Published in final edited form as:

Pteridines. 2013 August ; 23(1): 81–89. doi:10.1515/pteridines.2012.23.1.81.

Use of a Pteridine Moiety to Track DNA Uptake in Cells

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Abstract

Fluorescence labeled oligonucleotides have a long history of being used to monitor nucleic acid transport and uptake. However, it is not known if the fluorescent moiety itself physically limits the number of pathways that can be used by the cell due to steric, hydrophobic, or other chemical characteristics. Here, we report a method for comparing the uptake kinetics of oligonucleotides labeled either with the fluorescent pteridine, 3-methyl-8-(2-deoxy- β -D-ribofuranosyl) isoxanthopterin (3MI), or the common fluorophore 5-carboxyfluorescein (5-FAM). We use a multiphoton microscopic technique to monitor nucleic acid uptake LLC-PK₁, a pig renal tubular cell line that is known to have multiple uptake pathways. We find that the two fluorophores enter the cells at different rates, suggesting that choice of fluorescent moiety influences the uptake pathway used by a cell. Finally, we reconstituted an LLC-PK₁ membrane channel that is selective for nucleic acids in planar lipid bilayers, and tested the ability of the labeled nucleic acids to permeate the channel. We find that 3MI, and not 5-FAM labeled oligonucleotides can traverse the plasma membrane through the channel. These results have implications for future studies aimed at delivering pteridine moieties to cells and for tracking nucleic acid transport into tissues.

Keywords

DNA uptake monitoring; fluorescence label; methylisoxanthopterin

Introduction

Despite the long history of techniques developed to monitor internalization of oligonucleotides (ON) by cells and tissues, most have considerable limitations. For instance, measurements of internalized radiolabeled-ON cannot be reliably quantified without cell fractionation because ONs bind non-selectively to the plasma membrane and some membrane proteins. This can greatly distort the signal-to-noise ratio, where noise in the

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Author Discloser Statement

The National Cancer Institute holds patents on the pteridine nucleoside analog phosphoramidites. As an inventor, M. E. H. receives royalty payments. None of the other authors have competing financial interests

system arises from membrane bound ON (1–3). As an alternative, many investigators have turned to microscopic approaches using fluorescently labeled nucleic acids to distinguish between membrane adherent and internalized ON. Microscopic approaches allow one to differentiate between membrane bound and internalized ON signals, but the size of most fluorescent moieties add considerable steric dimensions to the ON, and it is not known if this alters or limits uptake.

Although these limitations are considerable, nucleic acid uptake studies have still managed to identify a number of membrane dependent internalization pathways for ONs such as pinocytosis (receptor independent) (1), endocytosis (receptor dependent) (2), and phagocytosis (scavenging) (1). Furthermore, an increasing body of literature points to a variety of uptake pathways in various cells. For instance, several members of the toll-like receptor family are known to bind nucleic acids for internalization as part of immune surveillance (4, 5), and members of the systemic RNAi defective (Sid) proteins are believed to function as channels or transporters of nucleic acids across the cell membrane in *Caenorhabditis elegans* (6, 7). Our laboratory has identified and characterized a plasma membrane channel that selectively conducts single stranded RNA and DNA. This nucleic acid conducting channel (NACH), first isolated from rat kidney, is non-rectifying, has an optimal ON length of approximately 20 bases, and is blocked by double stranded nucleic acids. While the steric hindrance imparted by addition of fluorescent moieties may not greatly alter some pathways, for instance endocytosis and pinocytosis, others (e.g., Sid and NACH) may be sensitive to RNA/DNA length and structure. In consideration of such size/conformation sensitivity, we have hypothesized that ON uptake is altered by the structure of the fluorescent probes used.

One of the most common fluorescent molecules used for labeling oligonucleotides and proteins is 5-carboxyfluorescein (5-FAM). Although fluorescein derivatives are typically large, rather bulky compounds, there are numerous examples where the size and chemistry of these molecules does not perturb the experimental system. For example, oligonucleotides can be readily labeled with 5-FAM for fluorescence *in situ* hybridization (8), and unnatural amino acids carrying fluorescein derivatives are accepted by the *E. coli* ribosome and are commonly used for labeling synthesized proteins *in vitro*. Despite these examples, 5-FAM is a relatively hydrophobic molecule that adds distinct chemical features to a nucleic acid polymer, which almost certainly changes interactions between nucleic acids and the plasma membrane or integral membrane proteins. While this may not cause obvious problems for endocytic internalization pathways, other delivery pathways may not function properly in the presence of 5-FAM labeled oligonucleotides.

We have searched for a suitable alternative to fluorescein derivatives for labeling nucleic acids. One such fluorophore is 3-methyl-8-(2-deoxy- β -D-ribofuranosyl) isoxanthopterin (3MI). 3MI is a pteridine analog of guanosine with much smaller dimensions than 5-FAM, and, as such, might be able to circumvent steric limitations inherent to more traditional fluorescent moieties (9–11). We therefore initiated a series of experiments to test the hypothesis that uptake is significantly different in ONs labeled with 5-FAM compared to those labeled with 3-MI. If true, this study would confirm that the choice of fluorophore would limit the available pathways of nucleic acid uptake. To test this hypothesis, we developed a method to monitor 3MI-internalization using multiphoton laser-scanning fluorescence microscopy and compared uptake in renal epithelial cells exposed to 5-FAM- or 3MI-ON. We also assessed the effect of these tags on activity of one of the size/conformation sensitive pathways by employing electrophysiological methods to analyze differences in NACH function when presented with 5-FAM- or 3MI-ON.

Materials and Methods

Cell culture

LLC-PK1 cells were purchased from ATCC and propagated in culture on a plastic growth surface as recommended. These cells are derived from pig kidney, they have a polarized epithelial phenotype when grown on collagen-coated growth surfaces (12), and we have previously shown that they express NACH (13). Three days prior to study, cells were treated with trypsin-EDTA solution to release them from the growth surface and approximately 5000 cells were seeded onto collagen-coated (type-1 collagen from rat tail, Collaborative Biomedical Products, Bedford, MA) glass-bottom culture dishes (MatTek Corp., Ashland, MA). Uptake studies were performed when cells reached 80–90% confluence.

Uptake of labeled ON in cultured cells

Immediately prior to study, cells were washed with serum free culture medium and equilibrated for 15 minutes on a heated microscope stage (37°C). A field of view was selected randomly using a 60X immersion lens. The laser and microscope were calibrated, a time-zero image was collected, and labeled ON was added (5 μ M). Labeled oligonucleotides consisted of a 22-mer oligodeoxynucleotide (5'-gtc cgc tgc tct gct cgt tgc c-3') to which a 3' 5-FAM was added (FAM-ON) or all guanines were substituted with 3MI (3MI-ON). All labeled oligonucleotides were purchased from Fidelity Systems (Gaithersburg, MD). Data acquisition began immediately with images acquired every 30 seconds for 10 minutes. Four to six cells in each field were analyzed as described below and averaged to provide a single data set.

Microscope

Multiphoton laser-scanning microscopy was performed on living cells using the BioRad Radiance 2000 system (BioRad Laboratories, Hercules, CA) with a Mira 900F titanium:sapphire laser (Coherent, Inc., Santa Clara, CA).

Image acquisition and analysis

Transport studies utilized a multiphoton laser-scanning microscope, which minimizes phototoxicity and photobleaching. This is particularly important when using 3MI, which has an excitation maximum of approximately 348nm (10). Multiphoton laser-scanning images were collected under conditions that were maintained between samples and between experiments. Detector (PMT) voltage, gain, offset settings (as indicated by the microscope software) and laser power were maintained throughout data collection. Laser power output was monitored via a wavelength selectable power meter located immediately before the beam entered the microscope. Images were acquired every 30 seconds for 10 minutes. Image analysis was performed using Image-J software (<http://rsbweb.nih.gov/ij/index.html>). Dead cells were first eliminated from analysis, 4–6 cells were then randomly selected from each field, quantified, and averaged to provide a single observation from each experiment. Regions of interest (ROI) were defined within the cytoplasm of each cell and data were analyzed using Image-J. During the initial optimization of this multiphoton approach, we observed minimal photobleaching of 3MI and FAM. Data adjustments were made using Image-J and were applied uniformly to all images within an experiment. The intensity of autofluorescence in unlabeled control cells was measured in order to determine the threshold for subsequent analysis of experimental images. Once threshold was determined and set, ImageJ was used to determine fluorescence intensity of an ROI.

Electrophysiology

Electrophysiological measurements in planar lipid bilayers were carried out as previously described (14, 15). Briefly, a 50/50 mixture of phosphatidylserine and phosphatidylethanolamine (Avanti) were prepared in n-decane to a concentration of 50mg/ml and painted over a hole in a Teflon film. Proteoliposomes were prepared as described previously and added to the cis chamber (14, 15). All recordings were carried out using an Axopatch 200B amplifier connected to a CV203BU head stage (Axon instruments). CLAMPX 9.2 and CLAMPFIT software was used to record and analyze ionic current.

Statistical analysis

All reported values are mean $\pm$ SEM. Statistical analysis was performed by two-way analysis of variance (ANOVA) as the test for significance. If $p < 0.05$ by two-way ANOVA, a least significant difference (LSD) test was performed to identify the significant differences.

Results

Since a multiphoton microscopic approach has not been used to image 3MI, we first performed spectral analysis by tuning the laser across a 200nm spectrum and monitoring light emission at each wavelength. 3MI-ON emitted light between 710 nm and 740 nm with an excitation maximum of 725 nm (Figure 1). These data demonstrate that 3MI can be excited by a multiphoton approach and provided a rationale for developing an optical method to measure nucleic acid uptake using 3MI-labeled ON.

LLC-PK1 cells, which are known to have NACH as well as rapid membrane turnover via endocytosis, were incubated with 5-FAM-ON or 3MI-ON and cytoplasmic accumulation was measured as described in the Methods (Figure 2). The first two minutes of accumulation are not shown in Figure 2 because addition of the nucleic acids to the experimental system introduced a significant amount of noise; however, after a brief (2 minute) period, the image stabilized and data were collected. Figure 2 shows the cytoplasmic accumulation of labeled ON over time. To compensate for the difference in the number of fluorescent moieties between the two ONs (six 3MI versus one 5-FAM) the data were normalized and are presented as relative fluorescence units. As can be seen, 3MI-ON (triangles, $n = 4$) accumulation in cells was detectable approximately 4 min after addition, and showed an average increase of 2.8 relative fluorescence units per minute. Within the 10 min collection period, 3MI-ON cellular accumulation appears to be slowing and may have reached a plateau. 5-FAM-ON uptake (circles, $n = 3$) was not detectable until 7 min after addition and accumulated at a rate of only 0.6 relative fluorescence units per minute. 5-FAM-ON did not reach a plateau throughout the duration of these studies, but rather accumulated steadily during the data collection period.

3MI and 5-FAM have substantially different chemical structures (Figure 3). While 5-FAM is a fluore-observed between the two labeled-oligonucleotides might be attributed to the difference in steric dimensions between the two fluorophores. As 3MI-ON is relatively congruent with purine size and is internalized more quickly than 5-FAM-ON, this indicated that our choice of 3MI might be illuminating alternative uptake pathways used by the cell for sequestering nucleic acids.

Previous electrophysiological studies in planar lipid bilayers have demonstrated that NACH functions as a gated, non-rectifying, heteromultimeric channel that is hundreds-fold more selective for single stranded nucleic acids than any other ion. As channel selectivity is conferred through structural recognition and charge interactions between the channel pore and its substrate, alterations in either the pore or its substrate can significantly alter, if not

inhibit, permeation through a channel. Thus, we hypothesized that the significant structural differences in ON substrate would allow NACH to conduct 3MI-ON but not 5-FAM-ON. To test this, we reconstituted the NACH complex in a model planar lipid bilayer and analyzed the ability of 3MI-ON and 5-FAM-ON to traverse the channel by a derivative that is covalently attached to the nucleic acid backbone through a hydrocarbon chain, 3MI is a guanosine analogue that is incorporated into the oligonucleotide strand as a base analog. We reasoned that the significant difference in uptake kinetics measuring the current generated by the movement of the negatively charged nucleic acid polymers. Figure 4A shows the experimental results of 5-FAM-ON in the lipid bilayer with NACH. As expected, current was not observed under two control settings: with the lipid bilayer alone (I), or once the NACH complex had been fused into the bilayer but ON was not present (II). Upon addition of a single-stranded 20 base homomultimer of thymidine (poly dT-20), a current ranging from 1–3 pA was detected (III). It is important to note that this planar lipid bilayer contains multiple channels (3 or more) and as such, transitions between multiple levels of activity are seen. Upon addition of equimolar 5-FAM-ON to the experimental system, channel gating and ionic current were no longer observed (IV). This observation is consistent with the hypothesis that NACH does not transport 5-FAM-ON, and we conclude that 5-FAM-ON actually induces a blockade of the channel, preventing unlabeled nucleic acid permeation through NACH.

Similar experiments were performed using 3MI-ON. As was the case in Figure 4A, channel activity was not observed under control conditions (i.e. with lipids alone or when NACH was fused with the bilayer but ON was not present, Figure 4B traces I and II, respectively). Addition of poly dT-20 revealed a single channel passing about 0.75 pA of current (Figure 4B-III). Transitions to zero current were frequent but very short in duration. Unlike when 5-FAM-ON was added, addition of 3MI-ON did not result in channel blockade (Figure 4B-IV). In fact, channel activity appears to increase in the presence of 3MI-ON. This increase can most likely be interpreted to suggest subsequent insertion of more channels into the bilayer as the experiment progressed, rather than activation of latent channels in response to the addition of 3MI-ON. Nonetheless, 3MI-ON does not appear to inhibit NACH activity, as current is still clearly measurable in the presence of the 3MI-modified oligonucleotide.

Figures 4C and 4D summarize channel open probability data derived from experiments with 5-FAM-ON ($n = 3$) and 3MI-ON ($n = 3$). Open probability was essentially zero with the bilayer alone (Figure 4C-I) and the bilayer with NACH fused but no ON (Figure 4C-II), indicating the channel is closed or unable to pass any ionic species in the buffer. Open probability increased significantly to approximately 0.08 in the presence of poly-dT20 (Figure 4C-III) and fell to zero when 5-FAM-ON was added (Figure 4C-IV), indicating the channel is blocked. In contrast, NACH open probability was not reduced when 3MI-ON was added (Figure 4D-III compared to Figure 4D-IV). In different experiments where NACH was reconstituted in the absence of poly-dT20, channel activity was observed in the presence of 3MI-ON but not when 5-FAM-ON was added alone, indicating 3MI-ON permeates the channel but 5-FAM-ON does not (data not shown). Together, these data indicate that 3MI-ON permeates the membrane through NACH while 5-FAM-ON does not.

Discussion

We compared the uptake kinetics of two fluorescently labeled oligonucleotides by multiphoton microscopy in LLC-PK₁ cells, a renal epithelial line. We chose this experimental system because we had previously isolated NACH from the plasma membrane of these cells and knew *a priori* that multiple nucleic acid internalization pathways exist for this cell line. Our decision to use a multiphoton approach had several logistical and technical advantages. To begin with, the experimental design required data acquisition every thirty

seconds over a ten-minute period. A multiphoton microscope drastically reduces the effects of photobleaching that would otherwise have been problematic using more traditional single photon excitation techniques. Multiphoton microscopy also allows for very precise determination of fluorescence intensity inside well defined, spatially restricted volumes of a cell (11). Furthermore, the fluorescent pteridine used in this study has an excitation maximum of 348nm (10). The fact that this wavelength lies well within the ultraviolet spectrum, raised concern that prolonged exposure at this wavelength would be phototoxic.

The discovery of NACH suggests that exogenous nucleic acid uptake might be accomplished in some cells through an endocytosis or pinocytosis independent pathway. However, most studies examining internalization of naked nucleic acid conclude the mechanism of uptake is through endocytosis or pinocytosis. This disparity led us to formulate a working hypothesis that the various fluorescent tags used in previous nucleic acid uptake studies might have inadvertently dictated which internalization pathways are utilized by the cell. It was reasonable to suspect that a nucleic acid selective channel would not conduct alternate molecules with significantly disparate structure, and therefore any studies using such indicators would not elucidate a contribution by NACH. This theory was reinforced by a wide variety of studies that support uptake pathways in addition to endocytosis. For example, numerous studies have reported that fluorescently labeled nucleic acid probes become sequestered in endosomes (3, 16–19), whereas other studies that have examined radiolabeled oligonucleotides show distribution throughout the cytoplasm, nucleus and various intracellular organelles (20–22). While it has been shown that some tissues and organs such as spleen and liver internalize nucleic acids primarily through scavenger receptor binding and endocytosis (3, 23–25), experiments in the rat kidney suggest alternative pathways also exist (2, 26, 27). There are numerous reports of other mechanisms of nucleic acid uptake (7, 28, 29), and a variety of conflicting reports on the cellular fate of the internalized nucleic acids (6, 7, 17, 22, 29, 30). The experiments detailed in this report demonstrate that choice of fluorescent moiety can limit the uptake pathways that can be studied, and perhaps in some cases may mask one or more uptake conduits that are also contributing to cellular accumulation. These results may also help to explain the diversity of conclusions regarding the cellular fate of the internalized nucleic acids, as different uptake pathways undoubtedly lead to different compartments of the cell. This last point is of particular importance when the role of a nucleic acid permeable channel in the membrane is considered, as this pathway leads directly to ON delivery to nucleic acid processing components in the cytosol, such as ribosomes or the RNA interference machinery. These observations might be useful to consider for any future studies in the field of cellular nucleic acid uptake.

A careful analysis of the results in Figure 2 led us to conclude that LLC-PK1 cells were utilizing two different internalization pathways, evident by the differing rates of cellular accumulation. While the uptake kinetics of 5-FAM were consistent with the reported rates of endocytosis (31, 32) or pinocytosis (33, 34), the rapid rate of 3MI accumulation exceeds the expected uptake kinetics of typical endocytic mechanisms. The different rates of accumulation suggest that more than one uptake mechanism exists. These data support the hypothesis of other investigators, notably Wu-Pong and colleagues (35), that nucleic acid uptake in cells and tissues results from at least two mechanisms: endocytosis and an endocytosis-independent “channel-like” process. To date NACH is the only nucleic acid channel described in LLC-PK₁ cells which leads us to propose that NACH is this “channel-like” transporter previously postulated by other groups.

It is of interest to note that pteridine labeled DNA did not show an identical current to unlabeled DNA in the planar bilayer system. This suggests that, even with a much smaller fluorescent tag, the experimental system was still perturbed to a measurable degree. While

the results from the bilayer indicate that 3MI-ON does not block NACH, the difference in kinetics between native ON and 3MI-ON demonstrate differences in the way each molecule interacts with the channel. Consequently, despite the advantages over FITC-labeled ONs, 3MI-ON has its own intrinsic physio-chemical limitations that contribute to altered conductance through NACH.

An important aspect of these results is to underscore the importance of NACH in delivering biologically active nucleic acids to the cytoplasm of cells. To date, NACH has been identified in rat brain (36) and porcine, canine, rat and mouse kidneys, and a growing body of literature indicates that its role may not be solely to scavenge and recycle extracellular nucleic acids from the environment. Given the density and complexity of genetic information contained in nucleic acids, it is not surprising that some cells have evolved elaborate transport mechanisms to exploit the significant quantities of circulating extracellular nucleic acids present in the vertebrate plasma milieu (37). Several reports in worms and flies indicate that nucleic acids might serve as paracrine signaling molecules; and considering the increasingly important roles of the RNAi and miRNA pathways in gene regulation, it has been proposed that NACH and other nucleic acid transporters like Sid-1 (6, 7) and Sid-2 (38) may serve to feed exogenous nucleic acids into cells to be processed for genetic and epigenetic regulatory applications. In light of the results reported here, we conclude that future studies aimed at optically tracking the delivery of naked DNA and RNA into cells should take into account the contribution of NACH as an additional conduit for nucleic acid internalization.

In conclusion, our data demonstrate the utility of a pteridine fluorescent guanosine analog for measuring cellular uptake of oligodeoxynucleotides. Furthermore, our data support our hypothesis that oligonucleotides labeled with fluorescein derivatives are not transported by NACH and, as such, studies measuring ON uptake with these moieties would be biased against observing NACH and possibly other nucleic acid channels/transporters.

Acknowledgments

Microscopy was performed at the MSSM-Microscopy Shared Resource Facility, supported with funding from NIH-NCI shared resources grant (5R24 CA095823), NSF Major Research Instrumentation grant (DBI-9724504) and NIH shared instrumentation grant (1S10 RR0 9145). These studies were supported by a grant from the NIDDK/NIH to BH (DK065838). Justin Costa is a trainee in the Integrated Pharmacological Sciences Training Program supported by grant T32GM062754 from the National Institute of General Medical Sciences.

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Figure 1. Spectral analysis of 3MI excitation by multiphoton microscopy - The fluorophore 3MI has a narrow spectral range of excitation, with an excitation maximum of 725nm.

Figure 2.

Internalization dynamics of labeled ON -Intracellular accumulation of 3MI-ON (triangles), 5-FAM-ON (circles), and unlabeled oligonucleotide (time control, squares) was monitored over time. 3MI-ON begins to accumulate inside the cell with a rapid upward slope (2.8 RFU/min) beginning at about 4 minutes after addition of the probe. The beginning of the upward trend of 5-FAM-ON does not begin until 7 minutes after addition of the probe, and has a lower slope (0.6 RFU/min) compared to 3MI-ON.

Figure 3.

Chemical structures of the two fluorophores and guanosine: Chemical structure of 5-FAM (showing its linkage to guanosine), 3MI, and guanosine.

Figure 4.

The effect of 3MI-ON and 5-FAM-ON on NACH activity: A) Effect of 5-FAM-ON on NACH activity. NACH was reconstituted as described. Electrical activity of the bilayer was monitored before (I) or after (II) the NACH complex was fused into the bilayer. These two traces represent negative controls and demonstrate the baseline electrical activity of the bilayer. Upon addition of a single-stranded 20 base homomultimer of thymidine (poly dT-20), 1–3 pA of current was detected (III). This activity was blocked by addition of 5-FAM-ON (IV). B) Effect of 3MI-ON on NACH activity. As with Panel A, traces I and II reflect electrical activity of the bilayer before and after fusion of NACH complex with the bilayer, respectively. Trace III was recorded after addition of poly dT-20 and shows channel current flowing consistently at 0.75 pA. Addition of 3MI-ON (trace IV) did not block NACH activity, although there are clearly more transitions between the open and closed state and more active channel present in the membrane. C) Mean open probability of NACH under the four conditions depicted in Panel A ($n=3$). D) Mean open probability of NACH under the four conditions depicted in Panel B ($n=3$).