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Synergistic effect of chemical penetration enhancer and iontophoresis on transappendageal transport of oligodeoxynucleotides

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ABSTRACT

Gap junction protein connexin43 (Cx43) specific antisense oligodeoxynucleotides (AsODN) have been shown to improve a number of inflammatory conditions and may therefore offer a novel strategy for persistent pain management. However, for such molecules to be clinically effective, delivery challenges owing to the molecules' high molecular weight, negative charge and hydrophilicity have to be overcome. In this study, the effect of various chemical penetration enhancers and cathodal iontophoresis on transdermal delivery was evaluated. Initial skin permeation studies revealed only a slight increase in the passive flux of the model anionic drug sodium fluorescein using limonene/ethanol. Applying cathodal iontophoresis, the amount of the model drug permeated through untreated skin was tripled, while a combination of chemical and physical penetration enhancement resulted in a fourfold increase in the fluorescein amount permeated. However, even the synergistic effect of limonene/ethanol and iontophoresis was insufficient to achieve complete permeation of Cy3-labeled Cx43 AsODN across the entire skin thickness. Instead, molecules were trapped in the epidermis or permeated deeply into the hair follicles. These results suggest that the synergistic effect of chemical and physical penetration enhancement increases intradermal delivery of oligonucleotides but is insufficient to deliver such large molecules across intact skin.

1. Introduction

Owing to the increasing number of diseases associated with inappropriate protein production, there has been a remarkable growth of nucleotide-based therapeutics. Connexin43 (Cx43) has been found to play a role in a number of inflammatory conditions (Green and Nicholson, 2008). An antisense approach to knock down this protein has been reported to accelerate the rate of skin epithelial (Coutinho et al., 2005; Qiu et al., 2003) and corneal wound healing (Grupcheva et al., 2012; Rupenthal et al., 2011), as well as spinal cord (Cronin et al., 2008; Zhang et al., 2010) and optic nerve (Chew et al., 2010; Danesh-Meyer et al., 2008) recovery after injury. In all these studies, the antisense oligodeoxynucleotide (AsODN) was applied directly to the site of injury without the need for the molecules to penetrate major delivery barriers. A recent review by Wu et al. (2012) discussed the role of connexin gap junction proteins in chronic pain and suggested that targeting such proteins could offer novel strategies for the management of persistent pain. However, for a topical formulation to be effective the nucleic acid would need to penetrate the skin barriers in order to reach its target site.

A number of researchers have reviewed the transdermal delivery of oligonucleotides (Brand and Iversen, 2000, 2005; Lin et al., 2004) with a major focus on chemical penetration enhancers such as surfactants (Som et al., 2012) or terpenes (Aqil et al., 2007), which may alter the skin morphology to enhance passive diffusion of drug molecules, as well as physical methods such as ultrasound (Lavon and Kost, 2004), electroporation (Escobar-Chavez et al., 2009) and iontophoresis (Aramaki et al., 2003; Brand et al., 1998; Brus et al., 2002; Hashim et al., 2010; Sakamoto et al., 2004). While nucleic-acid based therapeutics are generally known to have difficulty permeating across the entire skin thickness due to their high molecular weight, hydrophilicity and negative charge, recent studies have demonstrated successful delivery of such macromolecules into the dermal tissue. An IL-10 antisense oligonucleotide was successfully delivered using pulse depolarization (PDP) iontophoresis (Aramaki et al., 2003; Sakamoto et al., 2004), a method also

Abbreviations: Cx43, connexin43; AsODN, antisense oligodeoxynucleotide(s); PBS, phosphate buffered saline; H&E, hematoxylin & eosin; SLS, sodium lauryl sulphate; PG, propylene glycol; IPM, isopropyl myristate.

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recently employed to achieve penetration of a FITC-NF-κB decoy oligonucleotide across the *stratum corneum* (Hashim et al., 2010). Moreover, a topically applied anti-IL-10 siRNA reduced IL-10 mRNA levels after iontophoretic delivery into the epidermis (Kigasawa et al., 2010).

Since a combination of two or more delivery approaches has generally proven more efficient than an individual penetration enhancing method alone, this study aimed to explore the synergistic effect of chemical penetration enhancers and cathodal iontophoresis. Initial studies compared a number of chemical penetration enhancers for their potential to increase the passive flux of the negatively charged model drug sodium fluorescein. The most effective chemical penetration enhancer was then used in combination with cathodal iontophoresis to evaluate the in vitro transdermal delivery of sodium fluorescein and Cy3-labeled Cx43 AsODN across porcine ear skin. Finally, the effect of the penetration enhancing methods on the skin morphology was evaluated using histological methods and the intradermal penetration pathway of a Cy3-labeled Cx43 AsODN was visualized using confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Pluronic F127 (Poloxamer 407) was a gift from BASF Ltd. (Auckland, New Zealand). Sodium lauryl sulphate (SLS) and propylene glycol (PG) were purchased from Ajax Finechem (Auckland, New Zealand). Isopropyl myristate (IPM) was given by Chemcolour Ltd. (Auckland, New Zealand) and 70% ethanol was obtained from Scharlau Chemie (Barcelona, Spain). (R)-(+)-Limonene, sodium fluorescein and silver wire with diameter of 0.5 mm were purchased from Sigma–Aldrich (Sydney, Australia). Cy3-tagged mouse anti-Cx43 ODN (5′-Cy3-GTA-ATT-GCG-GCA-GGA-GGA-ATT-GTT-TCT-GTC-3′) was purchased from Sigma Genosys (Sydney, Australia). Na_2HPO_4 and KH_2PO_4 used for the preparation of PBS were ordered from Scharlau Chemie (Barcelona, Spain).

2.2. Skin preparation

Fresh male adult pig ears (*S. s. domesticus*, Auckland Meat Processors Ltd., Auckland, New Zealand) were washed under running cold water and epidermis and dermis were separated from the underlying fat and cartilage using a scalpel blade. Excised skin was cut into 2 cm × 2 cm squares and soaked in PBS or enhancer solution overnight prior to the permeation experiment.

2.3. Chemical penetration enhancers

The following enhancer solutions were prepared and five parts were further diluted in 95 parts of PBS to obtain the final working solutions: 0.1% SLS, 70% ethanol, PG/70% ethanol (1:1), IPM/70% ethanol (1:1) and limonene/70% ethanol (1:1). Enhancer-soaked skin pieces were washed with PBS before mounting them onto standard Franz diffusion cells (Logan Instrument Corp., Somerset, NJ, USA) with the *stratum corneum* facing upwards. The receptor chamber was filled with 12 ml of PBS. A volume of 1 ml of sodium fluorescein (6 μg/ml) in Pluronic gel (30%, w/v) was loaded into the donor chamber, which was then occluded with parafilm to prevent any solution loss by evaporation. Cells were maintained at 32 ± 0.5 °C by the VTC-220 heat circulator and the receptor medium was constantly stirred at 600 rpm by small magnetic bars. At predetermined time points (1, 2, 3, 4, 6, 8 and 12 h) a volume of 0.4 ml was withdrawn from the receptor chamber and replaced with an equivalent volume of PBS. All tests were performed in triplicate and the sodium fluorescein content was analyzed using a fluorometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) using 490 and 510 nm as the excitation and emission wavelength respectively. Cumulative amounts of sodium fluorescein permeated where calculated and plotted against time.

2.4. Cathodal iontophoresis

Franz diffusion cells were set up as described above with skin pieces soaked either in PBS (control) or limonene/ethanol. Electrodes were prepared by soaking a silver wire in concentrated chlorine solution for 30 min to generate an AgCl coating. A volume of 1 ml of sodium fluorescein (6 μg/ml) or Cy3-tagged Cx43 AsODN (1 μM) in Pluronic gel (30%, w/v) was loaded into the donor chamber. Since both of these are negatively charged compounds, cathodal iontophoresis was applied. Therefore, the cathode (−) was placed in the donor compartment containing the drug formulation without touching the dorsal surface of the skin, while the anode (+) was placed in the receptor compartment. A constant current of 5 mA (corresponding to 1.25 mA/cm^2) was applied for the first 4 h of the permeation studies. At predetermined time points (1, 2, 3, 4, 6, 8 and 12 h) a volume of 0.4 ml was withdrawn from the receptor chamber and replaced with an equivalent volume of PBS to maintain sink conditions. All tests were performed in triplicate and the sodium fluorescein or Cx43 AsODN content was analyzed using a fluorometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) with 490 and 510 nm or 543 and 570 nm as the excitation and emission wavelength respectively. Cumulative amounts (CA) permeated were calculated and plotted against time and the steady-state flux (J_{ss}) was determined from the slope of the cumulative penetration curve. The enhancement ratio (ER) was calculated from the J_{ss} across the chemical enhancer and/or iontophoresis treated skin divided by the J_{ss} across untreated skin soaked in PBS. Statistically significant differences between data sets were determined using a Student's independent two samples *t*-test with *p*-values <0.05 denoting statistical significance.

2.5. Histological analysis of skin sections

After 12 h of drug permeation, skin pieces were washed with PBS, embedded in Tissue-Tek® O.C.T. and frozen in liquid nitrogen. Frozen tissue blocks were stored at −80 °C until further processed. Sections (16 μm) were cut perpendicular to the skin surface using a Zeiss Microm HM550 cryostat (Carl Zeiss NZ Ltd., Auckland, New Zealand) and Hematoxylin and Eosin (H&E) staining was performed. Sections were viewed under the light microscope (Leica DMRA, Leica Microsystems, Heidelberg, Germany) and images were recorded with a digital camera (Nikon Digital Sight DS-U1) using the NIS-Elements BR imaging software (Version 2.10). The histology of untreated, enhancer-treated and iontophoresis-exposed skin was compared in terms of tissue thickness (measured using ImageJ) and integrity.

2.6. Skin permeation using confocal laser scanning microscopy

After 12 h of Cy3-tagged Cx43 AsODN permeation, skin pieces were removed from the Franz cells, attached to the bottom of a Petri dish and covered with PBS. The oligonucleotide penetration depth was assessed by performing z-scans on a Leica confocal laser scanning microscope (Leica DMRXA 2 microscope fitted with a TCS-SP2 scanhead, Leica Microsystems, Heidelberg, Germany) using a 20× water immersion lens, a zoom of 1 and a pinhole of 2 Airy disk diameter. Optical slices were taken in 20 μm steps to a total depth of 800 μm. Three dimensional reconstructions of the z-stacks were generated using AMIRA® visualizing and analyzing software (Version 3.1, Visage Imaging, San Diego, CA, USA). Penetration depths