Iontophoretic Delivery of a Telomeric Oligonucleotide

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Purpose. To evaluate the feasibility of iontophoretically enhanced transdermal delivery of a phosphorothioate oligonucleotide across hairless mouse skin.

Methods. The phosphorothioate sequence, 5'-d(TTAGGG)-3' (TAG-6) which mimics the repeat sequence of the telomere was used as a model compound. Iontophoresis was performed on hairless mouse skin using an *in vitro* flow-through diffusion system. Both 5'-FITC and uniformly ^{35}S labeled oligonucleotide were used to monitor transdermal flux.

Results. Cathodal delivery of TAG-6 resulted in substantial oligonucleotide flux. The molecular label did not alter transport properties. No flux was measured with either anodal or passive delivery. The oligonucleotide was not degraded as it crossed the skin. Molecular transport was donor condition dependent, with pH and salt concentration both having significant effects. Pre-treating the skin with ethanol reduced iontophoretic transport.

Conclusions. These data demonstrate that iontophoresis can enhance transdermal flux of an intact phosphorothioate oligonucleotide and that this penetration is donor condition dependent. Furthermore, iontophoretically enhanced transdermal delivery is a feasible apprach to the administration of phosphorothioate oligonucleotides.

KEY WORDS: iontophoresis; transdermal drug delivery; oligonucleotides; telomerase; hairless mouse skin.

BACKGROUND

The advantages of transdermal drug delivery over other modes of administration have been well documented. Oligonucleotides are not expected to be orally active due to the acidic environment of the stomach and endonuclease enzymes in the gastrointestinal tract. Additionally, any molecules which are absorbed may have their effectiveness reduced by extensive hepatic first-pass elimination. While charged or polar molecules do not cross the skin passively, molecules such as insulin, thyrotropin releasing hormone, gonadotropin releasing hormone and argenine vasopressin have been successfully delivered transdermally using iontophoretic enhancement (1). Iontophoretic flux is a function of molecular size and charge, with smaller and positively charged species being transported preferentially (2).

Antisense oligonucleotides can serve as potent therapeutic agents by interfering with gene expression. This has led to interest in a variety of drug delivery techniques to ensure adequate cellular availability (3). Oligonucleotide flux has been reported into hair follicles (4). Preliminary experiments have demonstrated trace amounts of oligonucleotides delivered iontophoretically in mice (5,6). More recently, iontophoretic delivery of oligonucleotides *in vitro* across hairless mouse skin has been reported (7). Electroporation has also been successfully used for transporting phosphodiester oligonucleotides across human cadaver stratum corneum (8).

Telomeres are the ends of the linear eukaryotic chromosomes and are essential for chromosome stability. DNA replication leads to progressive shortening of the chromosome from its ends. Telomerase synthesizes 6-base repeats of telomeric DNA during replication thereby inhibiting chromosomal shortening. The telomeric sequence is highly conserved among eukaryotes, consisting of a G-rich repeating sequence. Telomerase is present in tumor cells, but not in most normal somatic cells and therefore, the synthesis of telomers by telomerase may be an essential component in the imortalization of cancer cells (9).

If an oligonucleotide which mimics the telomere sequence is present in a cell, it can, in theory, bind to the enzyme telomerase, rendering it ineffective. This could limit DNA replication in tumor cells and might have potential as an anticancer drug (10). Because the telomere sequence (TAG-6) is relatively small (only six bases long -TTAGGG), and since transdermal delivery is related to molecular size, it is an excellent molecule to begin studying the feasibility of oligonucleotide delivery through the skin.

This work examines whether phosphorothioate oligonucleotides can be iontophoretically delivered intact across hairless mouse skin and studies the effect of donor solution composition on transdermal transport.

MATERIALS AND METHODS

Chemicals

The six base sequence of TAG-6 (5'-d(TTAGGG)-3') was synthesized on a 1 μmol column as a phosphorothioate using the Applied Biosystems DNA Synthesizer (Model 391A) (MW = 1927) as previously described (11). It was either plain, S^{35} or FITC labeled (MW = 2316). The oligonucleotide was diluted in 25 mM HEPES and 133 mM NaCl at pH 7.4. This buffer also served as anode and receptor solutions. Assay solutions were either 50 mM EDTA pH 8, with a 5:1 ratio of Formamide added to it or Econolume Scintillation Cocktail (ICN Biochemicals, Costa Mesa, CA).

Iontophoretic Studies

Dorsal skin from male hairless mice CRL:SK1 ages 8–20 weeks old was removed from a recently sacrificed animal. The skin was placed in a flow-through diffusion cell system based on the design of Glickfeld (12). These cells allowed both the anodal and cathodal chambers to be located on the epidermal side of the skin. The electrode chamber contained approximately 150 μL of donor solution. The receptor compartment and flow rates were approximately 200 μL and 250 μL/hr. As TAG-6 is negatively charged at neutral pH, the oligonucleotide was placed at the cathodal chamber and the anodal side was filled with buffer. The skin was allowed to equilibrate for 90 minutes

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prior to current introduction. Ag/AgCl electrodes were then connected to a Biorad Model 1000/500 Power Supply, in constant current mode. The current was set to 0.5 mA/cm^2 which has been reported in the literature to be the maximum acceptable current for producing minimal skin damage and irritation (13). Salt bridges made from 3% Agarose in 1M NaCl were used to prevent oligonucleotide from binding to the electrodes. Standard delivery conditions consisted of 65 μM TAG-6 in a 25 mM Hepes, 133 mM NaCl buffer solution at pH 7.4. The receptor chamber was perfused with a saline buffer at physiological pH which then passed to a fraction collector. The efflux was collected in 90 minute intervals.

Assays and Analysis

Samples from the FITC labeled TAG-6 flux experiments were diluted 1:1 with a formamide/EDTA buffer (50 mM EDTA pH 8, with a 5:1 ratio of Formamide added to it) and assayed using an Applied Biosystems Modal 672 GeneScanner. This instrument uses an electrophoretic separation to resolve bands according to molecular size. Fluorescence combined with size was used to determine if the molecule was delivered across the skin intact. The area under the curve for intact oligonucleotide was then quantitated and used for flux calculations. Transport from the ^{35}S experiments was determined by measuring radioactivity in a liquid scintillation counter (Packard: 1600TR, Liquid Scintillation Analyzer).

The data from each sample were algebraically adjusted to account for the amount of oligonucleotide which had crossed the skin during the sampling period but which had not yet reached the sampling vial. All values were expressed as mean ± standard error. When comparisons between experiments were conducted, significant differences were assessed with analysis of variance and unpaired t-tests at the level $p < 0.05$.

RESULTS AND DISCUSSION

The goal of this work was to determine if iontophoresis can enhance transdermal oligonucleotide flux while maintaining molecular integrity. Because, oligonucleotides carry a negative charge at neutral pH, iontophoretic flux of TAG-6 was expected to be greater when the drug was placed at the cathode. Figure 1 demonstrates the influence of 12 hours of cathodal iontophoresis on 65 μM FITC-labeled and ^{35}S labeled TAG-6. Steady state fluxes are 273 ± 65, and 285 ± 71 ng/cm^2-hr respectively. The similarity between the two data sets indicate that FITC-labeling does not alter TAG-6 transport properties, and gives indirect evidence that the oligonucleotide is penetrating the skin intact.

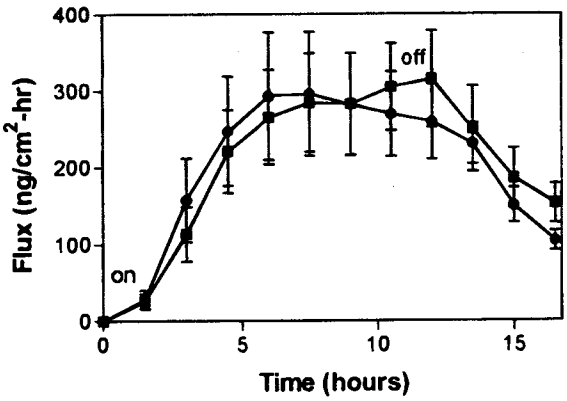

Fig. 1. Cathodal delivery of either FITC (square, n = 4), or ^{35}S (circle, n = 3), TAG-6 across hairless mouse skin. Donor solutions were 65 μM. Current was turned on at 1.5 hours and lasted for 12 hours.

Lack of degradation is further supported by examing the output from the Applied Biosystems Model 672 GeneScanner. Figure 2 contains two examples of GeneScanner data for TAG-6. Part a) is the result for a 5* 10^{-7} M standard solution, and b) is receptor after 12 hours of current. If molecular breakdown was occurring, it would be seen between 300 and 700 minutes of elution time. Examination of the data in this range indicates that there is minimal degradation of this phosphorothioate oligonucleotide after iontophoretic transport. These results in combination with the data from Figure 1 demonstrate that most TAG-6 remains intact as it crosses the skin.

The other major aspect of this work is to examine the influence of different donor conditions on TAG-6 transport, The two largest components of iontophoretic, flux are electrorepulsion and electroosmosis. Net electroosmotic flow is from anode to cathode, resulting in the successful delivery of neutral molecules from the anodal chamber. Pikal reported that two large anions, carboxy inulin (MW = 5,200) and BSA (MW = 69,000) had greater anodal enhancement despite their net negative charge (14). Because of their size, these molecules were not capable of electrorepulsive transport through the skin. Electroosmosis became the predominant flux mechanism, resulting in greater flow from the anodal chamber. We therefore examined the anodal delivery of TAG-6 at pH 7.4, to determine if the oligonucleotide had similar properties. There was no measurable oligonucleotide in the receptor chamber after 12 hours of anodal iontophoresis (data not shown). The lack of flux, therefore, indicates that this oligonucleotide can be transported, at least partially, by electrorepulsive forces. No oligonucleotide flux was measured after 24 hours of passive or anodal delivery at pH 7.4 (data not shown). This is not surprising given the size and charge of the molecule.

Changing donor solution pH can influence iontophoretic flux by altering either molecular charge or electroosmotic flux. The magnitude of the electroosmotic flux which normally flows from anode to cathode becomes lower as pH decreases and may reverse at very low pH (15). Therefore, with less electroosmotic flux interfering with cathodal transport, oligonucleotide transport can be expected to be greater at lower pH.

The influence of donor pH on TAG-6 flux was, therefore, examined. Iontophoretic transport was determined with a donor solution of 65 μM at pH 5, 7.4 and 9 using ^{35}S-labeled oligonucleotide (Figure 4). Steady state flux levels were 124 ± 38 ng/cm^2-hr at pH 5, 285 ± 71 ng/cm^2-hr at pH 7.4, and 203 ± 55 ng/cm^2-hr at pH 9. The decreased flux at lower pH is the opposite to what has been seen with other negatively charged compounds (16–18). We hypothesize that there are secondary structure changes in the oligonucleotide at low pH which lead to reduced transport. Decreased flux at higher pH may be explained by the fact that, ring nitrogens will lose their hydrogen ions near pH 9 or 10, leading to a larger negative charge on the TAG-6 molecule (19). Higher charges have been associated with lower transport numbers (20).

The idea that flux from higher pH solutions can result in better cathodal flux has been demonstrated with other oligonu-

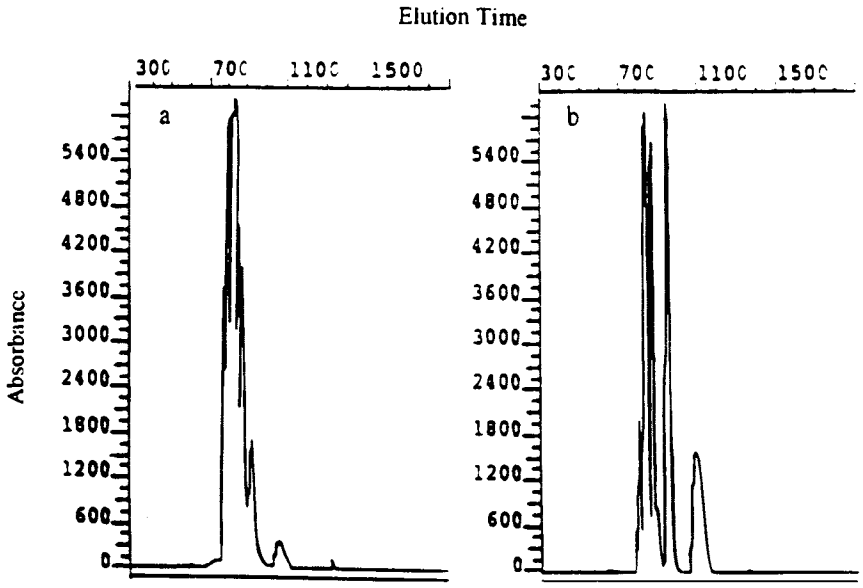

Fig. 2. Sample outputs from the Applied Biosystems Model 672 GeneScanner used for assaying FITC labelled oligonucleotide a) 5* 10^{-7} M TAG-6 standard solution, b) receptor fluid at 12 hours. Units are arbitrary fluoresence and time. TAG-6 degradation would be expected between 300 and 700 minutes of elution time.

cleotides (7). Olenberg et. al reported that maximal delivery for a 20 base phosphodiester oligonucleotide occurred at both pH 5.5 and 9.0. The data from Oldenberg et al differs from this work in several ways: 1. the oligonuclotides have a phosphodiester backbone instead of phosphorothioate, and it is not clear if the backbone will effect iontophoretic flux; 2. they used a random 20-mer and the influence of base composition and molecular size on pH sensitivity is not known; 3. pH control was maintained with different buffers, and oligonucleotides may interact differently with each of these buffers. This type of interaction could potentially lead to altered transport at each pH. In spite of these minor differences, both studies demonstrate that pH is a major factor in iontophoretic transport of oligonucleotides, and should be examined for each molecule to be delivered iontophoretically.

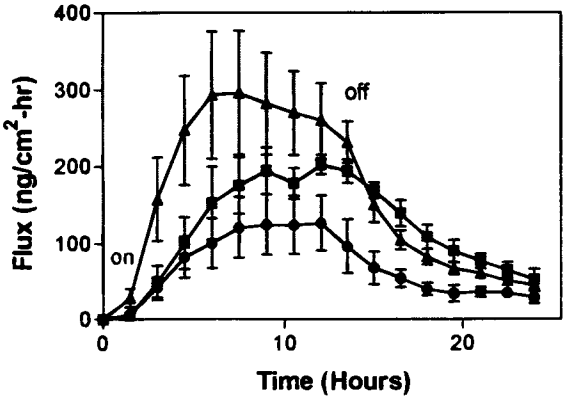

Fig. 3. Steady state flux as a function of donor pH. Transport of ^{35}S labelled TAG-6 at pH 5.0 (circle, n = 4), pH 7.4 (triangle, n = 3), and pH 9.0 (square, n = 3) are compared. Donor solutions were 65 μM. Current was turned on at 1.5 hours and lasted for 12 hours. Receptor solution was maintained at pH 7.4 for all experiments.

Current application leads to a specific charge which needs to be carried across the skin. Iontophoretic donor solutions frequently contain co-ions which are more electrically mobile than the drug of interest. Because of their enhanced mobility, these ions move more easily across the skin than the drug, thereby increasing their transport while reducing the desired drug flux. To test if this was the case for TAG-6, 65 μM ^{35}S-labeled oligonucleotide dissolved in 25 mM Hepes at pH 7.4 was placed in the cathodal chamber and a current of 0.5mA/cm^2 was applied for 12 hours. The steady-state flux increased from 286 ± 71 ng/cm^2-hr in the presence of NaCl to 829 ± 35 ng/cm^2-hr without the competing chloride ions (Table I). These data are consistent with the idea that co-ions present in donor solutions compete with the oligonucleotide to carry the charge across the skin, thus leading to a reduction in drug flux (14,20). Therefore, decreasing the amount of NaCl at the cathode led to less competition between the chloride ions and the oligonucleotide molecules, resulting in greater TAG-6 flux.

Combining iontophoresis with chemical penetration enhancers to synergistically improve transport has met with limited success. The most positive results have been with ethanol pretreatment (21). Therefore, we exposed the skin to 100% ethanol for 90 minutes prior to iontophoresis and then determined the steady-state flux of TAG-6. The oligonucleotide was placed in the cathode (65 μM), in the presence of 25 mM

Table I. Influence of Cathodal Salt Concentration and Ethanol Pretreatment on Steady State Flux

Cathode [NaCl]	Pretreatment	SS Flux (ng/cm^2-hr)
133 mM	—	286 ± 71
0 mM	—	829 ± 35
0 mM	ETOH	338 ± 31

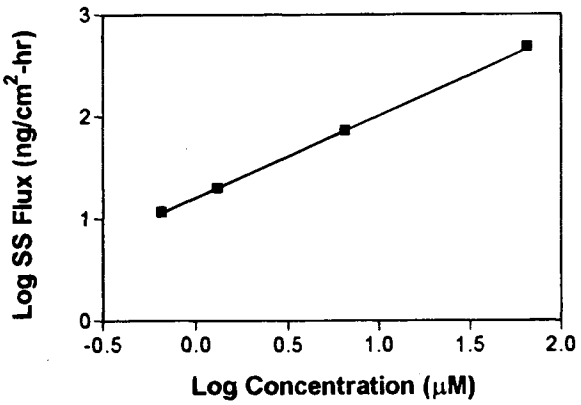

Fig. 4. Influence of concentration on steady state flux of FITC-labelled TAG-6. Donor solutions were 0.65 μM, 1.3 μM, 6.5 μM and 65 μM (n = 2).

HEPES (pH 7.4), but without NaCl. Transport was reduced from 829 ± 35.2 ng/cm^2-hr to 338 ± 30.8 ng/cm2-hr (Table I). These results are opposite of those expected. We hypothesize that because oligonucleotides can interact with ethanol, residual alcohol is probably altering oligonucleotide structure in a way that reduces iontophoretic transport.

The relationship of TAG-6 flux as a function of donor concentration was determined (Figure 3). FITC-labeled TAG-6 was placed in the cathodal chamber at 65 μM, 6.5 μM, 1.3 μM and 0.65 μM. Transport was monitored in 180 minute fractions and steady state flux calculated. The data demonstrate, as expected, that TAG-6 transport is greater with increasing concentration within the two orders of magnitude tested, and that saturation of delivery has not occurred.

As an understanding of iontophoretic transport of oligonucleotides is being developed, the question of whether therapeutic levels of these molecules can be delivered must be addressed. Zewert et. al (8) reported that 400 ng of DNA injected into the epidermis by gene guns have been shown to be effective vaccines. This is within the range of steady-state fluxes seen in these experiments. If necessary, quantities of oligonucleotide delivered iontophoretically may be increased substantially by increasing both the donor concentration and/or the patch size.

SUMMARY

Iontophoretic delivery TAG-6 resulted in substantial oligonucleotide flux. The molecule was not degraded as it crossed the skin. Molecular flux is donor condition dependent, with pH and salt concentration both having significant effects. Pretreating the skin with ethanol reduced iontophoretic transport. These data demonstrate that iontophoresis can enhance transdermal flux of phosphorothioate oligonucleotides and that further investigation is warranted.

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