from the sea anemone *S. helianthus*, is a peptide consisting of 35 amino acids and is a potent blocker of Kv1.3 channels [4]. In vitro studies have demonstrated that ShK and its analogues are not only able to block Kv1.3 channels at low picomolar (pM) concentrations but also exhibit high selectivity for Kv1.3 channels over other tested channels [4–7]. Pharmacokinetic studies with ShK analogues in rats and monkeys have demonstrated that, following a single subcutaneous dose, plasma concentrations remained above the Kv1.3-blocking IC_{50} for up to 7 days [6,8]. In addition, disease severity in three animal autoimmune disease models (i.e. delayed-type hypersensitivity, chronic relapsing–remitting experimental autoimmune encephalomyelitis and pristine-induced arthritis) was significantly reduced following repeated subcutaneous administration of ShK peptide analogues [8].

These preclinical studies have resulted in the development of a highly effective ShK analogue, ShK-186, which is currently being evaluated in phase I human clinical trials following subcutaneous administration. However, systemic delivery of peptides and proteins such as ShK presents challenges as the conventional oral route of administration often leads to poor bioavailability of these macromolecules [9]. In particular, the harsh conditions in the stomach and gastrointestinal tract, combined with the presence of potent peptidases and proteases, commonly result in significant degradation of peptides and proteins following oral administration, leading to the requirement for regular

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self-injections, as is the case for ShK-186. To achieve high patient acceptance and maximize the clinical utility of these potent macromolecules, alternative routes of delivery should therefore be exploited.

One such route, which has led to various clinical products containing prochlorperazine, testosterone and rizatriptan, is the buccal mucosa. The buccal mucosa is easily accessible, and applied formulations can be rapidly removed in the case of an emergency, which is a major benefit over other routes of administration [10]. In addition, the buccal mucosa has low enzymatic activity and a rich blood supply due to an extensive capillary network, and any therapeutic administered via this route can reach the systemic circulation rapidly because of direct drainage into the jugular vein, additionally bypassing first pass metabolism [11]. A key function of the buccal mucosa, however, is to protect the underlying tissues from exogenous substances, and as such, this membrane can act as a permeability barrier to hydrophilic macromolecules including peptides and proteins, even though it is more permeable than the skin [12]. Numerous approaches have therefore been explored in an attempt to improve the buccal mucosal delivery of small molecular weight drugs and macromolecules [13–15], and one of the most promising approaches involves the use of chemical penetration or absorption enhancers [16]. Surfactants and bile salts such as sodium taurodeoxycholate hydrate (STDC) and cetrimide have been found to facilitate the buccal mucosal delivery of peptides and proteins, including insulin and pituitary adenylate cyclase-activating polypeptide (PACAP) [17–19]. Although the exact mechanism by which these agents improve the buccal permeability is not completely characterized, it is suggested that surfactants and bile salts solubilize and extract the mucosal intercellular lipids, subsequently disrupting the paracellular route of diffusion and enhancing buccal mucosal absorption of hydrophilic macromolecules. While co-application of surfactants or bile salts could represent a promising approach to improve the buccal mucosal transport of peptides such as ShK, their use in a solution formulation may be limited as such solutions could theoretically be swallowed, decreasing the amount of peptide available for buccal mucosal absorption. To accurately control the amount of peptide administered to animals and humans, mucoadhesive gel formulations may be more practical. Chitosan and its modified derivatives have been employed to facilitate the buccal delivery of drugs, including peptides, by prolonging the retention time of the therapeutic on the buccal epithelium and the potential direct effect on buccal permeability of chitosan through disturbance of the intercellular lipids of the buccal epithelium [20–22].

In this study we have determined the effect of STDC and cetrimide on the buccal transport of 5-Fam-ShK (a fluorescent analogue of ShK) in an in vitro porcine buccal mucosa model. Confocal fluorescence microscopy was used to determine whether such penetration enhancers had the ability to increase the deposition of 5-Fam-ShK in the buccal mucosa. Finally, the plasma exposure of 5-Fam-ShK when formulated in a mucoadhesive chitosan gel formulation and applied to mice was assessed in order to determine whether therapeutically-relevant plasma concentrations of this peptide could be achieved with this route of administration.

2. Materials and methods

2.1. Materials

STDC was obtained from Sigma-Aldrich (St. Louis, MO) and cetrimide was purchased from Biotech Pharmaceuticals (Carole Park, Queensland, Australia). Krebs bicarbonate Ringer (KBR) buffer was prepared as described previously [23]. Chitosan of low molecular weight (ranging from 50,000 to 190,000 Da) with a deacetylation degree of 75–85% was obtained from Aldrich chemical company (Milwaukee, WI). Trifluoroacetic acid (TFA) was purchased from Merck KGaA (Darmstadt, Germany). HALT phosphatase Inhibitor Cocktail was obtained from Thermo Fisher Scientific Australia Pty Ltd. (Scoresby, Victoria, Australia). Solid-phase extraction (SPE) cartridges (tC_{18} Sep-Pak®, 50 mg) were purchased from Waters (Waters Corporation, Milford, MA). All other reagents were of HPLC grade and water was obtained from a Millipore purification system (Millipore Corporation, Billerica, MA).

2.2. Peptide synthesis

5-Fam-ShK was prepared by an Fmoc/tBu strategy using Rink-mBHA resin (Peptides International, Louisville, KY). All Cys residues were incorporated with a Trityl protecting group. All couplings were mediated by 6-Cl-HOBT/diisopropylcarbodimide. Following assembly of the peptide, a mini-PegTm linker was incorporated at the N-terminus for attachment of the 5-Fam. The single isomer 5-Fam was also coupled using 6-Cl-HOBT/diisopropylcarbodiimide. The peptide was simultaneously acidolytically cleaved from the solid support and deprotected on the side chains using TFA with cationic scavengers trisopropylsilane, thioanisole, water and anisole. The crude peptide was isolated from the spent resin beads with a sintered glass filter and precipitated in ice cold diethyl ether. The product was oxidatively folded to the biologically active form and purified as described previously [24].

2.3. In vitro permeation experiments

Buccal tissue from pigs was freshly obtained from a local abattoir immediately after slaughter and the epithelium was removed from underlying tissue by forceps and scissors. The buccal epithelium was then mounted into modified Ussing chambers with a diffusional area of 0.64 cm^2. After incubating the buccal mucosa for 30 min at 37 °C with 1.5 mL of KBR in both the donor and receptor chambers, the physiological buffer was removed and replaced with either 1.5 mL of KBR in the receptor chamber or 1.5 mL of 5-Fam-ShK (0.022 mM in KBR) with or without STDC (1% or 5%, w/v) or cetrimide (5% w/v) in the donor chamber ($n = 4$). Both donor and receptor chambers were maintained at 37 °C and supplied with carbogen to provide agitation. Samples were collected from the donor (10 μL) and receptor (200 μL) chambers at pre-determined time points over 5 h, with the receptor chamber replenished with 200 μL of fresh KBR immediately after each receptor chamber sample was taken. Both donor and receptor chamber samples were assayed for 5-Fam-ShK by a high-performance liquid chromatography (HPLC)-fluorescence coupled technique. In separate experiments, the buccal mucosa was removed at predetermined time points over a 5 h period, and the tissue deposition of 5-Fam-ShK quantified using confocal microscopy as described below.

2.4. Confocal laser scanning microscopy

An A1 confocal system (Nikon, Tokyo, Japan) equipped with an inverted Nikon Eclipse Ti microscope with 10× objective was used to quantify fluorescence deposition in buccal mucosa following application of 5-Fam-ShK (with and without enhancers). The argon laser was set at 488 nm. Optical sections were obtained (0.5 μm pixel size, 512 × 512 pixels) horizontally through the epithelium, from the apical towards the basal membrane. Data acquisition and collection were processed by NIS-Elements software platform (Version 3.22.14, Nikon, Tokyo, Japan) and the quantification of 5-Fam-ShK was determined using ImageJ (NIH, USA). Briefly, four random sections in each tissue (a total of 16 sections) were quantified and the average value was obtained to represent the accumulation of 5-Fam-ShK in the buccal mucosal tissue at that particular time point.

2.5. Chitosan gel preparation

A 3% (w/v) chitosan buccal gel containing 0.6% (w/w) 5-Fam-ShK was prepared with and without the addition of 5% (w/w) cetrimide. The purpose of adding this concentration of 5-Fam-ShK is that when a