Endo-Porter: A Novel Reagent For Safe, Effective Delivery Of Substances Into Cells

James E. Summerton

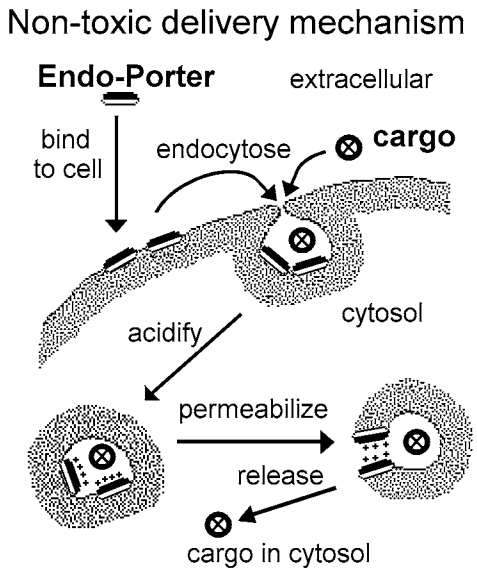
GENE TOOLS, LLC, One Summerton Way, Philomath, OR 97370
Phone: (541) 929-7840, Fax: (541) 929-7841, email: jsummerton@gene-tools.com

ABSTRACT

Delivering large molecules into the cytosol of animal cells without damaging the cells has been one of the toughest challenges in biology. Endo-Porter is a weak-base amphiphilic peptide that was designed to deliver Morpholino antisense oligos and other non-ionic substances into the cytosol/nuclear compartment of cells by an endocytosis-mediated process that avoids damaging the plasma membrane of the cell. This prevents the loss of vital cell contents and the attendant high cell toxicity typical of most delivery systems.

To deliver a substance into cells simply add that substance to the medium covering the cells, followed by addition of the pre-formulated Endo-Porter solution and swirl to mix. The mechanism of delivery, illustrated in Figure 1, entails rapid adsorption of Endo-Porter to cell surfaces, but this adsorption to the cell surface does not damage the plasma membrane. The membrane-bound Endo-Porter is rapidly endocytosed, along with any substances present in the medium (ie., the cargo one wishes to deliver). Upon subsequent acidification of the endosome (a natural process) the Endo-Porter contained within that endosome is converted to its poly-cationic form, which acts to permeabilize the endosomal membrane. This acid-induced permeabilization of the endosomal membrane allows any co-endocytosed cargo to pass from the endosome into the cytosol of the cell.

Figure 1.



This paper describes the basic design strategy used to develop Endo-Porter, test systems used to guide that development, and the effects of various structural parameters, including size and composition of the lipophilic face, size and composition of the weak-base face, and the relationship between peptide length and delivery efficiency in the presence of serum.

Ann N Y Acad Sci. 2005 Nov;1058:62-75.

INTRODUCTION

Sequence-specific oligomers for modifying gene expression (oligos) include antisense oligos developed in the 1980s, ribozymes developed in the 1990s, and most recently RNAi and siRNA. One of the principal challenges in using such oligos for modifying gene expression has been in achieving their safe, effective delivery into the cytosol/nuclear compartment of cells where their targeted RNA sequences reside.

Direct Delivery Across Plasma Membrane

The most widely used methods for delivering poly-anionic oligos (ie., DNA, RNA, S-DNA, 2'O-Methyl RNA, Ribozymes, RNAi and siRNA) generally entail forming electrostatic complexes with poly-cations, such as polyethyleneimine,[1] or forming electrostatic complexes with cationic lipids,[2] and then adding the resultant complexes to cells. Such complexes help protect these nuclease-sensitive oligos from enzymatic degradation, and they are moderately effective for delivering the oligos into cells. However, such delivery reagents are typically complicated and time consuming to use. More important, they are generally rather toxic to cells, and so must be removed from the culture medium after just a few hours. They typically also work well only in serum-free medium.

Advanced non-ionic antisense oligos, such as Peptide Nucleic Acids and Morpholinos,[3,4] have been developed to overcome the problems of nuclease sensitivity and off-target effects which plague the more conventional poly-anionic oligos. Because these novel oligos are non-ionic they do not form complexes with the widely used poly-cations and cationic lipids. Accordingly, in the last few years a number of "protein transduction domain (PTD)" peptides have been used to deliver such non-ionic antisense oligos.[5,6] Such PTD delivery peptides evolved as follows. A number of viral and excreted cellular proteins are known to penetrate animal cell membranes by virtue of a short strong-base (ie., poly-cationic at neutral pH) amphiphilic segment of the protein, typically with a segment length of around 12 to 18 amino acids. Representative well-studied examples include a short segment of tat protein of the HIV virus,[7] a short segment of the VP22 protein of Herpes Virus,[8] and a short segment of an excreted neural protein of the fruit fly.[9] Synthetic versions and close analogs of these membrane penetrating PTD peptide sequences have been developed and used to deliver a variety of substances into animal cells. Such membrane-penetrating peptides typically contain a lipophilic face or end, plus a face or end containing multiple strong-base amino acids selected from arginine and lysine. This basic design consisting of a lipophilic face and a strong-base face has been further modified by Prochiantz and coworkers to give a novel peptide having a lipophilic tryptophan face and a strong-base arginine face, which provides substantially better membrane penetrating capacity than the natural peptide sequences.[10] Somewhat similar peptides, developed in the late 1990s at GENE TOOLS, contain a lipophilic face of leucines and a strong-base face of lysines, and these are even more effective for entering cells (unpublished results). While such strong-base amphiphilic peptides can be exceptionally effective for delivering oligos into the cytosol of cells, most are of limited utility because of their considerable toxicity to cells, and poor efficiency in the presence of serum.

I believe it likely that much of the toxicity characteristic of delivery reagents which contain strongly basic amine moieties is a consequence of their compromising the permeability barrier of the plasma membrane – evidenced by their causing release of hemoglobin from red cells at neutral pH (assay described later herein). This compromising of the plasma membrane allows escape of vital cell contents into the extracellular medium. I and others have postulated that such toxicity should be largely avoided by developing delivery systems which utilize an indirect endocytosis-mediated delivery mechanism wherein the plasma membrane is never permeabilized.

Indirect Delivery Via Endosomes

1. Osmotic delivery system:

Osmotic delivery is an endocytosis-mediated system for delivering large polar molecules, particularly Morpholino antisense oligos, into the cytosol of cells. An osmotic delivery system, optimized and provided commercially by GENE TOOLS,[11] entails exposing cultured cells to a mixture of the substance to be delivered (cargo) combined with a high-osmolarity solution of sorbitol and polyethylene glycol 600 (PEG-600). After a specified period of time to allow for endocytosis of the cargo-containing solution, the delivery

procedure is completed simply by removing the mixture from the cells and replacing it with normal cell culture medium. The mechanism underlying this delivery process entails: 1) incorporation of the PEG-600 into the plasma membrane, rendering said membrane somewhat fragile; 2) uptake of both cargo and high-osmolarity sorbitol solution into newly-forming endosomes - which now have membranes rendered fragile by the PEG-600; and, 3) upon return to normal osmolarity cell culture medium the high osmolarity solution within the endosomes causes a net inflow of water into the cargo-containing endosomes - resulting in buildup of pressure within the endosome. Because of their fragile membranes (due to the PEG-600), that osmotic pressure within the endosomes results in release of the cargo into the cytosol of the cell. While this osmotic delivery system does achieve the desired delivery, the amount of delivery is modest because of the limited time which the cells can be exposed to the high-osmolarity solution without incurring undue cell toxicity.

2. Mixed-base delivery system:

The relatively low delivery efficiency of the osmotic system provided the stimulus for development of a newer more effective endocytosis-mediated mixed-base delivery system, referred to as Special Delivery.[12] This newer mixed-base delivery system entails forming a hydrogen-bonded complex between a non-ionic Morpholino antisense oligo (the cargo to be delivered) and a defined-structure degradable poly-anion, and then forming an electrostatic complex between that poly-anion and the mixed-base, ethoxylated polyethyleneimine (EPEI). EPEI was selected because it contains in roughly equal portions both moderately strong base moieties having pKa values above 7 (so largely ionized at neutral pH), and weaker-base moieties having pKa values in the range of about 5.5 to 6.5 (so largely non-ionized at neutral pH). In this delivery system the higher pKa base moieties of the EPEI serve both to bind electrostatically to the cargo-containing poly-anionic complex and to bind the poly-anionic surface of the plasma membrane of cells. Subsequent endocytosis and acidification within endosomes serve to ionize the weaker-base moieties of the EPEI, resulting in a high composite charge density on the EPEI sufficient to permeabilize the endosomal membrane, thereby allowing co-endocytosed cargo to pass from the endosome through the permeabilized endosomal membrane into the cytosol of the cell. While this indirect-entry mixed-base delivery system is indeed less toxic to cells than the widely-used strong-base direct-entry delivery systems described above, nonetheless, the EPEI component has been found to cause some release of hemoglobin from red blood cells at neutral pH (indicating some permeabilization of cell membranes) and has been seen to cause some toxicity to cultured cells if they are exposed to this substance for more than a few hours - presumably due to the slight permeabilization of the plasma membrane by the EPEI. In addition, the delivery efficiency of this mixed-base system is much reduced in the presence of just a few percent of serum. This poor activity in the presence of serum, a problem shared with most other delivery systems, is a significant limitation because numerous cell types, particularly primaries, grow poorly or are damaged in the absence of serum.

ENDO-PORTER DELIVERY REAGENT

Design Strategy

The improved delivery efficiency and reduced toxicity seen with the above-described mixed-base indirect-delivery system suggested the possibility that simply deleting most or all of the strong-base moieties (cationic at neutral pH) while keeping the weak-base moieties (non-ionic at neutral pH, but ionized when acidified within endosomes) might alleviate the cell toxicity problem. However, tests with peptides containing just weak-base histidines gave no significant delivery, suggesting that deleting the strong-base moieties necessitates the addition of some alternative means to achieve initial binding to cell surfaces. It was postulated that such cell binding might be achieved without concomitant toxicity by incorporating suitable lipophilic moieties or a lipophilic face. Finally, to provide rigorous control over positioning in space of the various components of the delivery reagent it was decided to go with a peptide structure designed to exist preferentially in an alpha helical conformation. These considerations led to preparation and testing of a large number of synthetic peptides characterized by having a lipophilic face composed primarily of amino acids selected from leucine, isoleucine, and valine, and a weak-base face composed primarily of histidines, with up to a few lysines for improved aqueous solubility.

The focus in our Endo-Porter development program was to design and optimize a safe and effective reagent for delivering non-ionic substances into the cytosol/nuclear compartment of cells. However, it should be noted that because of their substantially non-ionic nature at neutral pH, and their mechanism of disrupting membranes of acidified endosomes, these amphiphilic weak-base Endo-Porter peptides are not suitable for delivering poly-anionic oligos, such as siRNA.

Test Systems

1. Assessing membrane permeabilization by hemoglobin release from red cells at pH 5, 6 and 7.2:

A key requirement for a reagent effective for indirect endocytosis-mediated delivery is that it not permeabilize cell membranes at pH 7.2, but strongly permeabilize cell membranes at the pH within acidified endosomes (ranging from about pH 5 to 6). Prospective delivery peptides were initially tested in the following hemoglobin release assay to provide a quantitative measure of membrane permeabilization as a function of pH.

Add about 10 drops of fresh blood to 14 ml of isotonic saline (9 g NaCl in 1 L of water), mix, and centrifuge out red blood cells. Wash the cells by resuspending the cell pellet in another 14 ml of isotonic saline and centrifuge. Repeat wash once more. To pelleted red cells add 0.3 ml of isotonic saline, mix thoroughly, and add 10 microLiters of this red cell suspension to 990 microLiters of water to cause release of hemoglobin from the cells by osmotic lysis. Mix well and scan the lysate from 600 nm to 300 nm in a spectrophotometer. Use the absorbance value at 414 nm to calculate how much the red cell suspension should be diluted with isotonic saline in order to give an absorbance of 2.0 at 414 nm if all the hemoglobin were to be released from the red cells. Dilute the red cell suspension with this calculated amount of isotonic saline. Store this final stock red cell suspension in an ice bath during the course of the experiments and use on the same day it is prepared.

Prepare three isotonic buffers.
0.02 M Malic acid (pKa 3.4 and 5.11), 0.15 M NaCl, adjust to pH 5.0 with 5 M NaOH
0.02 M MES (pKa 6.1), 0.15 M NaCl, adjust to pH 6.0 with 5 M NaOH
0.02 M MOPS (pKa 7.2), 0.15 M NaCl, adjust to pH 7.2 with 5 M NaOH

To a 2-ml centrifuge tube add 10 microLiters of a 1 milliMolar solution of the delivery peptide in DMSO. Forcefully pipette in 1 ml of selected isotonic buffer to rapidly disperse the peptide solution, followed by addition of another 0.475 ml of the same isotonic buffer. Next, add 15 microLiters of the isotonic red cell suspension. Cap, mix by inverting the tube 3 times, and then let stand at room temperature for 30 minutes. Centrifuge, preferably in a swinging bucket rotor, to pellet the red cells. Next, carefully take 1 ml of supernatant from the upper portion of the tube and assess its absorbance at 414 nm to quantitate the amount of released hemoglobin. For the no-release value do an identical preparation with isotonic buffer, but instead of stock peptide solution, use only DMSO. For the 100%-release value do an identical preparation, but use water instead of isotonic buffer.

For a peptide to be both safe and effective for indirect endocytosis-mediated delivery it should give near quantitative hemoglobin release at pH 5.0, at least moderate hemoglobin release at pH 6, but virtually no hemoglobin release at pH 7.2.

2. Quantitation of delivery into cytosol of cultured cells using a splice correction assay:

The most definitive assessment of cytosolic delivery is one wherein when a cargo is delivered into the cytosol of a cell it generates a quantifiable signal proportional to the amount of cargo delivered to the cytosol of the cell. Also, for definitive results that cargo should be completely stable within the cell so that increases due to continuing delivery are not underestimated due to losses from ongoing degradation.

The splice-correction system developed by Kole and co-workers,[13] coupled with a Morpholino antisense oligo (which is completely stable within cells[14]) targeted against the splicing error site, has been shown to satisfy these requirements for quantitative assessment of cytosolic delivery. The Kole test system (commercially available from GENE TOOLS, LLC, Philomath, Oregon) comprises a cell line containing a stably-transfected gene that codes for an RNA transcript which contains a mutation that generates a splicing error that, in turn, acts to prevent translation of the luciferase coded by that RNA transcript. When

a properly-targeted Morpholino antisense oligo (commercially available from GENE TOOLS) is delivered into the cytosol/nuclear compartment of such cells, the Morpholino blocks the mutant site. This serves to correct the splicing error, which leads to normal translation of the luciferase. Measurement in a luminometer of the light emitted from that luciferase provides a quantitative measure of the amount of Morpholino oligo delivered into the cytosol/nuclear compartment of the cells.[15]

A typical assay for cytosolic delivery using the above functional assay system is as follows. Seed the stably-transfected cells in a 75 cm tissue culture flask and allow cells to grow to confluency. Split the cells into 24-well plates and allow cells to grow to confluency in each well containing 1 ml of DMEM-F12 media (Catalog # 11330-032, Gibco BRL, Gaithersburg, MD) 5% to 10% in fetal bovine serum. On the day of the experiment, replace the culture medium with 1 ml of fresh medium of the same type. To the test well add a suitable volume of stock solution of the cargo to give the desired final concentration of cargo in the culture well. For a Morpholino antisense oligo this is typically 1 to 5 microLiters of a 1 milliMolar stock solution of Morpholino oligo in water - giving a final 1 to 5 microMolar concentration of Morpholino oligo in the well. Swirl briefly to mix. Next, add a suitable volume of 1 mM stock solution of delivery peptide in DMSO to give the desired final concentration of delivery peptide. Immediately after adding the stock solution of delivery peptide swirl the plate to mix. For optimal delivery of Morpholino antisense oligos typically about 6 microLiters of a 1 milliMolar stock solution of delivery peptide in DMSO provides excellent delivery. An alternative to the above sequential addition of cargo and delivery peptide is to use a premix procedure. This entails mixing 1 volume of the 1 milliMolar stock Morpholino solution and 6 volumes of the 1 milliMolar stock delivery peptide solution, and then adding 7 microLiters of this mixture to the test well of cells, and immediately swirling to mix. While this premix procedure gives the same final concentrations of cargo and delivery peptide as above, it has been found to give somewhat greater delivery (typically about a 20% to 30% increase) than achieved by the sequential addition procedure.

After addition of the cargo and delivery peptide, return the culture plate to the CO_2 incubator and incubate at 37ºC for the desired delivery time. Because these indirect-delivery peptides are relatively non-toxic, cells are typically incubated in the presence of both cargo and delivery peptide for 24 hours, but this can be extended to 48 hours without undue damage to the cells, after which the cells are lysed and assayed for both luciferase and total cell protein. Quantitation of luciferase in the cells treated with Morpholino antisense oligo and delivery peptide entails lysis of the cells with Glo Lysis Buffer (Catalog # E2661, Promega, Madison, WI) and then adding 10 microLiters of that cell lysate to 40 microLiters of solution from the Steady-Glo Luciferase Assay System (Catalog # E2520, Promega, Madison, WI), mixing 5 seconds and then monitoring light emission for 10 seconds. Quantitation of protein from the cells is carried out by adding 10 microLiters of the above cell lysate to 990 microLiters of a 5-fold dilution of protein dye from the Bio-Rad Protein Assay Kit (Catalog # 500-0001, Bio-Rad Laboratories, Hercules, CA), and then measuring Absorbance at 595 nm. To correct for variations in cell density from well to well in the culture plate, light emission values are divided by protein absorbance values to give normalized luciferase values, which provide a measure of cytosolic delivery achieved by the delivery peptide.

3. Assessments of toxicity of delivery reagent in cultured cells:

Several currently popular methods for assessing damage to cells entail adding to the cell culture polar and/or ionic substances which do not readily enter the cytosol or nucleus of an animal cell unless the plasma membrane of that cell is damaged. Such substances include the vital dyes, and more recently, ionic dyes, such as ethidium homodimer, which only become fluorescent when intercalated into duplex DNA in the nucleus of the cell. However, it should be appreciated that such substances are not suitable for assessing cell toxicity in the presence of delivery peptides and other delivery systems. This is because delivery reagents are explicitly designed to deliver just such types of substances into the cytosol/nuclear compartment of cells. As a consequence, vital dyes and ionic DNA-intercalating agents give an inherently false indication of toxicity for delivery reagents.

The method I prefer for determining cell toxicity due to the presence of a delivery agent is to plate cells at low to medium cell density into a number of wells of a 24-well culture plate. Half of the wells are then treated with an appropriate concentration of the delivery reagent, and the other half of the wells serve as the untreated controls. After a suitable treatment period (as short as 12 hours and as long as 48 hours), the cells in each well are washed three times with serum-free medium, and then lysed as described

above, and the protein in each lysate quantitated using the Bio-Rad Protein Assay Kit, also described above. Delivery agents which are toxic to the treated cells cause a marked reduction in cell growth or, more often, extensive cell death (often within just a few hours), resulting in substantially reduced protein concentrations in the final cell lysates relative to the lysates from the untreated control wells.

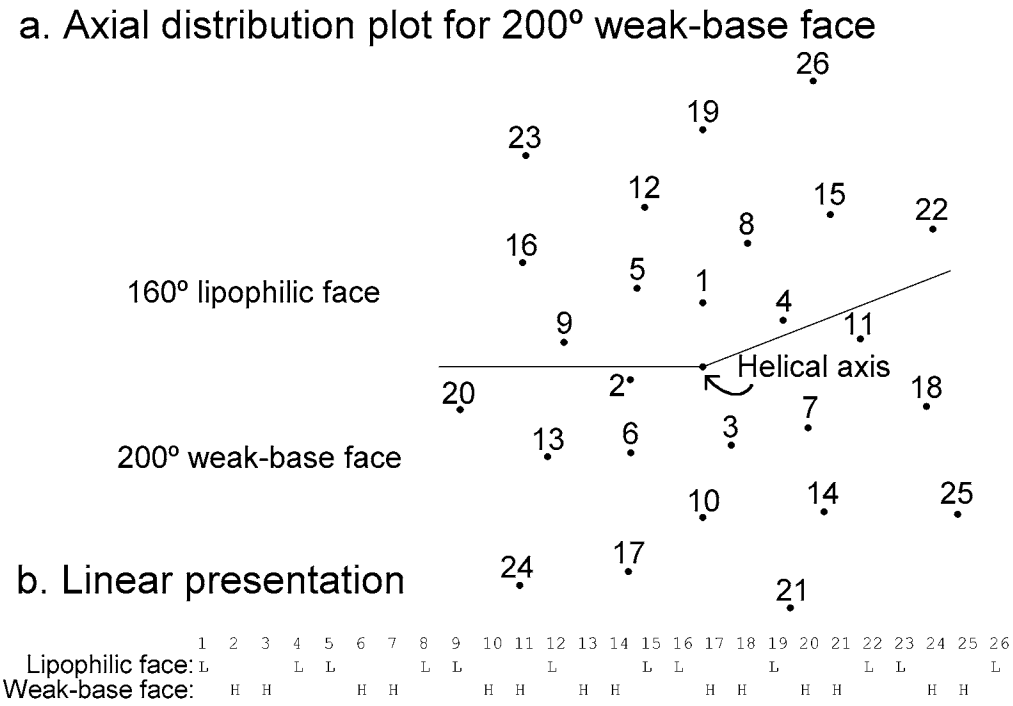
Using such a toxicity assay, it is commonly found that most delivery agents completely kill the cells over the course of a 12 to 24 hour exposure. In contrast, in culture medium containing 10% serum these indirect-delivery peptides, and particularly the optimized Endo-Porter, generally have little detrimental effect on cell growth and division during the first 24 hours, and only moderate effects by 48 hours.

Effects Of Various Structural Parameters

1. Faces of delivery peptide

In designing these weak-base amphiphilic delivery peptides an alpha helix backbone structure was utilized in order to effectively position the various moieties relative to each other. This is achieved both by incorporating amino acids known to favor an alpha helix conformation, and by avoiding amino acids known to disfavor or disrupt alpha helices.[16] By designing for an alpha helix conformation, one can then readily select sequences having defined faces. This is based on the well-characterized structure of the peptide alpha helix wherein each amino acid side chain is rotated a constant 100 degrees about the helical axis relative to the previous amino acid side chain in the peptide. To facilitate peptide design, side chain positions were plotted on a spiral graph wherein each successive numbered amino acid side chain is rotated 100 degrees about the helical axis relative to the previous amino acid of the chain, and each successive amino acid side chain is plotted a standard increment further out from the helical axis relative to the previous amino acid side chain in the peptide. Thus, the axial distribution of a given amino acid side chain indicates its face position, and its radial distance from the helical axis indicates its distance along the peptide chain. Such an axial distribution plot is shown in Figure 2a, where the numbers indicate the relative positions of the consecutive amino acid side chains, both with respect to their axial distribution and to their position along the length of the peptide chain.

Figure 2: Axial distribution plot and linear presentation of amino acid sequence for an amphiphilic peptide having a 200º weak-base face.



Using this axial distribution plot, designing a two-face peptide simply entails selecting the number of degrees to comprise one of the faces. To illustrate, Figure 2a shows a delivery peptide having a 160 degree lipophilic face and a 200 degree weak-base face. Figure 2b shows a corresponding linear presentation of the sequence of this same delivery peptide. This linear presentation better illustrates consecutive alpha helical turns of the peptide. Experimental results suggest that delivery efficiency is relatively independent of whether the peptide sequence starts in the lipophilic or the weak-base face, and whether the peptide sequence ends in the lipophilic or the weak-base face. It also makes little difference whether the 1 position corresponds to the N-terminal or the C-terminal amino acid of the peptide. However, in contrast to the above design flexibility, the respective sizes of the lipophilic and weak-base faces of the delivery peptide have a substantial impact on that peptide's delivery efficiency, as will be discussed later herein.

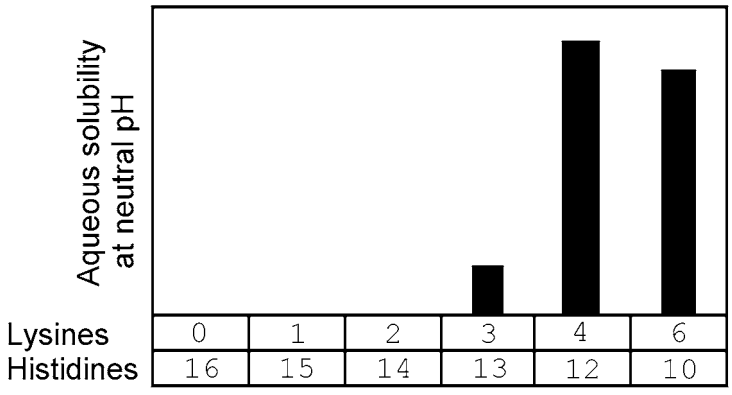
2. The lipophilic face:

In early unpublished work bearing on the design of these weak-base amphiphilic delivery peptides it was found that optimal lipophilicity is achieved when at least most, but preferably all of the amino acid side chains comprising the lipophilic face are both aliphatic and quite lipophilic. The importance of using quite lipophilic side chains is illustrated by my finding that when a relatively few leucines of the lipophilic face were replaced by alanines, the resultant peptide had substantially less affinity for cell membranes, and even at pH 5 such peptides were relatively ineffective for permeabilizing red cell membranes. The prospective natural amino acids for use in the lipophilic face are valine, isoleucine, and leucine. However, for practical reasons the preferred amino acid for the lipophilic face is leucine. This is because while valine and isoleucine were found to give peptides with fair to good delivery efficiencies, respectively, leucine gives good delivery efficiencies and is preferred over valine and isoleucine because leucine's lesser steric hinderance about the alpha carbon results in better coupling efficiencies during peptide synthesis - affording higher peptide quality and lower production costs.

3. The weak-base face:

Histidine is the amino acid of choice for the weak-base face because its pKa of 6.0 for the imidazole side chain is ideal for peptides which are to exist in a non-ionic state in the extracellular medium (about pH 7.2 to 7.5), but then gain a high density of cationic sites upon acidification within endosomes (pH ranging from about 5.0 to 6.0). However, weak-base amphiphilic peptides wherein the weak-base face is all histidines have poor aqueous solubility at neutral pH. Therefore, in an attempt to improve their aqueous solubilities, and possibly also increase their cell binding properties, a number of amphiphilic peptides were prepared and tested wherein an increasing number of the histidines of the weak-base face were replaced by strong-base lysines. The hope was that adding just a few lysines would provide improved solubility without causing significant membrane permeabilization at pH 7.2.

Figure 3: Aqueous solubility of delivery peptides with varying numbers of lysines.



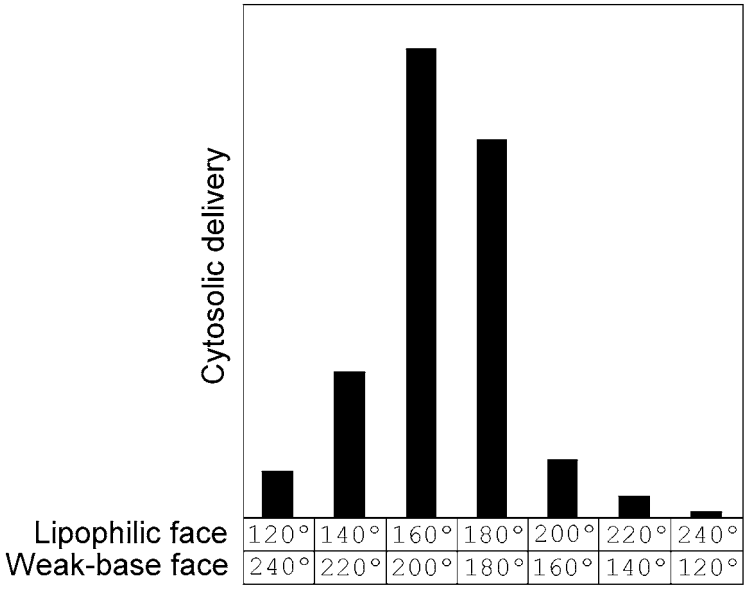
As shown in Figure 3, at least 3 lysines are required to give even modest aqueous solubility for a 33-mer

peptide containing a 180 degree lipophilic face, and 4 lysines are required for good aqueous solubility. However, it was also found that as few as two lysines in the weak-base face were sufficient to cause membrane permeabilization in the hemoglobin release assay, as well as toxicity in the cultured cell assay. These results led to a focus on weak-base faces containing only histidines.

4. Sizes of the lipophilic and weak-base faces:

The respective sizes of the lipophilic and weak-base faces of a delivery peptide have a substantial impact on that peptide's delivery efficiency. This is illustrated in Figure 4, which shows delivery efficiencies for peptides with varying face sizes in an 18-hour functional delivery assay in the presence of 5% serum. The results in this figure show that reasonable delivery is achieved when the lipophilic face ranges from 140 to 180 degrees and the corresponding weak-base face ranges in size from 220 to 180 degrees, with maximal delivery in this test being achieved when the lipophilic face is 160 degrees and the weak-base face is 200 degrees.

Figure 4: Cytosolic delivery by peptides of varying face sizes in 5% serum.

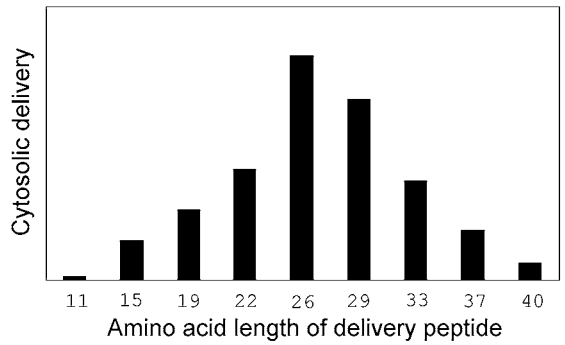


5. Effect of peptide length on delivery efficiency in the presence of serum:

While strong-base delivery peptides reported in the literature are commonly in the 12 to 18-amino acid length range, early test results indicated that 11-mer and 15-mer peptides of this new weak-base amphiphilic peptide type afforded little delivery in the presence of serum. It was postulated that serum might be inhibiting delivery because the delivery peptide was inserting into one of the hydrophobic clefts of serum albumin, which commonly serves to transport fatty acids and a host of other lipophilic substances. If this were the case, then making the peptide substantially longer than the albumin cleft is deep might serve to extend a portion of the peptide out of the cleft sufficient to allow effective binding to nearby cell surfaces. It was also postulated that delivery peptides might achieve greater activity if they were at least as long as the plasma membrane is thick (about 36 angstroms, which corresponds to an alpha helix 24 amino acids long), thereby affording the possibility of a "staves in a barrel" type of membrane permeabilization structure. In light of these postulates, a length series of these weak-base amphiphilic delivery peptides was prepared to test whether peptides longer than 15 amino acids might provide substantially improved delivery activity in the presence of serum.

Figure 5 shows representative delivery efficiencies in cultured cells in the presence of 5% serum as a function of peptide length for peptides varying in length from 11 amino acids through 40 amino acids. The results shown in this figure suggest that weak-base amphiphilic delivery peptides with lengths ranging from about 22 amino acids to about 33 amino acids give good delivery efficiencies, with peptide lengths of 26 and 29 amino acids giving the best delivery efficiencies.

Figure 5: Cytosolic delivery as a function of the length of the delivery peptide.



Representative Delivery Results With Endo-Porter

A typical delivery test was carried out with the optimized Endo-Porter (commercially available from GENE TOOLS, www.gene-tools.com) in Hela cells cultured in medium containing 10% serum. The cells contained Kole's splice-correction test gene, which is capable of coding for luciferase when its splicing error is corrected by a Morpholino oligo that has been delivered into the cytosol of the cell. The suitably-targeted Morpholino was added to the culture medium to give a final concentration of 3 microMolar, and Endo-Porter stock solution (1 milliMolar in DMSO) was added to give a final concentration of 6 microMolar. Incubations were carried out for 24 hours and for 48 hours, after which the cells were processed and assayed as described in the section above titled: "Quantitation of delivery into cytosol of cultured cells using a splice correction assay".

Figure 6: Effect of duration of treatment with delivery peptide on luciferase activity and cell protein.

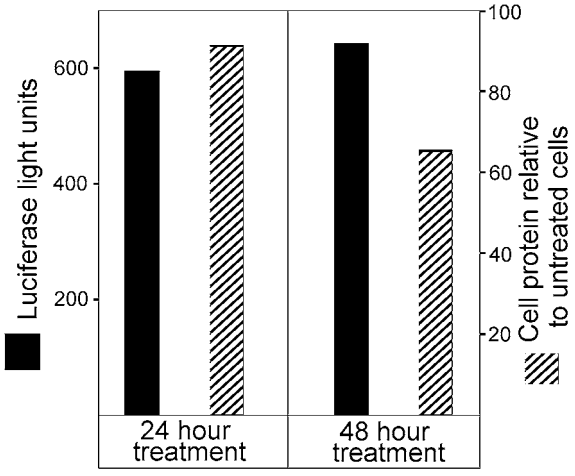


Figure 6 shows the results from that test. As can be seen, after 24 hours the luciferase values are near maximal and cell protein values are 92% relative to untreated control cells - indicating virtually no toxicity from the Endo-Porter delivery reagent. This level of delivery is about 10 fold higher than typically

achieved with osmotic delivery, described earlier herein, and about 3 fold higher than typically achieved with the mixed-base Special Delivery system, also described earlier herein. Figure 6 further shows that after 48 hours the luciferase values have risen another 8%, but the cell protein values have gone down to 66% relative to untreated control cells - suggesting some toxicity from the Endo-Porter delivery reagent after this long exposure time. However, for comparison it should be appreciated that most other delivery reagents will typically have caused the death of all cells within about the first 12 hours of exposure to the delivery reagent.

In summary, relative to the older mixed-base Special Delivery system, Endo-Porter is simpler to use, provides markedly higher delivery efficiency, is much less toxic, and achieves excellent activity in medium containing 10% serum.

ACKNOWLEDGMENTS

The following persons have contributed to our Endo-Porter development program. Chandell Terwilliger and Jeannine Earnest assisted in programming and preparing the peptide synthesizer runs, and carried out the mass spectral analysis of the finished peptides. James P. Summerton carried out the hemoglobin release assays. Shannon Knuth and Paul Morcos carried out the cultured cell assays. Jon Moulton prepared the graphics and formatted the manuscript.

REFERENCES

1. Boussif O., F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix & J.P. Behr. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethyleneimine. Proc. Natl. Acad. Sci. U. S. A. 92: 7297.

2. Thierry A., A. Rahman, A. Dritschilo. 1992. Liposomal delivery as a new approach to transport antisense oligonucleotides. In Gene Regulation: Biology of Antisense RNA and DNA. R. Erickson & J. Izant, Eds.: 147-157. Raven Press, New York.

3. Summerton J. 1999. Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim. Biophys. Acta 1489: 141-158.

4. Summerton J. & D. Weller. 1997. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev. 7:187-195.

5. Simmons C.G., A.E. Pitts, L.D. Mayfield, J.W. Shay & D.R. Corey. 1997. Synthesis and membrane permeability of PNA-peptide conjugates. Bioorg. Med. Chem. Lett. 7 3001.

6. Moulton H.M., & J.D. Moulton. 2003. Peptide-assisted delivery of steric-blocking antisense oligomers. Curr. Opin. Mol. Ther. 5(2):123-32.

7. Mann D. & A. Frankel. 1991. Endocytosis and targeting of exogenous HIV-1 tat protein. EMBO (Eur. Mol. Biol. Organ.) J. 10: 1733.

8. Elliott G.& P. O'Hare. 1997. Intracellular trafficking and protein delivery by a herpesvirus structural protein. Cell 88: 223.

9. Derossi D., A.H. Joliot, G. Chassaing & A. Prochiantz. 1994. The third helix of the Antennapedia homeodomain translocates through biological membranes. J. Biol. Chem. 269: 10444.

10. Chassaing G. & A. Prochiantz. 1997. Peptides usable as vectors for the intracellular addressing of active molecules. PCT published patent application WO 97/12912.

11. Morcos P., J.E. Summerton & J.P. Summerton .2001. Osmotic delivery composition, solution, and method. US Patent 6,228,392.

12. Morcos P.. 2001. Achieving efficient delivery of Morpholino oligos in cultured cells. GENESIS: The Journal of Genetics and Development 30(3): 94-102.

13. Kang S., R. Cho & R. Kole. 1998. Up-regulation of luciferase gene expression with antisense oligonucleotides: Implications and applications in functional assay development. Biochemistry 37(18):6235-9.

14. Hudziak R.M., E. Barofsky, D.F. Barofsky, D.L. Weller, S.B. Huang & D.D. Weller. 1996. Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev. 6(4):267-72.

15. McKeon J., M.J. Cho, & M.G. Khaledi. 2001. Quantitation of Intracellular Concentration of a Delivered Morpholino Oligomer by Capillary Electrophoresis-Laser- Induced Fluorescence: Correlation with Upregulation of Luciferase Gene Expression. Anal. Biochem. 293(1):1-7.

16. Chou P. & G. Fasman. 1974. Prediction of Protein Conformation. Biochemistry 13: 222.

GENE TOOLS, LLC

Website: www.gene-tools.com
Phone: (541) 929-7840
Fax: (541) 929-7841
Mail: One Summerton Way
Philomath, OR 97370 USA