Cellular Uptake of Multistranded Oligonucleotides-DNA Frayed Wires

By

Won-Sang Lee

A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Pharmaceutical Sciences

Faculty of Pharmacy

University of Toronto

2001
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-63186-9
Title of the thesis: Cellular Uptake of Multistranded Oligonucleotides: DNA Frayed Wires

Degree and Year of convocation: Master of Science, November, 2001, By: Won-Sang Lee
Department: Department of Pharmaceutical Sciences, University: University of Toronto

ABSTRACT

Cellular uptake of this multistranded DNA species called DNA Frayed Wires were studied in HepG2 and CNE1 cell lines, in comparison with non-complex-forming single-stranded DNA. Cellular uptake of FW was greater than that of single-stranded DNA by both HepG2 and CNE1 cells with a greater difference observed in HepG2 cells. This uptake process exhibited saturation, temperature dependence, substrate concentration dependence and inhibition by metabolic inhibitors. The initial rate of FW uptake was concentration-dependent. Uptake of both FW and single-stranded DNA was reduced by heparin but more significant inhibition was observed in FW uptake. FW derived from other parent strands demonstrated similar cellular uptake pattern as those from d(A15G15), indicating the importance of the guanine stem rather than the arms in determining cellular uptake. Electrophoretic structural analysis of extracellular and internalized FW indicated that they were not metabolized and maintained their structures after uptake.
ACKNOWLEDGEMENT

First and foremost, I would like to sincerely thank my Lord, Jesus Christ for His unconditional love and guidance day by day throughout this project. And especially for allowing me to meet and work with these wonderful people in this laboratory.

I would like to express my sincere gratitude and appreciation to the following:

My supervisors, Dr. Robert B. Macgregor, Jr. and Dr. Micheline Piquette-Miller for their patience, amazing support and guidance during this project.

My fellow graduate students, Gregory Man-kai Poon and David Noah Dubins for all the technical and emotional help they gave me so willingly without any hesitation and also for wonderful friendship.

Dr. Mike Rauth, Dr. Reina Bendayan, Dr. Peter J. O’Brien and Dr. Shirley Wu, my advisory committee members and examiners for their patience and support throughout this project.

My family for their amazing support with constant prayer throughout my life and this degree.

Mahadeo Sukhai, Gigi Lee, Georgy Hartmann and Julie Kalitsky for all their technical support and also for wonderful friendship.
TABLE OF CONTENTS

ABSTRACT.......................................................................................................................... ii
ACKNOWLEDGEMENT........................................................................................................ iii
LIST OF FIGURES................................................................................................................ vii
LIST OF ABBREVIATIONS ..................................................................................................... ix

CHAPTER 1:

INTRODUCTION
1.1. Background .................................................................................................................... 1
1.2. Oligonucleotides: Current Clinical Use ........................................................................ 3
1.3. Transport Across The Cell Membranes: General Overview ........................................ 5
1.4. Endocytosis: General Overview .................................................................................... 9
1.5. Endocytosis and Oligonucleotides .............................................................................. 11
1.6. Oligonucleotide Binding Receptors ........................................................................... 12
1.7. Delivery Problems and Delivery Strategies .................................................................. 14
   a) Liposomes ................................................................................................................... 15
   b) Protein and Peptide Delivery Vehicles ..................................................................... 16
   c) Dendrimers ............................................................................................................... 17
1.8. Recent Interest in Higher-order Structure DNA ......................................................... 18
1.9. DNA Frayed Wires ..................................................................................................... 19

CHAPTER 2:

RATIONALE
2.1. Rationale ...................................................................................................................... 25
### CHAPTER 3:

**HYPOTHESIS**

3.1 Hypothesis ................................................................. 29

### CHAPTER 4:

**MATERIALS AND METHODS**

4.1. Oligonucleotide Preparation ........................................... 33
4.2. Oligonucleotide Labeling and DNA Frayed Wire Formation .......... 34
4.3. Cells ........................................................................... 35
4.4. Tissue Culture ................................................................. 35
4.5. Time Course of Cellular Uptake ......................................... 37
4.6. Temperature Dependence Studies ....................................... 38
4.7. Concentration Dependence Studies ...................................... 39
4.8. Early Time Intracellular Accumulation Studies ....................... 40
4.9. Heparin Inhibition ........................................................... 40
4.10. Metabolic Inhibitors .......................................................... 41
4.11. Cell Viability Test .............................................................. 41
4.12. Intracellular FW ............................................................... 42
4.13. Polyacrylamide Gel Electrophoresis (PAGE) Analysis .............. 44
4.14. Uptake Pattern of Different FW ......................................... 45
4.15. Intact FW Vs. Cleaved FW ................................................ 46
4.16. Data Analysis ................................................................. 46
CHAPTER 5:

RESULTS

5.1 Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA in HepG2 at 25°C......48
5.2 Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA in CNE1 at 25°C......48
5.3 Initial Rate of Uptake into CNE1 for DNA Frayed Wires and Single Stranded DNA.....52
5.4 Temperature Dependence of CNE1 Cellular Uptake of DNA Frayed Wires and Single Stranded DNA...................................................................................................................54
5.5 Concentration Dependence of DNA Frayed Wire Cellular Uptake..........................58
5.6 Effects of Heparin on Cellular Uptake of DNA Frayed Wires and Single Stranded DNA... ......................................................................................................................................64
5.7 Effects of Metabolic Inhibitors on Cellular Uptake of DNA Frayed Wires ..........67
5.8 Cellular Uptake of DNA Frayed Wires Derived from Different Sequences .........70
5.9 Structural Analysis of Intracellular DNA Frayed Wires.........................................73
5.10 Cellular Uptake of Intact DNA Frayed Wires Vs. Cleaved DNA Frayed Wires ......76
5.11 Cell Viability Test....................................................................................................80

CHAPTER 6:

DISCUSSION

6.1. Discussion..............................................................................................................81
6.2. Future Experiments..............................................................................................89

CHAPTER 7:

REFERENCES

7.1. References.............................................................................................................94
# List of Figures

| Figure 1: | HepG2 Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA ..........49 |
| Figure 2: | Cellular Uptake of DNA Frayed Wire Uptake Vs. Single Stranded DNA in CNE1 Cells ........................................................................................................51 |
| Figure 3: | Early Time Point CNE1 Uptake of DNA Frayed Wires Vs. Single Stranded DNA ........................................................................................................53 |
| Figure 4: | CNE1 Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA at 4°C.... ........................................................................................................55 |
| Figure 5: | DNA Frayed Wires Cellular Uptake at Three Different Temperatures ..........57 |
| Figure 6: | Total Intracellular Accumulation of Different Concentration of DNA Frayed Wired after 120 Minutes in CNE1 Cells ........................................................................................................59 |
| Figure 7: | Intracellular Accumulation by Different Concentrations of DNA Frayed Wires after 30 Minutes of Incubation with CNE1 Cells ........................................................................................................61 |
| Figure 8: | Initial Intracellular Accumulations of Different Concentration of DNA Frayed Wires within 15 Minutes ........................................................................................................62 |
| Figure 9: | Initial Cellular Uptake Rate of Different Concentration of DNA Frayed Wires ...63 |
| Figure 10: | Effect of Heparin on Cellular Uptake of DNA Frayed Wires and Single Stranded DNA ........................................................................................................65 |
Figure 11: Effect of Metabolic Inhibitors on Cellular Uptake of DNA Frayed Wires in CNE1 Cells

Figure 12: CNE1 Cellular Uptake of DNA Frayed Wires Derived from Different Sequences

Figure 13: DNA Frayed Wire Formation by Different Guanine-Rich Strands

Figure 14: Structural Analysis of DNA Frayed Wire Stock Vs. Extracellular and Intracellular DNA Frayed Wires

Figure 15: Intracellular DNA Frayed Wire Vs. Intracellular Single Stranded DNA

Figure 16: Cellular Uptake of Intact DNA Frayed Wires Vs. Cleaved DNA Frayed Wires

Figure 17: Intact DNA Frayed Wire Vs. Cleaved DNA Frayed Wires
List of Abbreviations

CD  circular dichroism

CPM  counts per minute

EDTA  ethylenediamine tetraacetic acid

FW  DNA Frayed Wires

G  guanine

PBS  phosphate buffered saline

PNA  peptide nucleic acid

PNK  polynucleotide kinase

s-DNA  phosphorothioate-DNA

ssDNA  single stranded DNA

TBE  90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0

Tris  tris(hydroxymethyl)aminomethane
Chapter 1: Introduction

1.1. Background

DNA was discovered only four years after the publication of Mendel’s work, the first quantitative studies of inheritance. In 1869, a German chemist, Friedrich Miescher, extracted a white substance from the cell nuclei of human pus and from fish sperm nuclei. The proportion of nitrogen and phosphorous was different from any other known cell constituents at the time which convinced Miescher he had discovered a new biological substance (James, 1970). Because this new substance seemed to be specifically associated with the cell nucleus, he called this white substance “nuclein” and due to its slight acidity it came to be called nucleic acid (Raven and Johnson, 1995).

For many years, very little work was done on DNA by biologists because nothing was known of its function in cells. In the 1920’s, a biochemist, P.A. Levine, determined the basic chemistry of nucleic acids. Levine suggested that DNA contain three basic elements: 1) phosphate group, 2) five carbon sugar and 3) one of four nitrogen containing base: adenine, guanine, cytosine or thymine (Portin, 1993). It was also reported that all four types of bases were present in roughly the same amount which was the finding that led to the mistaken belief that DNA was a simple repeating polymers with no sequence variation. This belief made DNA seem like a very unlikely material to contain hereditary information. In 1944, Avery suggested that hereditary information is contained in DNA and not in protein (McCarty, 1994; Chambers, 1995). In the 1950’s, a study by Erwin Chargaff showed that the four nucleotide bases are not present in equal proportion in DNA molecules and that differences exist in DNA nucleotide composition from one
organism to another. Most importantly, they observed an approximately equal proportion of purines and pyrimidines (Magasanik and Chargaff, 1989). Then in 1952, Hershey and Chase performed an experiment using DNA containing T2 virus and showed that it is not protein but DNA that is responsible for carrying hereditary information (Xie et al., 1996; Stahl, 1998). Subsequent studies on the structure of DNA led Watson and Crick (1953) to the proposal of the double-helical structure of DNA; their proposal and other studies began to lead to a better understanding of molecular basis of inheritance.

Over the past 50 years, a great deal of research has been devoted toward the understanding DNA and its biological role in man and other organisms. With the advent of recent advances in biotechnology, scientists have gained significant and important knowledge of DNA and its related structures (Rich, 1993). This has led toward a better scientific understanding of the involvement of DNA in organ development and disease etiology. More importantly this information has led to the identification of possible therapeutic targets for a number of diseases and to the development of novel drugs and therapies (Javed et al., 1995; Steinberg and Raso, 1998). In addition to the extensive amount of DNA research that is part of the human genome project and genomic based research, recent interest has been in DNA-based nanotechnology. Seeman (1998) demonstrated that certain sequences of DNA could be used as building blocks to create a defined structure on the nanometer scale. This has opened up a new aspect of DNA research in nanotechnology.
1.2. Oligonucleotides: Current Clinical Use

Oligonucleotides are short nucleic acid sequences which have important applications in research and therapy. Much research is currently devoted to development of new therapies using oligonucleotides. Historically, studies performed in the 1960's lead scientists to realize possible pharmaceutical uses of short nucleic acid sequences. One of the first significant studies was carried out by Koch and Bishop in 1968, they demonstrated that naked poliovirus mRNA readily infected cells in tissue culture. This finding suggested that oligonucleotides are able to gain access into cells and be expressed such dictated by their sequence. Ultimately this knowledge led to gene-targeted therapeutic approaches based upon modulation of gene-expression by exogenous administration of short synthetic oligonucleotides (Zamecnick and Stephson, 1978). This approach became known as the 'antisense approach'.

![Figure 1: Antisense approach mechanism to oligonucleotide therapy.](image-url)
In this approach, a synthetic deoxy-oligomer complementary to a segment of targeted mRNA hybridizes with the target cellular mRNA in a sequence specific manner by Watson-Crick base-pairing (Fig. 1). This DNA-mRNA hybridization complex physically blocks translation of mRNA by cellular ribosome machinery or leads to RNase-H-mediated cleavage of the RNA/DNA duplex which inhibits protein translation of the gene. In order to exert an antisense effect, the oligomer must gain access to intracellular spaces of their target cells (Lonnberg and Vuoro, 1996). Leonetti et al. (1991) demonstrated, using microinjection of fluorescently labeled-oligomers into the cytoplasm, that there is a rapid translocation of oligomers into the nucleus which is completed within one minute after microinjection. This study indicated that the nuclear membrane is readily permeable to oligomers, likely due to the presence of highly permeable nuclear pores.

Figure 2: Typical model of plasma membrane structure.
1.3. Transport Across Cell Membrane: General Overview

The cell membrane or plasma membrane (Fig. 2) of a cell, is crucial for cell viability, it encloses the cell, defines its boundaries and maintains the essential differences between the cytosol and the extracellular environment (Miller and Ray, 1992). It acts as a highly selective permeability barrier rather than an impervious wall. In general, it is impermeable to large, charged molecules such as oligonucleotides. Selective permeability gives cells protection from undesired materials and surroundings and at the same time, it ensures that essential molecules such as glucose, amino acids, lipids and essential metabolites readily enter and remain in the cell, while waste compounds are capable of exiting the cell (Schultz, 1971). Molecules get across cell membranes via passive diffusion, facilitated diffusion or active transport. Materials such as lipids, lipid-soluble metabolites and gases enter cells via passive diffusion. This process is driven by the concentration gradient and occurs by first order rate processes. Essentially, the molecule diffuses from a region of high concentration to a region of lower concentration. Hydrophilic metabolites such as glucose and amino acids enter cells via facilitated diffusion. This process is passive and concentration dependent but requires the presence of protein carriers thus this process is partially dependent on these carriers.

Unlike the two previously mentioned processes, active transport does not require a concentration gradient, often occurring against an extreme concentration gradient (Oh and Amidon, 1999). In general, active transport involves translocation of the substrate by a transmembrane protein and requires cellular energy. This transport is dependent on cellular energy thus it is readily inhibited by well-known metabolic poisons such as
sodium azide and 2-deoxy-D-glucose which prevent cellular energy production. General characteristics of active transport include: 1) energy dependence, 2) temperature dependence, 3) ability to transport against concentration gradient, 4) saturability and 5) inhibition. Perhaps the best-characterized active transport system is Na⁺/K⁺-ATPase. This is a transmembrane protein pump that transports sodium ions from cells into extracellular space against a concentration gradient, by using energy stored as ATP (Therien and Blostein, 2000).

For most drugs, entry into cells involves diffusion across the lipid bilayer of the plasma membrane. However, large polyanionic DNA-oligonucleotides do not readily diffuse across membranes. The relative impermeability of cell membranes to oligonucleotides has led chemists to develop a number of modified oligonucleotides with lower electrostatic charge with the aim of enhancing cellular permeation of oligonucleotides via passive diffusion (Monteith and Levin, 1999).

![Diagram of modified oligonucleotides](image)

**Figure 3: Modified Oligonucleotides:** DNA = Phosphodiester, S-DNA = Phosphorothioate, PNA = Peptide Nucleic Acids.
Examples of modified oligonucleotides are shown in Figure 3. In phosphothioate linkage (S-DNA), one non-bridging oxygen atom was exchanged with a sulfur atom. Similar to S-DNA, methylphosphonate oligonucleotides (Methylphosphonate) have internucleotidic linkages that are non-ionic in contrast to the natural polyanionic phosphodiester backbone, methylphosphonate oligonucleotides have one non-bridging oxygen atom exchanged with a methyl group thus eliminating the negative charge (Miller et al., 1981). Another example is Peptide Nucleic Acids (PNA). In this modification, the deoxyribose phosphate backbone is replaced with a chiral polyamide backbone (Nielsen et al., 1991) thereby losing the polyanionic characteristic. Lastly, in morpholino oligonucleotides, the ribose moieties are replaced with morpholino groups and by replacing one of non-bridging oxygen with nitrogen which makes these oligonucleotides less charged (Taylor et al., 1996).

It is widely accepted that unmodified oligonucleotide containing phosphodiester linkages are rapidly degraded in biological fluids by a combination of both endo- and exonucleases (Wickstrom, 1986; Akhtar et al., 1991). Besides reducing electrostatic charge, these modifications illustrated in Figure 3 have been shown to increase the nuclease stability of oligonucleotides. The increased nuclease stability of phosphorothioate internucleotide linkage relative to phosphodiester linkage was first demonstrated by De Clerq et al. (1970) in cell culture experiments with interferon-inducing polynucleotides. A number of other studies have reported increased nuclease resistance of these modified oligonucleotides relative to unmodified oligonucleotides (Zon, 1988; Kashihara et al., 1998).
Another interesting finding observed by Shoji et al. (1991) indicated that uncharged methylphosphonate oligonucleotides gain access into the cell cytoplasm via a mechanism other than passive diffusion. Akhtar et al. (1991) compared the permeation characteristics of normal phosphodiester oligonucleotides with modified oligonucleotides such as methylphosphonate and phosphorothioate oligonucleotides. In this study, charged and uncharged oligonucleotides were placed inside a synthesized liposome and allowed to diffuse out through the membranes of liposomes suspended in water. They found that both charged and uncharged oligonucleotides exhibited an extremely low diffusion rate across liposome membranes, indicating that the polyanionic nature of oligonucleotides is likely not the only factor which impedes their diffusion across membranes. Their results also showed that abolishment of electrostatic charge would not lead to absorption via passive diffusion process. The results of studies using other uncharged conjugated oligonucleotides such as peptide nucleic acids, morpholino compounds, and cholesterol or alkyl conjugated oligonucleotides were consistent with these studies. Modifications increased cell membrane binding of oligonucleotides but it did not necessarily increase intracellular accumulation of oligonucleotides. Moreover, it was reported that their diffusion rates across synthetic lipid bilayers was very low (Wittung et al., 1995; Hughes et al., 1994). Results of these studies using modified oligonucleotides led to the conclusion that cellular uptake of natural and modified oligonucleotides probably does not involve passive diffusion through cell membranes. However, as oligonucleotides are capable of gaining entry into the cell cytoplasm the results implies another mechanism is involved.
1.4. Endocytosis: General Overview

A common mechanism by which larger materials gain access into cells is via endocytosis (Fig. 4). There are two types of endocytosis: pinocytosis and receptor-mediated endocytosis. There are only slight differences between these two processes. In pinocytosis or fluid-phase endocytosis, small invaginations of the cell membrane form around the material to be taken in and entrap extracellular fluid with the material. This results in formation of a vesicle and this pinocytotic vesicle pinches off from the cell surface and enters the intracellular space (Junquiera et al., 1998).

As the name implies, receptor-mediated endocytosis involves cell surface receptors that exhibit high substrate specificity and affinity (Smythe and Warren, 1991). Generally, receptors are either originally widely dispersed over the surface or aggregated in special regions called coated pits. The binding of a ligand to a receptor causes the widely spread receptors to accumulate to one site. Similar to pinocytosis, the cell membrane then forms a vesicle around the receptor-bound ligand. The vesicle containing both ligand and receptor pinches off from the cell membrane into the cytoplasm. The resulting vesicle is called an endosome. Once internalized, the receptors are recycled back to the cell membrane surface (Besterman and Low, 1983). A common fate of endosomes is it fuses with a primary lysosome.
A lysosome contains a mixture of ~ 40 different acid hydrolases in a low pH environment. Among these enzymes are acid phosphatase, ribonuclease, deoxyribonucleases, proteases, sulfatases, lipases and β-glucuronidase. Upon fusion of the endosome with primary lysosomes, a secondary lysosome is formed in which subsequent digestion of intake material takes place (Shepherd, 1989). For example, proteins and complex sugars get digested by the degrading enzymes into smaller units. In the case of DNA and RNA, they get degraded into mononucleotide building blocks.
1.5. Endocytosis and Oligonucleotides

There are several proposed mechanisms of oligonucleotide internalization such as receptor-mediated endocytosis, protein-independent potocytotic (Zamecnik et al., 1994) and porin-mediated (Wu-Pong, 2000) mechanism. One of the well-characterized mechanisms of cellular uptake of oligonucleotides is endocytosis. Some early work suggested that the uptake of phosphodiester oligonucleotide into cells involved endocytosis and not diffusion across the membrane. Studies reported that the uptake characteristic of oligonucleotides closely resemble the typical endocytosis pattern (Juliano and Yoo, 2000). When oligonucleotides were exposed to cells extracellularly, uptake occurred in a saturable manner and the accumulation of oligonucleotide was reduced by lowering the temperature of cells and by treating cells with endocytosis inhibitors such as sodium azide, deoxyglucose and cytochalasin B (Shoji et al., 1991; Loke et al., 1989; Yakubov et al., 1989). Further evidence arrived from electron microscopy studies. All the steps typical of endocytosis were found on the electronmicrograph: absorption of DNA at the membrane surface, formation of deep invaginations of the sites of the DNA binding and formation of internal vesicles containing DNA surrounded by a membrane inside the liposome (Vlassov et al., 1994). In addition, using fluorescein-labeled oligonucleotides they detected accumulations of the labeled oligonucleotides in an acidic environment that was endosomes fused with acidic lysosomes (Tonkinson et al., 1994). These data implied that cellular uptake of oligonucleotide involves endocytosis.
1.6. Oligonucleotide Binding Receptors

More evidence that cellular uptake of oligonucleotides involves endocytosis came when a number of cell surface receptors were shown to bind to oligonucleotides. For receptor-mediated endocytosis of an oligonucleotide to occur efficiently, the oligonucleotide needs to bind to cell surface receptors that exhibit affinity to that oligonucleotide. The binding to cell surface does not always initiate the endocytic pathway but it has been shown to be correlated with cellular uptake.

Numerous oligonucleotide-binding cell surface receptors have been isolated from different cell types which are likely involved in their receptor-mediated endocytosis (De Diesbach et al., 2000; Yakubov et al., 1989; Loke et al., 1989; Rappaport et al., 1995; Beltinger et al., 1995; Wu-Pong et al., 1994; Yao et al., 1996). Currently there are two cell-surface oligomer-binding proteins that have been well-characterized and a host of others which have been identified. Bennett et al. (1998) identified a 30 kDa DNA receptor protein in human hematopoietic cells including neutrophils, monocytes, T and B lymphocytes but not erythrocytes. In the late 1980s, a family of oligonucleotide binding proteins of Mw = 75 - 80 kDa, termed the nucleic acid binding receptor-1 (NABR-1), was discovered (Loke et al., 1989). These proteins were found to be present in HL-60, cos-1, Vero, L-671, mouse fibroblast, hepatocytes, and Chinese hamster ovary cells (Yakubov et al., 1989). In 1993, Chan and co-workers identified several double-stranded DNA-binding proteins in the membrane, nuclear, and cytoplasmic fractions of HUVEC cells. In 1995, Beltinger and co-workers also identified several
phosphorothioate oligonucleotide binding proteins with widely different molecular weights on the surface membranes of K562 cells. For the majority of these cell-surface oligonucleotide binding receptors, lack of follow-up studies and characterization precludes elucidating their possible involvement in endocytosis.

In 1996, Yao et al. (1996) demonstrated that the rate of cellular internalization of oligonucleotides is correlated with protein expression of the oligonucleotide binding receptors (Mw = 100 and 110 kDa) on HepG2 cell membranes. Furthermore, a number of studies have also demonstrated that oligonucleotides bind, sometimes with high affinity, to heparin-binding proteins which are often present on the cell surface. Currently identified oligonucleotide-binding heparin proteins include epidermal growth factors receptors (EGFR), vascular endothelial growth factor receptors (KDR/flk-1), basic and acidic fibroblast growth factors (bFGF & aFGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), as well as to the VEGF receptors (Guvakova et al., 1995; Benimetskaya et al., 1997a). A well-characterized cell surface heparin and oligonucleotide binding receptor, Mac-1 (CD11b/CD18; CR3; αMβ2) is predominantly expressed on neutrophils and natural killer (NK) cells. Up-regulation of this receptor has been reported to increase cellular binding of oligonucleotides and this binding has a positive correlation with the rate of oligonucleotide internalization suggesting a role in the endocytosis of oligonucleotides.
1.7. Delivery Problems and Delivery Strategies

There are several well-recognized problems associated with delivering an oligonucleotide to its target. One key difficulty with oligonucleotide delivery is that oligonucleotides are relatively sensitive to enzymatic degradation. There are a number of sites where oligonucleotides face possible degradation into nucleotides thus hindering therapeutic effects. First of all, in serum, there are numerous nucleases present that may readily degrade oligonucleotides before they reach their intended target cells. Secondly, intracellular entry of oligonucleotides via endocytic pathways exposes the oligonucleotides to numerous degradation enzymes present within lysosomes (Wickstrom, 1986; Temin, 1990; Akhtar et al., 1991). In addition to problems with nuclease stability, as was already stated, cell membranes are impermeable to large polar molecule such as oligonucleotides. Therefore, once they are internalized via endocytosis and are inside an endosome, oligonucleotide must then penetrate yet another layer of membrane, the endosomal membrane, to gain access to cytoplasm. This will also contribute to low delivery efficiency.

In addition to the oligonucleotide modifications mentioned previously, a number of delivery strategies have been developed. The goals of these strategies are 1) to improve cell membrane penetration, 2) to improve protection of the oligonucleotide from extra- and intracellular enzymatic degradation, and 3) to promote release of oligonucleotides from the endosomal compartments into cytoplasm.
a) Liposomes.

Liposomes are composed of an aqueous compartment enclosed within a phospholipid bilayer and they have been utilized as drug carriers since the mid-1970's. When oligonucleotides are mixed with positively charged lipids, there is a spontaneous formation of a complex which occurs by electrostatic interaction. This results in a tightly packed oligonucleotide surrounded by lipids (Gershon et al., 1993). Studies have suggested that a cationic lipoplex (cationic lipid plus oligonucleotides) is a more effective delivery formulation than anionic and neutral liposomes (Alahari et al., 1996; Roh et al., 2000). Due to their positive charge, this delivery system has high affinity for most cellular membranes which are negatively charged under physiological conditions. Once bound to the membrane, similar to naked oligonucleotides, the liposome-oligonucleotide complexes are taken up in cells via endocytosis rather than by direct fusion of lipid-oligonucleotide particles with the plasma membrane (Zelphati et al., 1998; Roh et al., 2000). Once internalized and within the endosomal compartment, lipid mixing between lipid-oligonucleotide particles and the endosomal membranes takes place. This mixing eventually induces both the membranes to fuse and release the oligonucleotides into the cytoplasm. A study supporting this mechanism has shown that lipids which are capable of promoting this inter-membrane mixing are capable of disrupting the endosomal membrane (Bennett et al., 1998). In order to further enhance the disruption of endosomal membrane, helper lipids such as DOPE (Dioleoylphosphatidyl ethanolamine) have been added to the membrane of liposomes. For example, DOPE which is an inverted-cone-shaped lipid, forms non-bilayer phases thus destabilizing the bilayer (Farhood et al., 1995). Another example is a pH sensitive fusogenic liposome. These liposomes consist
of DOPE and a titratable acidic amphiphile such as oleic acid or cholesterylhemisuccinate. At pH 7, the acidic amphiphiles form a bilayer structure with DOPE. However, internalization of the liposome within endosomes results in the protonation of amphiphiles as the endosomes fuse with the acidic lyzosomes. This protonation causes the liposome to collapse and fuse with the endosomal membrane, resulting in oligonucleotide release into cytoplasm (De Oliveria et al., 1998). Unfortunately, cationic lipids have disadvantages such as cytotoxicity and decreased activity in the presence of serum and antibiotics (Zelphati et al., 1998).

b) Protein and Peptide Delivery Vehicles.

Recently, oligonucleotide delivery systems have been developed using proteins and peptides. Fusion of oligonucleotide with protein and peptide increases lipophilicity and hence penetration through cell membranes. Furthermore, this fusion affords oligonucleotides protection against nuclease degradation. In its most common form, this type of delivery system involves conjugation of oligonucleotides with poly-L-lysine (PLL) and cell-specific carrier molecules which are ligands for various cell surface receptors. Numerous specific carrier molecules exist and these include glycoproteins, transferrin, insulin, antibodies, EGF, and folic acids (Wu and Wu, 1988; Wagner et al., 1991; Hucket et al., 1990; Walker et al., 1995; Deshpande et al., 1966, Ginobbi et al., 1997). By conjugating an oligonucleotide with an appropriate carrier molecule, it has been shown that oligonucleotides accumulate in specific target cells and promote receptor-mediated endocytosis of these complexes into the cells. In the case of PNA (Peptide Nucleic Acid) bound antisense oligonucleotides, it has been demonstrated that
when they are conjugated with certain peptides, the efficiency of cellular uptake is greatly enhanced relative to unconjugated PNA; there is a parallel increase in antisense activity. (Aldrian-Herrada et al., 1998; Simmons et al., 1997; Pooga et al., 1998).

c) Dendrimers.

Dendrimers are highly branched three-dimensional macromolecules possessing a well defined molecular weight and a large number of controllable 'peripheral' functionalities. They have a tendency to adopt a globular shape once a certain size is reached. Dendrimers are synthesized via very defined polymerization reactions which result in a monodisperse, reproducible product. They can also be synthesized with several functional groups making them more versatile than other delivery systems. These features have made their application to drug delivery particularly attractive (Esfand and Tomalia, 2001). One commonly studied dendrimer is the polyamidoamine (PAMAM), it possess a hydrocarbon core with charged surface amino groups (Delong et al., 1997; Bielinska et al., 1996). These cationic dendrimers form stable complexes with oligonucleotides under a variety of conditions. Oligonucleotides complexed with these dendrimers have been demonstrated to exhibit enhanced cellular uptake, increased intracellular availability, enhanced nuclear translocation as well as increased protection against metabolic degradation in serum and in lysosomes (Haensler and Szoka, 1993)
1.8. Recent Interest in Higher-order Structure DNA

In recent antisense research, emphasis has also been placed on antisense oligonucleotides containing a G-quartet (Fig. 5). One of the early reports concerning G-quartet containing oligomers was by Pearson and his coworkers in 1993. They studied a number of oligonucleotides that were effective macrophage scavenger receptor inhibitors. These oligonucleotides were found to contain G-quartets that bind with high affinity to the macrophage scavenger receptor. Denaturation of the G-quartet was found to deactivate the macrophage inhibitory effects.

Burgess et al. (1995) examined the inhibition of smooth muscle cell proliferation by antisense oligonucleotide targeted to c-myb and noted that the majority of activity was
a function of this antisense sequence element. Bishop and co-workers in 1996 demonstrated that G-quartet forming oligonucleotides were relatively specific inhibitors of HIV-1 integrase. More importantly, they observed efficient cellular uptake only with G-quartet forming antisense oligonucleotide compared to the non G-quartet forming oligonucleotides with the same sequence suggesting the importance of this structure on antisense activity and cellular uptake. More recently, Benimetskaya et al. (1997)a have shown that antisense RelA oligonucleotides form a G-quartet structure near their 5’-termini and antisense activity is abolished when G-quartet formation was prevented by substitution of 7-deazaguanosine for guanosine at several positions, suggesting the importance of the G-quartet on antisense activity. However, it is still not clear whether the enhanced antisense activity of the G-quartets is due to increased DNA binding or due to an enhanced cellular delivery and uptake.

1.9. DNA Frayed Wires

DNA can form many structures other than the well-known Watson-Crick base paired, right-handed, anti-parallel double helix. Examples include triple-stranded DNA and parallel stranded duplexes. Studies have shown that guanines can form self-complementary structures via hydrogen bonding to yield guanine-guanine base pairs (Gellert et al., 1962; Panyutin et al., 1989), and that guanine-rich sequences form four-stranded tetraplexes. These structures have been implicated in a variety of biological roles such as the function of chromosome telomeres (Blackburn, 1991), site-specific recombination of immunoglobulin genes (Sen and Gilbert, 1988) and the dimerization of the human immunodeficiency virus RNA genome (Sundquist and Heaphy, 1993).
Studies have shown that the main structural motif of the guanine tetraplex is a planar G-quartet composed of four Hoogsteen-base paired guanines arranged in a cyclical array (Laughlan et al., 1994) and that these tetraplexes are stabilized by a cation positioned in a cavity between two planes of the G-quartet (Hardin et al., 1992). There have been several reports of high-molecular weight oligonucleotide complexes formed by guanine-rich sequences. It was reported that d(T9G3) and d(T12G3), in the presence of potassium cations, form complexes of 4-, 8-, 12- or 16 self-assembled strands (Sen and Gilbert, 1992). Marsh and Henderson (1994) have reported a rigid linear polymer called a G-wire, which arises from spontaneous self-assembly of d(G4T2G4) in the presence of magnesium or sodium ions. Dai et al. (1995) studied the self-association of oligonucleotides that contain one or two runs of four consecutive guanines (G4) at their 3' end and found only those with two consecutive guanine runs are capable of forming high molecular weight species, whereas oligonucleotides with a single G4 run form duplex hairpins or four stranded complexes.

Similar to the high molecular weight structures mentioned above, DNA Frayed Wires (FW) arise from guanine-rich oligonucleotides. Protozanova and Macgregor (1996) first reported that guanine-rich sequences d(A15G13), in the presence of cations, self-assemble to form high-molecular weight species. When this structure is resolved by denaturing gel electrophoresis, a ladder of bands appears. When the logarithm of mobilities of the bands is plotted against the band number, one obtains a straight line with a correlation coefficient greater than 0.99. This indicates the bands were similar
structures with respect to shape, charge density and flexibility suggesting the polymerization of FW occurs by the successive addition of a parent \( d(A_{15}G_{15}) \) strand. The authors reported that the most extensive polymerization was observed in presence of magnesium cations. Although monovalent cations such as \( \text{Na}^+ \), \( \text{K}^+ \) and \( \text{NH}_4^+ \) promote formation of complexes containing four, five and six strands of \( d(A_{15}G_{15}) \), they do not lead to the formation of higher-molecular weight species like divalent magnesium cations. Protozanova and Macgregor (1996) proposed that the guanine-runs of parent strands self-associate into an aggregate called a "stem" whereas adenine runs would stay single stranded and span out from the guanine aggregate forming "arms" (Fig. 6) and the arms can bind to their complementary strands. These structures were named DNA Frayed Wires.

![Diagram of DNA Frayed Wire](image)

**Figure 6: Proposed structure of DNA Frayed Wire:** \( G = \) guanine, \( A = \) adenine.
One of the most important characteristics of FW is their remarkable stability. It was first reported that FW are resistant to standard denaturation conditions, 50% formamide heated to 90°C for 2 minutes followed by electrophoresis in 7 M urea at 55°C. They also exhibit resistance towards enzymatic degradation by Mung bean nuclease, a single-strand specific endonuclease (Protozonova and Macgregor, 1996). In a subsequent study by Poon and Macgregor (1999), the stability of DNA Frayed Wires was compared to G-quartet containing complexes. They reported that FW exhibited higher resistance against digestion by a number of nucleases such as DNAse I, Mung Bean nuclease, S1 nuclease and exonuclease III relative to the complexes containing G-quartets.

In their original publication, Protozanova and Macgregor (1996) suggested that this self-assembled guanine aggregate would likely contain G-tetrads as the main structural motif, similar to previously reported self-complexed guanine aggregates. However, subsequent structural studies were inconsistent with this idea. Poon and Macgregor (1998) studied different guanine-rich sequences, one of which had been previously shown to form G-quartets, d(T15G4T4G4) and another one was d(T15G12) which was shown to readily form FW. Results showed that the guanines of the G-quartet containing complex formed by d(T15G4T4G4) were protected from methylation at the N-7 site whereas all guanines of FW were susceptible to methylation. Also, when d(T15G12) and d(T15G4T4G4) were methylated prior to polymerization, the methylation at N-7 site of guanine prevented formation of G-quartets whereas it had no effect on formation of FW. These results suggested that the guanine-guanine interaction in FW does not involve the
N-7 site of guanines and thus is different from the guanine-guanine interaction in G-quartet.

Further studies using circular dichroism (CD) spectroscopy (Protozanova and Macgregor, 1998a) reported that subtracting the CD spectrum of d(A₁₅) from that of FW formed by d(A₁₅G₁₅) results in a spectrum similar to poly(G). Subtracting the CD spectrum of d(A₁₅):d(T₁₅) from that of DNA Frayed Wire:d(T₁₅) showed that the spectrum of guanines of DNA Frayed-wires were not significantly altered and the helix transition temperatures were found to be identical. The CD spectrum of FW exhibited temperature independence indicating thermal stability of this structure. Therefore results of these studies provide evidence that supported the proposed structure, that the arms of FW are available for Watson-Crick base paring with a complementary strands which have no effect on the conformation of guanine aggregates and that FW exhibit thermal stability.

The two different structural domains, a stem and arms, of FW are independent according to previous reports, however the length of each domain plays an important role in the stability of the complex. Protozanova et al. (1999) studied the effect of the length of consecutive guanine-runs on the formation of the FW using oligonucleotides with six guanines and 10-15 guanines. They reported, oligonucleotides with six consecutive guanines were only able to form 4- and 8-stranded complexes without further polymerization; however, increasing the number of guanines to 10 - 15 leads to polymerization resulting in a ladder pattern of up to 9 bands and an intense top band at
the gel. It was suggested that the relative population of any given species in a FW sample is determined by the length of guanines in the parent strands (Protozanova et al., 1999). In a subsequent study using d(T15Gn) where n = 4 - 15 to assemble FW, it was reported that oligonucleotides with smaller numbers of contiguous guanines (n = 5 - 8) form primarily four-stranded tetraplexes whereas oligonucleotides with larger numbers of contiguous guanines assemble into FW structures (Poon and Macgregor, 2000). By varying the length of the non-guanine tract, they found solubility of FW increases with length of the arms and that the electrophoretic motion of the FW is primarily determined by the length of the arm (Protozanova and Macgregor, 1998a; Protozanova and Macgregor, 1998b).
CHAPTER 2: RATIONALE

2.1. Rationale

As discussed in the introduction, there are number of problems concerning the use of oligonucleotides in therapy. These include a lack of i) cellular uptake, ii) enzymatic stability, and iii) nuclear translocation. In particular, enzymatic stability and transport across biological barriers are two key difficulties (Vlassov et al., 1994). In terms of enzymatic stability, initial exposure of oligonucleotides to human serum results in rapid degradation by nucleases. They rapidly degrade them into simple nucleotides or cleave them in a way that the oligonucleotides no longer contain correct nucleotide sequences to exert their therapeutic effect (Wickstrom, 1986; Temin, 1990; Akhtar et al., 1991).

Secondly, it is widely accepted idea that oligonucleotides gain access to cytoplasm via endocytosis by forming an endosome (Budker et al., 1987). Thus, an oligonucleotide within an endosome not only needs to penetrate the endosome membrane again (Stein, 1999) but also needs to withstand degrading enzymes that may be introduced by lysosome.

In a therapeutic setting, an ideal therapeutic oligonucleotide would be highly resistant to nucleases and have high cellular uptake efficiency. These two requirements have lead to the development of a number of modified oligonucleotides such as phosphorothioate, methylphosphonate and peptide nucleic acids to improve resistance cleavages of the against phosphodiester linkage (Miller et al., 1981; Nielson et al., 1991;
Taylor et al., 1996). In addition, several oligonucleotide delivery strategies such as liposomes, peptide and protein delivery vehicles, and dendrimers have been (Lebedeva et al., 2000) developed to further enhance stability and, more importantly, to improve cellular delivery to therapeutic targets.

DNA Frayed Wires have important characteristics that may be advantageous to the therapeutic use of oligonucleotides. Previous studies from our laboratory have demonstrated that in comparison with other normal oligonucleotides, FW exhibit unusual stability. They were stable in standard denaturing conditions and they exhibited resistance to degradation by nucleases (Protozanova and Macgregor, 1996; Poon and Macgregor, 1999). Bishop and co-workers (1996) reported G-quartet containing anti-HIV oligonucleotides have higher serum nuclease resistance compared to non-G-quartet forming oligonucleotides and it was shown by Poon and Macgregor (1999) that FW are more nuclease resistant than G-quartet forming oligonucleotides. Thus these results indicated that FW might be highly nuclease resistant in vivo. The arms readily bind to complementary strands via Watson-Crick base pairing without disrupting the stems or compromising nuclease resistance (Protozanova and Macgregor, 1996; Protozanova and Macgregor 1998). This indicates that complementary strands, containing therapeutic sequences and/or attached to drugs, may be bound to the arms and be transported into cells, avoiding enzymatic degradation. Furthermore, the large number of arms per FW means that it may be able to carry multiple strands at once thus making this therapy more potent.
As mentioned previously, the notion that oligonucleotides are internalized into cells via endocytosis has been well supported by evidence such as identification of numerous oligonucleotide binding receptors, visual detection of oligonucleotides in endocytic steps and inhibition of uptake by endocytosis inhibitors (Vlassov et al., 1994; Beltinger et al., 1995; Deshpande et al., 1996; De Diesbach et al., 2000). Studies reported that G-quartet containing guanine-rich strands bind to a number of heparin-binding cell surface receptors with greater affinity than other oligonucleotides. The effect is enhanced when the G-quartet is located at either 5'- or 3'- terminus (Guvakova et al., 1995; Benimetskaya et al., 1997a). Recently, Bishop and co-workers (1996) have shown that HeLa cells display higher uptake for G-quartet containing oligonucleotides than non-G-quartet forming oligonucleotides.

The previous work on the uptake of G-rich oligonucleotides (Rando et al., 1995; Bishop et al., 1996; Bates et al., 1999) led us to predict that FW would show enhanced cellular stability and enhanced cellular uptake via endocytosis. Because FW contain a guanine stem at either the 5'- or 3'- terminus, they would be expected to bind to heparin-binding cell receptors with greater affinity (Benimetskaya et al., 1997a) and internalized via receptor-mediated endocytosis resulting in, overall, higher cellular uptake than normal DNA.

There have been many extensive studies on the structure of FW but absolutely no work has been done with cells. As described earlier, there have been a number of cellular studies of G-quartet containing oligonucleotides where their uptake and stability were
characterized but there has been no attempt to study the guanine-rich FW. Therefore in order to investigate the potential clinical usefulness of FW, we examined FW uptake and stability compared to normal DNA in human cell lines.
CHAPTER 3: HYPOTHESIS

3.1. Hypothesis

1) DNA Frayed Wires will show cellular uptake in different cell lines and the uptake pattern will be different from normal DNA.

In order to compare the cellular uptake of single stranded DNA (ssDNA) and DNA Frayed Wires (FW), the same concentration of radio-labeled DNA Frayed-wires and random sequenced DNA will be incubated with human hepatic and nasopharyngeal tumor cell lines over a period of time up to two hours. Then the total intracellular accumulation of DNA at each incubation time will be measured to determine the uptake patterns of both DNA species over time. Cellular uptake pattern of ssDNA and FW will be plotted against time and compared to each other. These experiments will be performed in two different cell lines to examine whether transport processes are cell specific for each of these DNA structures.

Studies have already shown that oligonucleotides are taken up by cells via endocytosis. Several high-molecular weight G-quartet containing antisense oligonucleotides (Rando et al., 1995; Bishop et al., 1996; Bates et al., 1999) have been shown to be readily internalized and exert biological activity. Perhaps, more importantly, Bishop et al. (1996) demonstrated cellular uptake of G-quartet containing oligonucleotides is non-sequence specific and possess greater cellular uptake than non-G-quartet forming oligonucleotides. This has been attributed to heparin-binding proteins
which mediate endocytosis and exhibit a higher affinity towards G-quartets especially when the G-quartet is located at either end of the oligonucleotides. Because FW contain a guanine stem at either the 5'– or 3'– terminus, we hypothesized that these structures would exhibit a high affinity to these heparin-binding cell receptors (Benimetskaya et al., 1997a). Hence interactions with these proteins would enable FW to be internalized via receptor-mediated endocytosis resulting in, overall, higher cellular uptake than normal DNA.

2) The cellular uptake of FW will occur via active transport mechanisms. This will likely involve heparin-binding receptor-mediated endocytosis. Thus cellular uptake of FW will likely be non-sequence specific, concentration dependent, temperature dependent and inhibitable by specific inhibitors of endocytosis and/or active transport.

As active transport processes are generally retarded at lower temperatures and enhanced at higher biological temperatures, we examined the temperature dependence of cellular uptake. The incubation temperature for both FW and ssDNA will be reduced to 4°C to see if the process is suppressed. Then cellular uptake of FW will be examined at 4, 25, and 37°C to see if the uptake is dependent on the temperature of cells. To examine the concentration dependence of this uptake, cells will be incubated with 5 different concentrations of FW (0.1 to 0.5 μM) for 30 minutes and 120 minutes to look for differences in overall intracellular accumulation at different concentrations. If these studies demonstrate differences in initial uptake rates, cells will be incubated for time
periods of less than 15 minutes to estimate the concentration dependence of the initial uptake rate. To further support the notion that cellular uptake of oligonucleotides occurs via active process and is inhibitable, cells will be pre-treated with several metabolic poisons which have been shown to inhibit endocytosis. The possible involvement of heparin-binding cell surface receptors will be determined by examining cellular uptake in cells pre-incubated with different concentrations of heparin. Also, in order to examine the dependence of uptake on sequence, cellular uptake studies of FW will be performed using different parent strands.

Studies have shown that the uptake of oligonucleotides occurs via active processes involving receptor-mediated endocytosis. It is thought that cellular uptake of FW would exhibit similar uptake characteristics to that of other oligonucleotides such that it will demonstrate concentration and temperature dependence and also inhibited by metabolic poisons (Goodchild et al., 1988; Loke et al., 1989; Yakubov et al., 1989; Shoji et al., 1991). As suggested for the uptake of G-quartet containing oligonucleotides (Benimetskaya et al., 1997a), uptake of FW will be triggered by high affinity binding to heparin-binding cell surface receptors thus the putative involvement of heparin-binding cell receptors in this uptake, implies that pre-occupying heparin binding cell surface receptors with heparins should decrease overall cellular accumulation of FW. Also, a study by Pearson et al. (1993) demonstrated that G-quartet binding to macrophage scavenger receptors was due to electrostatic interactions thus it is likely that FW could also bind to the receptors via electrostatic interactions which will cause FW derived from different parent strands to have similar cellular uptake pattern.
3) Due to unusually high thermal and nuclease stability in vitro, FW will demonstrate a higher stability to metabolic degradation in cells than normal DNA.

In order to examine the stability of FW after exposure to cells, the laddering patterns of FW will be compared in several samples. These samples are; 1) Stock - never exposed to cells, 2) Extracellular – the extracellular mixture after incubation and 3) Intracellular – cell lysates collected after washing the cells with buffer. In case of the intracellular samples, the lysates will be purified by phenol extraction and then concentrated by ethanol precipitation. Formamide will be added to the samples then samples will be heated at 90°C for 5 minutes then the samples will be resolved on denaturing 10% PAGE for visualization.

As characterized by previous studies, FW exhibit unusually high resistance to chemical, thermal and enzymatic degradations (Protozanova and Macgregor, 1996; Poon and Macgregor, 1999). Interestingly, Bishop and co-workers in 1996 already demonstrated that G-quartet motif containing anti-HIV oligonucleotides exhibited higher serum nuclease resistance in comparison to a non-G-quartet forming sequence variant. They also reported the detection of intact G-quartet structures after internalization. Previous work from our laboratory has already demonstrated that FW are indeed more stable and more nuclease resistant than G-quartet containing complex (Poon and Macgregor, 1999). Therefore, it is reasonable to hypothesize that FW will exhibit limited degradation after exposure to cellular enzymes and maintain their intact structure once internalized by cells.
CHAPTER 4: MATERIALS AND METHODS

4.1. Oligonucleotide Preparation

Oligonucleotides used in the experiments were purchased from ACGT Inc, Toronto. Using a computer sequence selecting program (designed in house) to minimize secondary structure, we selected an oligonucleotide that does not form self-complexed secondary structure as the control oligonucleotides. For FW forming strands, we chose strands containing 15 consecutive guanines because a previous study showed that 10 or more guanines are required to form stable FW (Protozanova et al., 1999). The length of arms are kept as 15 nucleotide long because the arms shorter than 5 nucleotides long have been shown to easily precipitate (Protozanova and Macgregor, 1998a). In order to ensure we form stable FW without precipitation problem, all FW forming strands had 15 consecutive guanines with 15 nucleotide long arms. The previous physicochemical studies of FW were done with the similar sequence thus FW from this sequence are well-characterized. For control, the length was chosen to be 30 nucleotide long because we are using the same length parent strands to form FW. The sequence of this random sequence oligonucleotide was:

5' - ACT/ TAG/ GCA/ CCA/ AGT/ ACT/ CTT/ TAC/ GTA/ TAA - 3'.

The parent DNA Frayed-wire forming oligonucleotides were:

d(A15G15)

5' - AAA/ AAA/ AAA/ AAA/ AAA/ GGG/ GGG/ GGG/ GGG/ GGG - 3'
d(T_{15}G_{15})
5'-TTT/TTT/TTT/TTT/TTT/GGG/GGG/GGG/GGG/GGG-3'
d(N_{15}G_{15}) , N= random sequence
5'-ATA/~T/GAG/TTA/TAT/GGG/GGG/GGG/GGG-3'
d(G_{15}A_{15})
5'-GGG/GGG/GGG/GGG/GGG/AAA/AAA/AAA/AAA/AAA-3'

Upon receipt, lyophilized oligonucleotides were dissolved in 200 μL of deionized water. The concentration of the oligonucleotide in the resulting solution was measured by spectrophotometer at 260 nm. A stock solution of the oligonucleotide at a concentration of 100 μM strands was prepared and further dilutions were made when necessary. The stock solutions were stored at -20°C until use.

4.2. Oligonucleotide Labeling and DNA Frayed Wire Formation

Oligonucleotides were 5'-end-labeled using [γ-\textsuperscript{32}P] ATP with T4 polynucleotide kinase at 37°C for 35 minutes. The enzymatic reaction was terminated by incubating the samples in a 65°C waterbath for 10 minutes. Unincorporated \textsuperscript{32}P was then removed from samples using P-6 Bio-Spin Gel Chromatography columns (Bio-Rad, Inc.). The purified samples were in a buffer containing 10 mM Tris-HCl and 0.02% sodium azide.

The DNA Frayed Wire forming reaction buffer containing 50 mM Tris-HCl and 10 mM MgCl\textsubscript{2} was added to radiolabeled oligonucleotides then vortexed for a few seconds. This reaction mixture was then incubated at 80°C for 30 minutes and left
overnight at room temperature. For control DNA, the same amount of the reaction buffer was added to make the conditions equal but the sample was not heated. Both FW and control DNA were stored at -20°C until use.

4.3. Cells

The two human cell lines used in these experiments were HepG2 (hepatoma cell line) and CNE1 (cranial nasopharyngeal carcinoma). The cells were routinely maintained by serial culture in alpha-Minimum Essential Media from Life Technologies, Inc. (Burlington) supplemented with 10% Fetal Bovine Serum (FBS) for HepG2 cell culture medium. For CNE1 cells, additional 1% Penicillin-Streptomycin was added to the culture medium. The cells were incubated at 37°C in 5% CO₂/95% O₂ atmosphere. HepG2 cell line was chosen because hepatocytes are considered the principle cells of metabolism cells in the body with number of functions including detoxification and deactivation of various drug and substances. They express many metabolic enzymes and are phenotypically similar to liver. CNE1 cell line was chosen due to the request of our financial supporter, who was interested in using FW as possible therapeutic agent carrier in nasal pharyngeal route.

4.4. Tissue Culture

Using a light microscope, the confluency of the cells in the tissue culture flasks was checked. Once the cells were confluent, they were washed using 1x Phosphate Buffered Saline (PBS) then detached from the flasks using 1x Trypsin-EDTA, purchased
from Life Technologies Inc. (Rockville, MD). After cells were detached completely, the enzymatic reaction of trypsin was terminated by adding cell culture media to the cell line.
The detached cells were pipetted out, collected in a 50 mL tube and spun at 1000 x g for 10 minutes. After centrifugation, the pellet of cells from one flask was reconstituted with 45 mL of cell culture media in order to maintain equal amounts of cells per well. Once homogenized, cells were plated out (0.274 ± 0.013 µg/µL protein) in 24-well culture dishes from Corning Inc. (NY). The dimensions of each well are: diameter = 15.6 mm, growth area = 1.9 cm². Upon plating, culture dishes were shaken lightly to make the cell distribution on the plating surface uniform and plates were checked under the microscope. Cells were incubated at 37°C in 5% CO₂/95% O₂ atmosphere for approximately 48 hours. On the day of the experiments, each well was carefully examined under the microscope to determine its confluency and look for any detachment of cells. Wells with any sign of cell detachment were not used in the experiments. The protein concentration of representative wells was determined using the Bio Rad protein quantitation.

*All experiments were repeated on separate days with similar findings unless otherwise stated. For uptake studies using time points, non-specific binding values were determined at 0 minutes and subtracted from subsequent uptake values.*
4.5. Time Course of Cellular Uptake

HepG2 and CNE1 cells were incubated with FW and ssDNA at 25°C over different periods of time to examine their cellular uptake over time. After incubation of cells and the oligonucleotides, cell media of each well was aspirated and 1 mL of 1 x PBS was added. The buffer was aspirated off and 150 μL of the reaction mixture was added immediately to each well and incubated for the desired amount of time. It is important to note that throughout the experiments, the washing buffer and the reaction mixture were maintained at the same temperature as the cell plates.

At the end of the incubation, the reaction mixture (FW in 1 x PBS) from each well was removed by pipetting and collected into microcentrifuge tubes (extracellular sample). Then the cells were carefully washed twice with 500 μL of ice-cold 1 x PBS. When adding ice-cold buffer to the well, pouring directly on to the cells was avoided to prevent cell detachment. After aspiration of the second wash buffer, 200 μL of 1% Triton X-100 (t-octylphenoxypoly-ethoxyethanol) was added to each well and incubated at room temperature for one hour to lyse cells. The wells were scraped and the lysed cells were collected in microcentrifuge tubes. Triton X-100, Sigma Chemical, Co. was purchased and diluted to 1% by 1 x PBS. It is important to note that while collecting samples for one time point, the wells for other time points were disturbed as little as possible by minimizing unnecessary shaking of the cell plates. Extra- and intracellular samples were vortexed vigorously for 5 minutes and 10 μL from each sample was added to a scintillation counter vial containing 5 mL of Cytoscint scintillation fluid (ICN Pharmaceutical, Inc). The vials were vortexed for 5 seconds and then counted in a
Beckmann LT5000 Scintillation Counter to measure their radioactivity as CPM (Counts Per Minute).

4.6. Temperature Dependence Studies

CNE1 cells (0.274 ± 0.013 µg/µL protein) were incubated with FW or ssDNA at temperatures of 4 ~ 37°C to examine the effect of temperature on cellular uptake. Similar to the time-course experiments (4.1 and 4.2), the cellular uptake of both types of DNA was examined as a function of time (0, 30, 60 and 120 minutes). Radio-labeled oligonucleotides were diluted with 1 x PBS so that a concentration of 0.3 µM strands constituted the ‘the reaction mixture’. Prior to the experiment, the reaction mixture, the cell culture dishes and washing buffer were all incubated at desired temperature (4, 25 or 37°C) for 20 minutes to ensure the cells had reached the appropriate temperature.

For the temperature dependency study, the cells were plated in such a way that each set of wells per time point were separated by at least one well from the wells used for another time point. This is important because after incubation, the cells are washed using ice-cold 1 x PBS buffer, in case of 25°C and 37°C incubation, this procedure will likely lower the temperature of the well next to the ones being washed and if the cells are still under incubation, it would likely change its incubation temperature.

For the 4°C incubation, the reaction mixture and the cell plates were incubated on ice within a Styrofoam box with a lid. During the uptake procedures, the cell plates, the washing buffer and the reaction mixture were all kept on ice to reduce temperature
fluctuation. Contact with hands was also kept to minimum in order to decrease heat transfer.

For the 25°C incubation, washing buffer, cell plates and the reaction mixture were kept at 25°C for 20 minutes to equalize the temperature. As with the 4°C experiments, touching the bottom of the cell culture plate was also avoided.

For the 37°C incubation, a 37°C water bath was used for incubating the reaction mixture, cell plates and wash buffer. It was ensured that the bottom of the cell plates was in contact with the warm water. During the uptake procedure, everything was kept in the water bath.

4.7. Concentration Dependence Studies

For these experiments, 5'-end labeled d(A15G15) were used to form FW. The stock solution of FW were diluted with 1 x PBS to form reaction mixtures of [0.1, 0.2, 0.3, 0.4 and 0.5] μM FW. CNE1 cells (n = 5 wells / concentration) were incubated with these concentrations of FW for 30 or 120 minutes at 25°C. After the incubation, cells were washed twice with ice-cold 1 x PBS and lysed using 1% Triton X-100. The final intracellular accumulation of radiolabeled FW was measured by the scintillation counter.

In order to determine initial uptake rates, CNE1 cells (n = 1 well / time point) were incubated with these concentrations (0.1, 0.2, 0.3, 0.4 or 0.5 μM) for 1 to 15 minutes incubation times. The incubation times were: 1, 2, 3, 5, 7, 10 and 15 minutes.
The accumulation at each time point was measured and plotted (CPM/μg/μL Vs. Time) to obtain the initial cellular uptake pattern at each concentration.

### 4.8. Earlier Time Intracellular Accumulation Study

In order to examine difference in cellular uptake of FW and ssDNA before 30 minutes, CNE1 cells (n = 5 wells / time point) were incubated with 0.3 μM FW or ssDNA at 25°C for 5, 10, 15, 30 or 60 minutes. All other procedures were the same as regular cellular uptake described above (4.5). The samples were collected in the same manner as the previous DNA Frayed-wires uptake study in CNE1 cells at 25°C.

### 4.9. Heparin Effect

Heparin (from porcine intestinal mucosa) (Sigma Chemical, Co.) was reconstituted with 1 x PBS to a concentration of 10 Units/μL and stored at -4°C. Prior to this study, aliquots of the Heparin solution were warmed to 25°C and diluted to 0.1, 1.0, 5.0 or 10.0 Units/μL of Heparin with 1 x PBS. The cell plates and 1xPBS wash buffer were incubated to adjust their temperatures to 25°C.

At the start of experiment, the CNE1 cells in each well were washed using 1 mL of 1 x PBS. The cells (n = 5 wells / concentration) were then incubated with 100 μL of different concentrations of heparin for 20 minutes. At the end of incubation, the heparin mixture was aspirated. Without washing, either 150 μL of FW (0.3 μM) or control single
stranded oligonucleotide (0.3 μM) were added to the cells and incubated for one hour at 25°C. Samples were collected and analyzed as previously described.

4.10. Effects of Metabolic Inhibitors

The metabolic inhibitors, sodium azide (NaN₃) and deoxyglucose (2-deoxy-D-glucose), were purchased from Sigma Chemical Co. All inhibitors were dissolved in 1 x PBS to appropriate concentrations and stored at 4°C until use. Seven different inhibition conditions were utilized for these experiments. They are: (1) control, no inhibitor added, (2) 50 mM NaN₃, (3) 50 mM NaN₃ and 0.1 U/μL heparin, (4) 50 mM NaN₃ and 10 mM Deoxyglucose, (5) 10 mM deoxyglucose, (6) 10 mM deoxyglucose and 0.1 U/μL heparin, (7) 0.1 U/μL heparin. It is important to note that the concentration of NaN₃ was chosen because preliminary study using 5, 10, 30 mM have failed.

After aspiration of cell media from the wells, CNE1 cells were washed once with 1 mL of 1 x PBS (25°C), aspirated and 100 μL of each mixture was added to each of 5 wells and incubated at 25°C for 20 minutes before being aspirated off. Then 150 μL of 0.3 μM ³²P labeled FW in 1 x PBS was added and left to incubate for 60 minutes. At the end of incubation, the FW mixture was aspirated and collected. The cells were washed twice with 1 mL of ice-cold 1 x PBS and lysed with 200 μL of 1%-Triton X-100. The radioactivity of the cell lysates was measured using the scintillation counter.

4.11. Cell Viability Test

Cell viability tests using 0.4% Trypan Blue Solution (Sigma Chemical Co.) were determined following treatment with FW, ssDNA or NaN₃. For 60 minutes, cells (n = 6
wells / treatment) were incubated with (1) 0.3 μM FW, (2) 0.3 μM ssDNA, and (3) Reaction mixture buffer only. Another set of cells (n = 6) was incubated with 50 mM NaN₃ for 20 minutes. Cells were placed on a hemacytometer (Hausser Scientific) after mixing with Trypan Blue solution and cells within area of 1 mm² were counted and recorded.

\[
\text{Cell viability (\%)} = \frac{(\text{Number of live cells in the area})}{(\text{Total number of cells in the area})} \times 100 \%
\]

4.12. Intracellular DNA Frayed Wires

Initial attempts to visualize intracellular DNA from cell lysate samples failed. The method used to obtain intracellular FW for denaturing PAGE was slightly different from the procedure given above because after numerous trials, we recognized that the 1% Triton X-100 was hindering phenol extraction. In order to obtain a maximum amount of FW for better visualization, instead of using 24-wells to collect the samples, one confluent tissue culture flask was used. The cells were cultured, washed and incubated with 0.3 μM FW or ssDNA in PBS at 25°C.

At the end of the incubation period, the extracellular media was aspirated and collected as 'External samples'. The cells were then washed twice with 10 mL of ice-cold 1 x PBS being careful not to disrupt or detach cells. The washing buffer was aspirated and then 4 mL of 1 x Trypsin-EDTA (Life Technologies) was added to detach the cells from the wells without rupturing them. The cells were incubated with Trypsin
for 10 minutes at 37°C and the sides of the cell culture flasks were gently tapped to enhance cell detachment.

Once cells were detached, 1 mL of sterile water was added to each flask then the trypsin mixture containing detached cells plus water were pipetted out. The mixture was then placed in a cryogenic vial and tightly sealed. The vial was then submerged into liquid nitrogen for approximately 30 seconds to ensure it was completely frozen. The vial was taken out and immediately submerged in a 80°C water bath. Once the mixture was completely thawed, the vial was vortexed vigorously for one minute then submerged into liquid nitrogen again. This freeze-thaw procedure was repeated a total of five times to ensure complete lysis of the cell membranes.

The cell mixture was poured into a 50 mL tube from the cryogenic vial. In order to extract DNA from cellular proteins, an equal amount of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma Chemical Co.) was added and vortexed for one minute. The tube was centrifuged at 1000 x g for 5 minutes to separate it into two phases. The top phase was collected into a new tube. The interface was carefully avoided. In order to remove all of the phenol from the sample, an amount of diethyl ether equal to three times the volume collected was added to the top phase. This mixture was vortexed for one minute and then centrifuged for 5 minutes. After centrifugation, the diethyl ether layer was discarded and the bottom phase was air-dried to evaporate remaining ether from the solution. To precipitate DNA from the solution, 0.1 times volumes of 3 M sodium acetate was added with 1 μL of glycogen (20 μg/μL) (MBI Fermentas, Inc.) to aid
precipitation. Then ice-cold 100% ethanol was added and the solution was kept at -80°C overnight. The sample was centrifuged at 14000 x g for 20 minutes to obtain a DNA pellet. After the supernatant was removed, the pellet was carefully washed using 70% ethanol, dried under vacuum and stored at -20°C.

4.13. **Polyacrylamide Gel Electrophoresis (PAGE) Analysis.**

For structural analysis of oligonucleotides, both 10% denaturing and native polyacrylamide gels were used. Loading buffer consisted of bromophenol blue in formamide (Sigma Chemical Co.) for denaturing gels and bromophenol blue in glycerol in native gels. 1 x TBE (Tris-Borate-EDTA) was used as running buffer. All the chemicals were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA)

For denaturing PAGE, 10% polyacrylamide (acrylamide : bisacrylamide = 19 : 1) gel containing 7 M urea was heated to 50°C. Prior to loading, the sample in formamide were heated to 95°C for 3 minutes and then run at 55 W for 45 minutes. For native PAGE, the samples were ran on 10% polyacrylamide gel at 150 volts. It is important to note that unless stated otherwise, each lane of the gels is loaded similar amount of radioactivity, total amount of radioactivity going into one lane was estimated using a Geiger counter.

After electrophoresis, the gels were dried under vacuum at 80°C using Bio-Rad Model 583 Gel Dryer. The dried gels were scanned and bands were visualized using an Ambis model 4000 Radioanalytic Imaging System (Scanalytics, Inc., Bellerica, MA).
**4.14. Uptake Pattern of Different DNA Frayed Wires**

Different sequences of oligonucleotides were purchased from ACGT Inc. The sequences used in this study were:

1) \( d(A_{15}G_{15}) \)
2) \( d(T_{15}G_{15}) \)
3) \( d(N_{15}G_{15}) \) where \( N = 5'\)-ATA/CTT/GAG/TTA/TAT-3' \)
4) \( d(G_{15}A_{15}) \)

Each of these oligonucleotides have been previously shown to form FW. These oligonucleotides were 5'-end-labeled using \([\gamma^{32P}]\) ATP with T4 polynucleotide kinase at 37°C for 35 minutes. As previously described, after labeling, the oligonucleotides were purified on the spin columns (Bio-Rad Laboratories, Hercules) and formation of DNA Frayed-wire was carried out in a reaction buffer containing 50 mM Tris-HCl and 10 mM MgCl₂. The reaction mixtures were heated to 80°C then cooled to room temperature overnight.

Cellular uptake of these structures was examined in CNE1 cells. The cells were subcultured and plated out in the same manner as the previous uptake studies. In this uptake, CNE1 cells (\( n = 3 \) wells / time point) were incubated at 25°C with different FW (0.3 \( \mu M \)) for 0, 15, 30 or 60 minutes. At the end of incubation, the extra- and intra-cellular samples were collected and counted in the same manner as described earlier.
4.15. Intact DNA Frayed Wires Vs. Cleaved DNA Frayed Wires

In order to test whether uptake of cleaved $^{32}\text{P}$-labeled arms from the FW contributed to our FW uptake, we examined cellular uptake of cleaved FW and intact FW. Using d(A$_{15}$G$_{15}$), FW were formed in the same manner as previous DNA Frayed-wire formation in earlier sections. From this, half of the FW samples were incubated with 5 μL of DNase I for 2 hours at 37°C. DNase I (127 U/μL, MBI Fermentas Inc). After incubation, an additional 5 μL of DNase I was added to this mixture and incubated at the same temperature for 24 hours. The other half of the DNA samples was incubated under the same conditions for the same amount of time, without DNase I.

Prior to cellular uptake studies, cleavage of FW was confirmed on native 10% PAGE and compared to intact FW. Once complete degradation of DNase I treated FW sample was observed, CNE1 cells were incubated with either Intact or Cleaved FW for 15, 30 and 60 minutes at 25°C. Final intracellular accumulation at each time point was measured and plotted against time.

4.16. Data Analysis

The intracellular accumulations of DNA were determined as percent uptake, i.e. the fraction of the oligonucleotide that is cell-associated relative to the total amount of oligonucleotides exposed to the cells. This was found to be a more accurate method of data presentation than simply reporting the radioactivity counts per amount of protein for the following reasons.
First of all, we end-label our oligonucleotide using T4 PNK. This enzyme does not always exhibit 100% labeling efficiency and exhibits some sequence dependence. Thus we ended up with different total amounts of radioactivity between the different oligonucleotides samples. For example, freshly radiolabeled control DNA and d(A15G15) had slightly different CPM even though the same number of moles of each oligonucleotides was labeled in exactly the same manner. Thus a better way of taking into account difference in specific activity is to assume that the total radioactivity exposed to the cells is equal to the total amount of oligonucleotides presented to cells. Secondly, the [\gamma^{32}\text{P}]ATP stock used for labeling also showed differences in its radioactivity, a fresh batch of [\gamma^{32}\text{P}]ATP exhibits higher radioactivity in comparison an older batch. Presenting raw counts (CPM) as internalized oligonucleotide is misleading because an oligonucleotide labeled with newer batch of [\gamma^{32}\text{P}]ATP will, of course, exhibit higher count. Lastly, the number of cells per well does not differ greatly from one well to another. Protein quantitation results have indicated that cell numbers per well are very consistent from well to well and from experiment to experiment. Normalizing CPM by cell counts is not necessary because cells were, first, plated 1 mL per well from a stock of cells and incubated for the same amount time in the same condition, second, cells remain as a monolayer in the wells therefore any overgrown cells would have been detached and washed away by the first wash, leaving a confluent well with a monolayer of cells.
CHAPTER 5: RESULTS

5.1. Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA in HepG2 at 25°C

For this preliminary uptake study, we chose \( d(A_{15}G_{15}) \) to form FW and as control, we chose a non-self complementary random sequence 30-mers (ssDNA). Initial uptake studies of FW and ssDNA (0.3 \( \mu \text{M} \)) in HepG2 cells at 25°C displayed significantly higher uptake of FW as compared to the control DNA (Fig. 1). From 30 to 120 minutes, the overall accumulation of FW in HepG2 was approximately 5 times more than that of the ssDNA. This indicated that either FW are more readily transported in HepG2 cells than ssDNA or that ssDNA is not able to penetrate the cellular membrane as efficiently as FW. The uptake of FW appeared saturable as the percent uptake plateaus off by 30 minutes and little additional accumulation occurs between 30 and 120 minutes. A similar pattern was observed with ssDNA but with much less initial uptake. Therefore, uptake in HepG2 cells suggested a saturable uptake of both FW and ssDNA. Moreover, this process appears to proceed more efficiently with FW than ssDNA.

5.2. Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA in CNE1 cells at 25°C

Initial studies in HepG2 cells indicated an increased cellular uptake of FW as compared to normal single-stranded DNA. There is interest in nasal delivery routes for oligonucleotides. Therefore, in order to elucidate whether enhanced cellular uptake of
Figure 1: HepG2 Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA. In this preliminary uptake study, HepG2 cells (n = 3 wells / time point) were incubated at 25°C with either 0.3 μM of FW or random 30mers (ssDNA) for four different times; 0, 30, 60 and 120 minutes. Both FW and ssDNA were $^{32}$P labeled at 5' termini. HepG2 showed overall higher uptake of FW than for ssDNA Total accumulation of FW in HepG2 cells after 120 minute incubation was close to 5 times higher than that for the control, ssDNA. Non-specific binding (time 0) was subtracted from these uptake. The values of non-specific binding were approximately 0.002631 % for FW and 0.003119 % for ssDNA. Each point is average of 3 wells with error bars indicating standard deviation.
FW also occurs in other epithelial membranes, such as that found in the nasal passages, we examined the cellular uptake of d(A₁₅G₁₅) and ssDNA (0.3 μM) in a nasopharyngeal cancer cell line (CNE1). In this study, similar research protocols to HepG2 uptake were followed. As seen with the HepG2 uptake study (Fig. 1) CNE1 cells also showed, an overall higher cellular uptake of FW than ssDNA (Fig. 2). However, it is clear that the difference in uptake was not as prominent as seen in HepG2 cells where the difference in the total accumulation after 120 minutes incubation was approximately 5 fold. Uptake of FW is similar in both cell lines. Examining the slope of the early time points (between 0-30 minutes), it is evident that uptake of FW was more rapid than that of ssDNA. CNE1 cellular uptake of ssDNA seemed to be saturated by 60 minutes of incubation whereas the rapid initial 30 minute uptake of FW was followed by a slower uptake. This suggested that FW cellular uptake may eventually reach saturation if they were to be incubated with cells for a longer period. In comparison, both HepG2 and CNE1 cells exhibited more favorable uptake of FW than for ssDNA but these uptakes reached saturation earlier on in HepG2 than in CNE1 cells.
Figure 2: Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA in CNE1 Cells. CNE1 cells (n = 3 wells / time point) were exposed to either 0.3 μM of FW or ssDNA for four different times at 25°C. The oligonucleotides were $^{32}\text{P}$ 5'-end labeled. Similar to the HepG2 uptake, CNE1 cells showed higher uptake for FW than for ssDNA. Total intracellular accumulation after 120 minutes differed approximately 45 percent. Cellular uptake of FW and ssDNA appears to be saturating with maximum uptake plateauing at approximately 60 minutes. Each point is average of 3 wells with error bar indicating standard deviation.
5.3. Initial Rate of Uptake into CNE1 Cells for DNA Frayed Wires and Single Stranded DNA

CNE1 cells once again showed higher uptake for FW than for ssDNA, displaying a 150% greater accumulation after 60 minutes. Estimation of slopes of initial uptake in Figure 2 between 0 ~ 30 minutes, we obtained, approximately, a rate of 0.4% / minute for FW and a rate of 0.2% / minute for ssDNA. Also, by estimating slopes between 0 ~ 5, 0 ~ 10, 0 ~ 15 and 0 ~ 30 minutes, it can be noted that the highest rates of uptake of both FW and ssDNA were observed within the first 5 minutes, with the uptake rate of FW (approx. 0.76% / min) higher than of ssDNA (approx. 0.36% / minute). These results indicated that cellular uptake of both DNA and ssDNA occurs rapidly and with FW being taken up more efficiently than ssDNA.

The earlier DNA Frayed-wire cellular uptake experiment (Fig.1 and 2) have already demonstrated that the highest rate of uptake was observed between 0 ~ 30 minutes. For HepG2 cells, the uptake of both FW and ssDNA plateaus off after 30 minutes and with CNE1 cells, uptake by CNE1 cells did not saturate until later but its uptake also showed the highest rate between 0 ~ 30 minutes. Therefore, it was reasonable to look at the uptake of both FW and ssDNA at time points earlier than 30 minutes and we observed a higher initial rates of uptake of FW at earlier times, suggesting that the overall higher uptake of FW may be due to a process that exhibits a higher rate of uptake for FW than for ssDNA.
Figure 3: Early Time Point CNE1 Uptake of DNA Frayed Wires Vs. Single Stranded DNA. CNE1 cells (n = 4 wells / time point) were exposed to 0.3 μM FW or ssDNA at 25°C. CNE1 cells were then incubated for six different times: 0, 5, 10, 15, 30 and 60 minutes. With earlier incubation times, cells showed a higher uptake of FW than ssDNA. The intracellular accumulation of FW at 60 minutes was approximately 150% greater than that observed with ssDNA. Each point is average of 4 wells with error bars indicating standard deviation.
5.4. Temperature Dependence of CNE1 Cellular Uptake of DNA Frayed Wires and Single Stranded DNA

In order to determine whether FW exhibits temperature dependence and to compare the temperature dependence of ssDNA, CNE1 cells (n = 3 wells / time point) were incubated at 4, 25 and 37 °C with 0.3 μM of either FW or ssDNA. In order to minimize temperature fluctuations, the cells and the reaction mixtures were equilibrated to these temperatures prior to the experiment. Cells (n = 3 wells / time point) were then incubated with 0.3 μM of FW or ssDNA for 0, 30, 60 or 120 minutes.

In a preliminary uptake study at 4°C, we observed significant decreases in uptake for both FW and ssDNA (Fig. 4), relative to the uptake at 25°C (Fig. 2). The total accumulation of both FW and ssDNA was approximately four times lower than seen at 25°C. However, even though there was overall decrease in uptake of FW and ssDNA at 4°C, we detected that FW uptake was ~ 290% greater than that of ssDNA. These results suggested a temperature dependence of cellular uptake of both FW and ssDNA.

In another experiment, three sets of CNE1 cells were incubated with a solution containing 0.3 μM FW for different times and each set was incubated at three different temperatures : 4, 25 and 37°C. There was the highest uptake for FW at 37°C and lowest uptake at 4°C (Fig. 5). The total cellular accumulation of FW at 37°C was approximately 4 times higher than at 4°C. Initial uptake rates (0 ~ 30 minutes) of FW at the three temperatures were estimated. The highest uptake rate was observed with 37°C
Figure 4: CNE1 Cellular Uptake of DNA Frayed Wires Vs. Single Stranded DNA at 4°C. CNE1 cells (n = 3 wells / time point) were exposed to 0.3 μM of FW or single stranded 30mers for four different amounts of time at 4°C. Prior to incubation, both cells and the reaction mixtures containing both DNA were cooled down to 4°C to minimize temperature differences. Overall, there was a higher uptake of FW in comparison to ssDNA uptake. In comparison to the previous uptake at 25°C (Fig. 2 and 3), total accumulation of both FW and ssDNA were reduced significantly. Each point is average of 3 wells with error bars indicating standard deviation.
and the lowest was at 4°C. The rates are approximately 0.04% / min at 4°C, 0.11% / min at 25°C and 0.23% / min at 37°C.

Previous uptake studies (Fig 1 - 3) of FW demonstrated that cellular uptake of this unusual DNA is higher in comparison to normal DNA and that the uptake becomes saturated after prolonged incubation. This suggested that uptake may occur through active processes (Besterman and Low, 1983). One of the characteristics of active or energy dependent cellular transport process is that this process is inhibited by lower temperature (Henthorn et al., 1999). A number of cellular uptake studies of oligonucleotides have demonstrated that the accumulation of oligonucleotides are reduced by lowering the incubation temperature (Shoji et al., 1991; Loke et al., 1989; Yakubov et al., 1989; Goodchild et al., 1989).

Our results clearly indicated that both total accumulations and initial uptake rates are positively correlated to temperature, suggesting that FW uptake is a temperature-dependent active transport process similar to that which has been described for cellular uptake of normal DNA (Juliano and Yoo, 2000). More interestingly, even at 4°C, a temperature at which minimal active transport occurs (Fig. 4), FW internalization was more efficient than ssDNA.
Figure 5. DNA Frayed Wires Cellular Uptake at Three Different Temperatures.
CNE1 cells (n = 5 wells / time point) were incubated with 0.3 μM of FW at three different temperatures to examine effect of temperatures on cellular uptake. The highest cellular uptake was observed with 37°C incubation whereas the lowest uptake was observed with 4°C incubation. Slope estimation of 0 ~ 30 minutes at three temperature indicated that initial rates of FW increased with temperature. Thus there was a positive correlation between temperature and total accumulations and also between temperature and initial rate of uptake. Each point is average of 3 wells with error bars indicating standard deviation.
5.5. Concentration Dependence of DNA Frayed Wire Cellular Uptake

The effect of the concentration of oligonucleotides on its cellular uptake was examined by incubating CNE1 cells (n = 5 wells / time point) with five different concentrations of FW. The concentrations used are as follows: 0.1, 0.2, 0.3, 0.4, 0.5 μM. The total accumulation of FW was measured after 120 minutes incubation at 25°C (Fig. 6). We found that cells incubated with 0.3, 0.4 and 0.5 μM FW exhibited similar final accumulations of FW, indicating a possible concentration dependent saturation of the cellular uptake process.

To examine this possibility further, a similar cellular uptake study using five different concentrations of FW was done but this time the incubation time was reduced to 30 minutes because of the possibility that the accumulation of FW after the 120-minute incubation might be simply due to the limited spatial capacity of CNE1 cells to take in oligonucleotides and thus may have little to do with the concentration. It was already shown by previous results in CNE1 cells (Figs. 2 and 3) that FW accumulation did not reach the maximum by 30 minutes.

After 30-minutes, cells incubated with 0.3, 0.4 and 0.5 μM of DNA Frayed-wires exhibited very similar final accumulations (Fig.7), further suggesting that this uptake process is concentration dependent. To investigate further, cells (n = 1 well / time point)
Figure 6: Total Intracellular Accumulation of Different Concentration of DNA Frayed Wires after 120 Minutes in CNE1 Cells. CNE1 cells (n = 5 wells / concentration) were incubated with five different concentrations of FW and incubated for 120 minutes at 25°C. Final accumulation of FW inside the cells was measured and the data was presented as counts per minutes per concentration of protein (CPM/µg/µL). 120 minutes incubation with 0.3, 0.4 and 0.5 µM of FW resulted in a similar total intracellular accumulation. A saturation of FW uptake was observed at a concentration of 0.3 µM. Each bar is average from 5 wells with error bars indicating standard deviation.
were incubated for even shorter amount of times (1, 2, 3, 5, 7, 10, and 15 minutes) with five different concentrations of FW. Care was taken during this procedure to assure the accuracy of these incubation times. Cellular uptake patterns at these early time points (Fig. 8) using different concentrations of FW showed that the pattern exhibited by 0.3, 0.4, and 0.5 \( \mu \)M FW were superimposable.

The results were then re-plotted and fit to a straight line using linear regression (Fig. 9A) to estimate slope or rate of uptake at each concentration of FW. The estimated rates of 0.1, 0.2, 0.3, 0.4, and 0.5 \( \mu \)M FW were as follows: 890, 1500, 2400, 2400, and 2600 CPM/\( \mu \)g/\( \mu \)L/minute. The estimated rates of 0.3, 0.4 and 0.5 \( \mu \)M were close to one another. The initial uptake rates were plotted against the concentration to obtain \( K_m \) and \( V_{max} \). They were 0.19 \( \pm \) 0.05 \( \mu \)M and 3100 \( \pm \) 590 cpm (\( \mu \)g/\( \mu \)L\(^{-1} \) min\(^{-1} \), respectively. These results further support the idea that cellular uptake process is concentration dependent and indicate that the maximum rate of initial cellular uptake in CNE1 cells was seen with concentrations of approximately 0.3 \( \mu \)M.
Figure 7: Intracellular Accumulation by Different Concentrations of DNA Frayed Wires after 30 Minutes of Incubation with CNE1 cells. CNE1 cells (n = 5 wells / concentration) were incubated for 30 minutes at 25°C with 5 different concentrations of FW. Final accumulation of FW was measured and the data was presented as count per minutes per concentration of protein (CPM/µg/µL). 30 minutes incubation by 0.3, 0.4 and 0.5 µM of FW resulted in a very similar total intracellular accumulation pattern as that seen at 120 minutes (Fig. 6). Each bar is average of 5 wells with error bars indicating standard deviation.
Figure 8: Initial Intracellular Accumulations by Different Concentrations of DNA Frayed Wires Within 15 minutes. CNE1 cells (n = 1 well / time point) were incubated with five different concentrations of FW (0.1, 0.2, 0.3, 0.4 and 0.5 μM). The incubation times were 1, 2, 3, 5, 7, 10, and 15 minutes. Final accumulation was measured after each incubation time and they were plotted against time to obtain the uptake pattern exhibited by each concentration. The uptake was saturable at concentrations greater than 0.3 μM.
Figure 9: Initial Cellular Uptake Rate of Different Concentration of DNA Frayed Wires. Data shown in Fig. 8 fit to straight lines with linear regression, initial uptake rate of different concentrations of FW in CNE1 cells (n = 1 well / time point) were estimated. The unit for estimated rate was cpm/μg/μL per minute. Initial cellular uptake rates of 0.3, 0.4 and 0.5 μM were not significantly different, indicating saturation of cellular uptake (A). The estimate initial rates were plotted against concentration to obtain $K_m = 0.19 \pm 0.05 \mu M$ and $V_{max} = 3100 \pm 590 \text{cpm(μg/μL protein)}^{-1} \text{min}^{-1}$. 
5.6. Effects of Heparin on Cellular Uptake of DNA Frayed Wires and Single Stranded DNA

In this experiment, CNE1 cells (n = 5 wells / concentration) were pre-incubated with five different concentrations of heparin from porcine intestinal mucosa. The concentration used were; 0, 0.1, 1, 5, and 10 Units/μL. Cells were then incubated with 0.3 μM of FW or ssDNA at 25°C for 60 minutes to allow intracellular accumulation. Relative to the total accumulation by untreated cells, the uptake of FW decreased by approximately 60% in cells treated with 0.1 U/μL heparin. Treatments with higher concentrations of heparin (1, 5, and 10 U/μL) also significantly reduced intracellular uptake; higher reductions were seen with increasing concentration. Compared to 0.1 U/μL, there was a ~ 10% further reduction in cells treated with 10 U/μL of heparin. In the case of single-stranded DNA, heparin treatment imposed a reduction in intracellular ssDNA but it was not as pronounced as that seen with the FW. Preincubation with 0.1 U/μL heparin reduced the accumulation by approximately 30% but higher concentrations did not further inhibit ssDNA uptake. These results demonstrate that pre-incubation with heparin decreased the uptake of FW and ssDNA although greater inhibitory effects are observed on the uptake of FW.

Studies have correlated expression with overall cellular uptake of oligonucleotides suggesting their possible involvement in receptor-mediated endocytosis of oligonucleotides (De Diesbach et al., 2000; Yakubov et al., 1989; Loke et al., 1989; Rappaport et al., 1995; Beltinger et al., 1995; Loike et al., 1997). Among these
Figure 10: Effects of Heparin on Cellular Uptake of DNA Frayed Wires and Single Stranded DNA. CNE1 cells (n = 5 wells / concentration) were pre-treated with heparin (0.1 to 10.0 U/μL) and then incubated with 0.3 μM of FW or ssDNA for 60 minutes. For FW, uptake was inhibited by 60% with 0.1U/μL Heparin and further inhibition, up to 70%, was observed with 10.0U/μL Heparin. In the case of ssDNA, a 30% inhibition of uptake was observed with 0.1U/μL Heparin, however higher concentrations did not further suppress cellular uptake, as seen with FW. Each bar is average from 5 wells with error bars indicating standard deviation.
receptors, several studies have demonstrated that oligonucleotides bind, sometimes with high affinity, to many heparin-binding proteins such epidermal growth factors receptors (EGFR), vascular endothelial growth factor receptors (KDR/flk-1), basic and acidic fibroblast growth factors (bFGF and aFGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), as well as to the VEGF receptors (Guvakova et al., 1995; Benimetskaya et al., 1997a). Our results suggest that heparin binding proteins or transporters are likely to be involved in the uptake of FW.

Recently, there were studies reporting that G-quartet containing oligonucleotides are readily taken up by cells (Burgess et al., 1995; Rando et al., 1995; Bates et al., 1999) and that their cellular uptake was higher than that of non G-quartet forming oligonucleotides (Bishop et al., 1996). More importantly, it was reported that cell surface heparin-binding proteins exhibited high affinity to G-quartet containing oligonucleotide and the binding is maximal when G-tetrad is located either 5'- or 3'- terminus (Benimetskaya et al., 1997). Structural studies of FW have already demonstrated that guanine-guanine interaction in the stem is different from one in G-quartet (Poon and Macgregor, 1998). It was reported that a guanine stem of FW is located 3'-terminus thus unless the high affinity exhibited by heparin-binding proteins towards the G-quartet is stereospecific to the 3-dimensional structure of G-quartet, heparin-binding cell surface receptors may also exhibit high affinity toward DNA Frayed-wires. Therefore, by preoccupying these receptors with heparin, a poly-anionic polysaccharide, cellular uptake of DNA Frayed-wires may be inhibited.
5.7. Effects of Metabolic Inhibitors on Cellular Uptake of DNA Frayed Wires

As previously discussed, one of the well-characterized cellular uptake mechanism of oligonucleotides is endocytosis. Numerous studies have reported oligonucleotides uptake via endocytic pathway (Budker and Sokolove, 1987; Budker et al., 1987) and many oligonucleotide binding cell surface proteins have been identified which are likely linked to receptor-mediated endocytosis (de Diesbach et al., 2000; Yakubov et al., 1989; Loke et al., 1989; Rappaport et al., 1995; Beltinger et al., 1995; Wu-Pong et al., 1994; Yao et al., 1996). As shown in previous results, intracellular accumulation of FW is saturable (Figs. 1 and 2), temperature dependent (Fig. 5) and concentration dependent (Fig. 8 and 9) and inhibitable; thus, it is likely to be an active process. As heparin is a good inhibitor of this uptake, it is likely that heparin-binding cell surface receptors are involved (Fig. 10).

In order to provide further evidence that this process is indeed an active transport process possibly involving receptor-mediated endocytosis, cells were treated with well-known inhibitors of active transport. It has been previously demonstrated that oligonucleotide uptake is reduced in the presence of sodium azide (NaN3), 2-deoxy-D-glucose and cytochalasin B (Loke et al., 1989; Yakubov et al., 1989). NaN3 and 2-deoxy-D-glucose are metabolic poisons; NaN3 blocks oxidative phosphorylation and 2-deoxy-D-glucose blocks glycolysis.
In this experiment, CNE1 cells (n = 5 wells / inhibitor mixture) were pre-incubated with one of the following inhibitor mixtures: (1) control, no inhibitor, (2) sodium azide, (3) sodium azide + heparin, (4) 2-deoxy-D-glucose, (5) 2-deoxy-D-glucose + heparin, (6) sodium azide + 2-deoxy-D-glucose and (7) sodium azide + 2-deoxy-D-glucose + heparin. It is important to note that previous inhibition study using 5, 10 and 30 mM sodium azide did not produce significant inhibition thus the concentration was increased to 50 mM. The cells were then incubated with 0.3 \mu M FW for 60 minutes and the final intracellular accumulations were measured. In all treated cells, there was a significant reduction (p < 0.05) in the intracellular uptake of FW. Cells treated with 50 mM sodium azide (2) showed more than 50% reduction in uptake and with 10 mM 2-deoxy-D-glucose (4), there was more than 60% reduction. In combination, these two chemicals reduced accumulation of FW somewhat more than sodium azide alone (2) but less than by 2-deoxy-D-glucose alone. It was already shown that pre-treatment of cells with heparin effectively decreased intracellular uptake of FW and normal DNA (Fig. 10) therefore in order to examine the effect of heparin with other inhibitors, heparin was mixed with either one of the inhibitors. For cells treated with sodium azide + heparin, there was a significant decrease in the accumulation of FW (3) greater than that seen with sodium azide alone (2). Addition of heparin to 2-deoxy-D-glucose (4) or the mixture of sodium azide + 2-deoxy-D-glucose (6), also had an additive inhibitor effect on FW uptake (5 and 7).

The inhibitory effect on the intracellular accumulation of FW by sodium azide and 2-deoxy-D-glucose further supports the notion that this uptake process is an energy-
Figure 11: Effect of Metabolic inhibitors on Cellular Uptake of DNA Frayed Wires in CNE1 Cells. CNE1 cells (n = 5 wells / inhibitor mixture) were treated with one of following chemicals prior to the incubation with FW for 60 minutes at 25°C. (1) Untreated, (2) 50 mM NaN₃, (3) 50 mM NaN₃ + 0.1 U/µL Heparin, (4) 10 mM Deoxyglucose, (5) 10 mM Deoxyglucose + 0.1 U/µL Heparin, (6) 50 mM NaN₃ + 10 mM Deoxyglucose, (7) 50 mM NaN₃ + 10 mM Deoxyglucose + 0.1 U/µL Heparin. There was significant reduction (p < 0.05) in total accumulation when cells were treated with each of these inhibitors and further inhibition was seen in the presence of heparin. (*) indicates the significance of the data. Each bar is average of 5 wells with error bars indicating standard deviation.
dependent active transport process. Furthermore, additional inhibitory effects observed with heparin strengthen the suggestion that DNA Frayed-wire cellular uptake occurs via receptor-mediated endocytosis. Receptors involved are likely to be heparin-binding cell surface proteins which have a high affinity for oligonucleotides containing guanine aggregates at either 5'- or 3'-termini or molecules with very high negative charge density.

5.8. Cellular Uptake of DNA Frayed Wires Derived from Different Parent Strands

In order to examine if enhanced cellular uptake of FW (Fig.1 and 2) is non-sequence specific and due to the receptors exhibiting high affinity to the guanine stems, we tested the uptake of FW which were composed of different nucleotide sequences. FW were formed using: d(A\textsubscript{15}G\textsubscript{15}), d(T\textsubscript{15}G\textsubscript{15}), d(G\textsubscript{15}A\textsubscript{15}) and d(N\textsubscript{15}G\textsubscript{15}) where N = random sequence. The formation of FW was verified by electrophoretic separation on a denaturing gel (Fig. 13).

The cellular uptake of FW made from four different parent strands (0.3 μM) was examined in CNE1 cells (n = 3 wells / time point) at 25°C. Overall, FW from different sequences exhibited similar cellular uptake patterns (Fig. 12). There was a small but significant difference in the uptake of d(T\textsubscript{15}G\textsubscript{15}) as compared to three other type of FW. The d(T\textsubscript{15}G\textsubscript{15}) FW had the lowest cellular uptake although the difference was minimal. The results imply that the cellular uptake of FW is not sequence specific and that it provides evidence that the superior cellular uptake of FW takes place via endocytosis.
Figure 12: CNE1 Cellular Uptake of DNA Frayed Wires Derived from Different Parent Strands. FW were derived from difference guanine-rich sequences: d(A₁₅G₁₅), d(T₁₅G₁₅), d(G₁₅A₁₅) and d(N₁₅G₁₅) where N = random sequence. CNE1 cells (n = 3 wells / time point) were incubated with 0.3μM of FW from different sequences for different amount of times at 25°C. Uptake patterns of FW derived from different sequences exhibited similar uptake patterns. Each point is average of 3 wells with error bars indicating standard deviation.
Figure 13: DNA Frayed Wire Formation by Different Guanine-rich Strands. All previous cellular uptake studies of FW used d(A\textsubscript{15}G\textsubscript{15}) as the parent oligonucleotides. In order to examine the effect of FW derived from different parent oligonucleotides on cellular uptake, other guanine-rich strands were used to form FW. They were: d(T\textsubscript{15}G\textsubscript{15}), d(G\textsubscript{15}A\textsubscript{15}) and d(N\textsubscript{15}G\textsubscript{15}) where N = random sequence. They were resolved by 10% denaturing PAGE containing 7 M urea. (1) d(A\textsubscript{15}G\textsubscript{15}), (2) d(T\textsubscript{15}G\textsubscript{15}), (3) d(N\textsubscript{15}G\textsubscript{15}), (4) d(G\textsubscript{15}A\textsubscript{15}). The ladder of bands appeared for all sequences, as seen with d(A\textsubscript{15}G\textsubscript{15}), indicating other guanine-rich strands were able to form FW. Smaller amount of the same oligonucleotides were loaded at (1)*, (2)*, (3)*, (4)* and (5)* for better visualization.
involving heparin binding cell surface receptors that exhibit a greater affinity for the guanine stems of FW in comparison to normal DNA.

As described for cellular uptake of G-quartet, our results also suggested that FW uptake is non-sequence specific (Bishop et al., 1996; Burgess et al., 1995; Rando et al., 1995; Bates et al., 1999) and likely involves electrostatic binding between the guanine stems and the receptors. As different arms resulted in similar uptakes, it appears that the arms do not play a significant role in the enhanced uptake.

5.9. Structural Analysis of Intracellular DNA Frayed Wires

Intracellular FW were resolved by denaturing 10% PAGE against stock and extracellular FW. The internalized FW exhibited the same ladder of bands as stock FW and extracellular FW (Fig. 14 and 15). The top band, which corresponds to the most polymerized species of FW, was detected near the top of the gel and the rest of the bands appeared as a successive ladder. Furthermore, both extra- and intracellular ssDNA were resolved on denaturing 10% gels against stock ssDNA. Both ssDNA samples exhibited different bands in comparison to their stock. Typical stock ssDNA, when separated, showed two bands near the bottom of the gel. However, more bands were detected near the bottom of the gel in both extra- and intracellular ssDNA, degradation of ssDNA was more apparent in the intracellular samples (Fig. 15).
Figure 14. Structural analysis of DNA Frayed Wires stock Vs. Extracellular and Intracellular DNA Frayed Wires. Both the extra- and intracellular FW were resolved by 10% denaturing PAGE (7 M urea at 50°C) against stock FW (not exposed to cells). (I) = Intracellular, (I*) = Intracellular but smaller amount, (E) = Extracellular and (S) = Stock. All three samples exhibited very similar ladder of bands (located by the arrows—→). The result indicated FW underwent little degradation in both extra and intracellular environment and kept their structure. (MON) indicates the monomer.
Figure 15: Intracellular DNA Frayed Wires Vs. Intracellular Single Stranded DNA. Intracellular FW [F(I)] and intracellular single stranded DNA [s(I)] were resolved on denaturing 10% PAGE to examine their structure in comparison with both their stocks (not exposed to cells) [F(S) & s(S)] and extracellular samples [F(E) & s(E)]. Intracellular FW, compared to both the stock and the extracellular sample, are not different whereas there was a structural change in intracellular ssDNA. The samples were collected by incubating cells with the DNA for 120 minutes. Similar amount of radioactivity was loaded in each well.
The main characteristic of FW is when they are resolved by a denaturing PAGE, they exhibit a characteristic ladder of bands with most of bands detected close to the top of the gel (Protozanova and Macgregor, 1996). This indicates that high-molecular weight species have been formed and that these complexes are resistant to the denaturing conditions of the gel (7 M urea, 50°C). To examine the extent of degradation of FW after incubation in the extra- and intracellular environment (after 120 minutes), on a denaturing 10% gel, both extra- and intracellular FW were run against the stock which was never exposed to cells. All three samples exhibited the same pattern of bands, suggesting FW remain intact structures and that little or no degradation occurred in the extra- or intracellular environment. However, in the case of ssDNA, as expected, more bands were detected near the bottom of the gel. These bands corresponded to fragments of ssDNA, suggesting some extent of degradation have occurred to ssDNA in extra- and intra-cellular environment. Therefore, the results of both Figure 14 and 15 suggested that no cellular degradation has occurred for FW. This likely occurs as a result of their higher thermal and nuclease stability whereas ssDNA underwent some cellular degradation by nucleases or by other cellular mechanisms.

5.10. Cellular Uptake of Intact DNA Frayed Wires Vs. Cleaved DNA

Frayed Wires

One potential disadvantage or drawback of using 5'- terminal radio-labeled oligonucleotides in cellular uptake studies is the possible loss of the radiolabeled 5'-end nucleotide by nucleases. Thus, cell associated radioactivity measured could be cleaved radiolabeled 5'-end nucleotides (32P-adenine) from the arms, instead of intact FW. In
order to examine this, CNE1 cells were incubated with either intact FW or cleaved FW over time, in the same manner as previous uptakes (Fig. 1, 2 and 3). FW were cleaved into simple nucleotides by repeated exposures and prolonged incubation with DNase I. Prior to the uptake, the cleaved FW were resolved by native 10% PAGE against the intact FW to ensure they were all cleaved into simple nucleotides (Fig. 17).

The cellular uptake pattern exhibited by cleaved FW was different from intact FW. There was higher uptake of cleaved FW in CNE1 cells than of intact FW. The uptake pattern of the intact FW exhibited saturation over time which is consistent with the data shown in Fig. 2 and 3 at the same temperature (25°C). In comparison, uptake of the cleaved FW showed no saturation and that the intracellular accumulation increased constantly with time. The final intracellular accumulation of the cleaved FW after 60 minutes was 270% greater than the intact FW.

The significant difference between uptake of the intact and the cleaved FW confirms that the cell-associated radioactivity we measured as intracellular FW in previous uptake studies is neither cleaved 5’ radiolabeled nucleotides nor cleaved arms of FW.
Figure 16: Cellular Uptake of Intact DNA Frayed Wires Vs. Cleaved DNA Frayed Wires. FW were formed by d(A₁₅G₁₅) and half of them were cleaved into simple nucleotides by prolonged exposure (24 hours) to DNase I at 37°C. CNE1 cells (n = 6 wells / time point) were incubated with either intact FW or cleaved FW at 25°C for three different time points; 0, 15, 30 and 60 minutes. The total cell associated radioactivity was presented in CPM. Cellular uptake of intact and cleaved FW exhibited a different pattern over time, with higher intracellular uptake recorded with cleaved FW. Each point is average of 6 wells with the error bar indicating standard deviation. For direct comparison, the data is presented in raw count (CPM, count per minute) instead of % uptake.
Figure 17: Intact DNA Frayed Wires Vs. Cleaved DNA Frayed Wires. d(A_{15}G_{15}) was 5'-end labeled using $^{32}$P and incubated at 80°C in the reaction buffer to form FW. Half of it was then incubated with DNase I for 24 hours to allow maximum degradation. The final product was resolved by 10% native PAGE (polyacrylamide gel electrophoresis) with intact FW. (1) Cleaved FW, (2) Intact FW. Intact FW exhibited expected typical ladder pattern whereas Cleaved FW ran off the gel.
5.11. Cell Viability Test

Cell viabilities were $98 \pm 2\%$ for FW, $99 \pm 2\%$ for ssDNA, $99 \pm 2\%$ for the reaction buffer and $97 \pm 2\%$ for NaN$_3$. The cell viability was tested, by trypan blue, in order to rule out the possibility that the uptake of FW and ssDNA are not due to catatonic effects of these treatments. Cells were incubated with either FW, ssDNA or the reaction buffer for 60 minutes but cells were only incubated with 20 minutes with 50 mM NaN$_3$. The results indicated that cell viability is maintained under these treatment conditions and the uptake of FW and ssDNA were not likely to result of passive diffusion through damaged or leaky membranes.
CHAPTER 6: DISCUSSION

6.1. Discussion

For my research project, I characterized the cellular uptake of DNA Frayed Wires (FW) and compared it to the uptake of normal DNA. As the control, we used non-self-complementary single stranded 30-nucleotide long oligonucleotide. We have demonstrated that, there is indeed cellular uptake of this unusual DNA structure, FW. Significantly, with both HepG2 and CNE1 cell lines, there tends to be a greater amount of FW being accumulated inside cells than of random sequenced single-stranded DNA over the same period of time (Fig. 1 and 2). This difference was greater in HepG2 than in CNE1 cells. This indicated that the cellular uptake of FW occurs in more efficient manner than that which occurs with normal DNA.

Other studies on the cellular uptake of oligonucleotides have reported that oligonucleotides can gain access to the intracellular space and exert their intended effects (Loke et al., 1989; Beltinger et al., 1995; Kalahari et al., 1996; Benimetskaya et al., 1997a; Hughes et al., 2001). Unfortunately, these studies also sited major problems such as low cellular uptake and high nuclease susceptibility which are possibly linked to one another (Wickstrom, 1986; Temin, 1990; Akhtar et al., 1991). It is not clear whether rapid degradation by nucleases or low cellular permeability is responsible for the low cellular uptake. Furthermore, a slow uptake process may allow increased oligonucleotide exposure to nucleases which would also contribute to low cellular uptakes. The approaches have been taken to overcome these problems have met with some success.
Uptake studies of the other well-characterized guanine-rich complexes, e.g. G-quartets, have demonstrated that these structures can readily enter the intracellular spaces and exert non-sequence specific biological effects (Lin et al., 1994; Burgess et al., 1995; Macaya et al., 1995; Bishop et al., 1996; Benimetskaya et al., 1997a; Bates et al., 1999). Interestingly, Bishop and co-workers (1996) reported a higher intracellular uptake for G-quartet-containing oligonucleotides in comparison to non-G-quartet forming sequence variant, similar to our observations between FW and ssDNA in HepG2 and CNE1 cells. In addition to the greater cellular uptake, they reported that the G-quartet is responsible for greater serum nuclease resistance. It has been demonstrated in previous works from our laboratory that FW and G-quartet arise from different guanine-guanine interactions (Poon and Macgregor, 1998). FW also exhibited greater nuclease resistance than normal DNA or oligonucleotides containing G-quartet structures (Protozanova et al., 1996; Poon and Macgregor, 1999). This suggests that the enhanced uptake of FW in HepG2 or CNE1 cells may be due to the following factors. 1) due to high stability, there were simply more intact FW than ssDNA to be internalized or 2) it is due to a transport mechanism that favors guanine aggregates compare to ssDNA or 3) uptake of FW and ssDNA were mediated by different mechanisms.

The cellular uptake of FW exhibited characteristics similar to those previously reported for other oligonucleotides (Yakubov et al., 1989; Loke et al., 1989; Shoji et al.,

(Capaccioli et al., 1993; Gershon et al., 1993; Beltinger et al., 1995; Taylor et al., 1996; Delong et al., 1997; Bennett et al., 1998; Nielsen et al., 1999).
Our results strongly suggest that uptake of FW occurs via an active transport mechanism. First of all, FW cellular uptake is saturable. Cellular uptake in HepG2 was saturated within 30 minutes and after about 60 minutes in CNE1 cells. The differences in the extent of uptake from one cell line to another may be attributable to differences in the expression of transport proteins and metabolic enzymes in addition to other cellular activities that govern cross-membrane transport in these two cell lines. This is one of the findings which supports the hypothesis that increased cellular uptake of FW is due to increased enzymatic stability. HepG2 expresses a much larger number of metabolic enzymes as well as a greater amount. Hence it would be expected that ssDNA would be metabolized to a large extent in HepG2 as compared to CNE1. Our finding of higher ssDNA uptake in CNE1 cells supports this hypothesis. Also, the saturation of this uptake can be attributed to either irreversible binding of FW to their transporters or limited quantity of transporters present that also may exhibit slow recycling.

Secondly, the uptake process is temperature dependent, displaying inhibition at low temperatures and enhanced uptake at higher temperatures (Fig. 4 and 5). The concentration dependence of the uptake also suggested that it is regulated by energy-dependent cellular transport processes that are saturable due to the limitation in cellular energy for the process and/or presence of a limited number of transporters.

The suggestion that uptake involves active transport was further supported by the experiments where the uptake of FW was inhibited by metabolic inhibitors such NaN₃.
and 2-deoxy-D-glucose (Fig. 11). In the present case, 2-deoxy-D-glucose was a more effective inhibitor than NaN₃. The difference in their effectiveness can be attributed to the fact that 2-deoxy-D-glucose is an inhibitor of glycolysis which blocks the early steps of cellular energy producing process, thus inhibiting ATP formation. As NaN₃ inhibits oxidative phosphorylation, which is later in the cellular energy production line, compensatory ATP formation is generated through increasing the rate of glycolysis. Hence glycolysis may fuel this energy-expending process for a short time.

Besides the involvement of active cellular process, our inhibition studies also provide evidence that uptake of FW occurs via receptor-mediated endocytosis in a manner similar to the cellular uptake of other oligonucleotides (Huïkett et al., 1990; Shoji et al., 1991; Deshpande et al., 1996). This notion was supported by identification of oligonucleotide-binding cell receptors, actual electron micrograph observations and more (Budker et al., 1987; Sandvig et al., 1987; Yakubov et al., 1989; Yao et al., 1996; de Diesbach et al., 2000). Among the supporting evidence was our observation that heparin inhibited the cellular uptake of both FW and ssDNA. Heparin-binding cell surface receptors have been identified as oligonucleotide-binding cell surface receptors and some are directly involved in receptor-mediated endocytosis of oligonucleotides (Guvakova et al., 1995; Benimetskaya et al., 1997b). They are reported to exhibit particularly high affinity towards G-quartet-containing oligonucleotides particularly when the G-quartet is located on the 5'- or 3'- terminal ends of an oligonucleotide (Benimetskaya et al., 1997a). Thus these receptors likely exhibit similar affinity towards FW which also contain guanine-aggregates at the 3'-termini.
The greater inhibition by heparin observed for the uptake of FW in comparison to single stranded DNA implies two things. One is that the greater cellular uptake of FW relative to single stranded DNA is due to the presence of heparin-binding cell surface receptors which exhibit higher affinity towards the 3'-guanine aggregate of FW. It is plausible that FW bind more efficiently to these receptors and trigger internalization in a much more efficient manner. This would imply that the binding of the receptors with DNA Frayed-wire is electrostatic and independent of the DNA sequence. A similar effect has been described by Pearson et al. (1993) with regards to the binding of G-quartet to macrophage scavenger receptors. In their study, they characterized binding of G-quartet containing oligonucleotides to macrophage scavenger receptors and suggested that this high affinity binding is due to the spatial distribution of negatively charged phosphates in polynucleotide quadruplexes forming a charged surface which electrostatically binds to the positively charged surface of a receptor domain. The electrostatic binding of G-quartets have also been described in the binding to HIV envelope protein gp-120 (Callahan et al., 1991; Wyatt et al., 1994; Suzuki et al., 1999). We propose that the negatively charged guanine-aggregate domain (the stem) of FW binds electrostatically to a positively charged domain or the positively charged surface created by overall structure of the receptors. This binding event triggers endocytosis. Using the same reasoning, the smaller uptake of single stranded DNA could also be attributed to its lower negative charge density than FW and the involvement of non-heparin binding receptors or cell membrane proteins which are more sparse or less efficient at uptake.
The results of the heparin inhibition study (Fig.10) showed that increasing the concentration of heparin exponentially did not result in a complete elimination of FW uptake. This can be attributed to the fact that uptake of FW likely occurs through heparin-binding receptors mediated endocytosis as well as other endocytosis or passive diffusion pathways.

The results of the study of the cellular uptake of FW derived from different parent-oligonucleotide sequences offered some important information. First, it allowed us to reconfirm that consecutive guanines are crucial in DNA Frayed-wire formations and this was demonstrated by forming FW using three different 15-guanine sequences other than d(A15G15) (Fig. 13). Secondly, we observed that their cellular uptake is similar regardless of the sequence of the arm region (Fig. 12) validating our previous statement that the electrostatic binding between the receptors and the stem of FW is likely responsible for their uptake. Using FW from d(G15A15), which has the stem at 5'-end rather than 3-end like the others, we were able to further emphasize the role of the guanine stem and also that the binding was not likely to be stereo-specific. This finding is similar to the binding between macrophage scavenger receptors and the G-quartet (Pearson et al., 1993).

Previous work from our laboratory has demonstrated that the arms and the stem of FW are independent and that the arms are able to bind to their complementary strands via Watson-Crick base pairing without disrupting the stem (Protozanova and Macgregor,
1998). Initial studies in HepG2 cells demonstrated a higher uptake for both complementary strand bound and unbound FW than for ssDNA (Piquette-Miller, 1999). This further support the previous suggestion that the guanine stem is mainly involved in the non-sequence specific receptor binding which triggers the uptake. More importantly, this suggested that because the arms are not involved in receptor binding, FW whose arms are complexed with either their complementary strands with potential therapeutic influences or with drugs are still able to bind to the receptors and be internalized.

An important piece of information further supporting the involvement of heparin-binding receptor is the additional inhibitory effect on FW uptake when metabolic poisons (sodium azide, 2-deoxy-D-glucose) were introduced to cells with heparin (Fig. 11). These chemicals, by themselves or in combination, reduced cellular uptake significantly but with these results only, it was difficult to make any conclusion beyond the fact that it was energy dependent. As stated, NaN₃ and 2-deoxy-D-glucose inhibit oxidative phosphorylation and glycolysis respectively (Sung and Silverstein, 1985; Sulliva et al., 1987) thus halting or slowing down these energy expending cellular process, such as endocytosis, but these inhibitors do not directly alter FW binding to cellular receptors. Therefore, further inhibition of FW uptake by heparin implies either that this additional effect is achieved by heparins interfering with FW binding to receptors or by heparin somehow affecting cellular metabolism.

It is evident from the chemical inhibition study (Fig. 11) that none of the conditions tested completely inhibited uptake. This may be attributable to inadequate
concentrations of inhibitors and/or pre-incubation with these chemicals. It is also possible that although the cellular processes are inhibited, there may have been sufficient energy left in cell energy reservoirs to fuel uptake of FW to a small extent or that it could be due to inhibitors exerting their effect slower than the uptake process thus allowing some internalization to occur (Nakai et al., 1996). It could also be due to passive diffusion. A small amount of uptake may occur through via passive diffusion. In contrast, it could be argued that the concentration of chemical inhibitors were cytotoxic thus allowing FW to passively diffuse through compromised or leaky membranes resulting in a small amount of intracellular accumulation. However, we demonstrated that cells treated with 50 mM NaN₃ had a greater than 98% viability at 25°C after 20 minutes. A study of endocytosis by Kock et al. (1996) showed that 50 mM 2-deoxy-D-glucose, 5 times the concentration we used, with one-hour incubation, also had no effect on cell viability. Thus these results rule out diffusion through leaky membranes. Cell viability studies in cells incubated with FW and our control single-stranded DNA for one hour also confirmed that it is very unlikely that their enhanced intracellular accumulation arise due to cytotoxicity.

It could be argued that cell-associated radioactivity, used to represent the presence of FW, represents internalized ³²P-label that was cleaved by phosphodiesterase or internalized ³²P labeled 5'-end nucleotides that was cleaved by nucleases rather than FW. Our results demonstrated that the uptake pattern of the latter was different from intact FW, suggesting that this is not the case (Fig. 14). Other laboratories have performed oligonucleotide uptake studies using the same type of labeling and claimed it
was not due to cleaved $^{32}$P label (Rando et al., 1995; Bishop et al, 1996; Benimetskaya et al, 1997a; Bates et al., 1999). More convincing evidence arrived when our intracellular samples were resolved by the denaturing PAGE where the samples exhibited the same ladder pattern as the stock FW that had never been exposed to cells. This indicated that high molecular weight species of FW populations are internalized and that they maintain their structures during this process. The lack of enzymatic degradation confirms their unusual stability.

### 6.2. Future Experiments

In addition to the studies we have carried out to characterize the cellular uptake of FW up to present, the following studies would surely provide further information that would allow us to generate a more concrete and detailed picture of this uptake. First, cell fractionation by centrifugation (reviewed by de Duve, 1975) would allow us to examine the internalized FW further and determine which cellular organelle FW are associated with. Additionally, by doing a time-course experiment using this procedure, we may be able to elucidate the intracellular trafficking of FW. In order to visually detect patterns of intracellular compartmentalization and trafficking, FW can be labeled at the 5' end with fluorescein and visualized by fluorescence microscopy, as Tonkinson and Stein (1994) did to visualize internalized oligonucleotides in HL60 cells.

Choosing other molecules as the control would provide additional information; 1) d(A$_{15}$G$_{15}$) with chemically modified guanines that prevent DNA Frayed Wire formation, 2) G-quartet forming oligonucleotide that have the same length of the parent strands, and
3) double stranded DNA. In order to look further into the involvement of receptor-mediated endocytosis, inhibition by cytochalasin B, which inhibits microtubular-microfilament system that is involved in intracellular vesicle transport, and by phenylarsine oxide, which is an inhibitor of internalization process, would be interesting (Nakai et al., 1996; Agnell et al., 1998). Inhibition of FW uptake by acidifying the cytoplasmic pH should tell us if uptake is indeed receptor-mediated and also able to exclude the possibility of fluid-phase endocytosis (Sandvig et al., 1987; van Deurs et al., 1989; Shoji et al., 1991). Another useful way of studying FW uptake would be to transfect cells transfected with genes which encodes for the heparin binding receptors. By comparing uptake of FW with transfected cells to non-transfected cells, we should be able to tell if uptake is indeed heparin-binding receptors mediated. More interestingly, by transfecting cells with genes which encode other receptor-mediated endocytosis pathways, we should be able to examine the uptake efficiency mediated by these pathways.

To further elucidate binding between heparin binding receptors and the guanine stem of FW, we would select a well-known heparin binding cell receptors with known competitor then calculate the binding constant for competitors, in presence of FW (Beninemtskaya et al., 1997a). To examine the involvement of electrostatic interaction between these two, if possible, we would neutralize the charges on FW after they formed and compare their cellular uptake with un-neutralized FW and also further convincing evidence may arise if treatment with dextran sulfate, which have been used with heparin
as a highly charged anionic polysaccharide (Zelphati and Szoka, 1996; Xu and Szoka, 1996), inhibit FW uptake in the same manner as heparin did.

Until now, we have been only concerned with FW entering the cells, therefore, we have radio-labeled our oligonucleotides and relied on detecting the radioactivity as a way of indicating their presence. In addition to radiolabeling, other means of detecting the presence of intracellular FW could involve detecting the alteration of the biological activity of cells, similar to other antisense studies (Burgess et al., 1995; Macaya et al., 1995; Alahari et al., 1996; Ginobbi et al., 1997). For example, by using anti-sense sequences of a highly expressed protein for the arms of FW or by attaching that sequence to the arms, the target protein expression level could be used to determine the presence of FW in cytoplasm.

Other than intracellular transport of therapeutics oligonucleotides or agents, there are other areas where the use of FW can be explored. One of these areas is concerned with human immunodeficiency virus research. Studies have reported that G-quartet containing oligonucleotides inhibit both cell-to-cell and virus-to-cell attachment by binding to HIV envelope protein gp120 at the V3 loop (Wyatt et al., 1994; Suzuki et al., 1999). The V3 loop maintains a high percentage of positively charged amino acids across all HIV strains (Callahan et al., 1991) and increased cationic composition of the V3 loop have been correlated with the extent of virus-mediated cell fusion and rapid viral replication (Fouchier et al., 1992). This suggests that binding with G-quartet is likely be
electrostatic rather than sequence specific thus there is the possibility that FW also bind
to the V3 loop of the gp120 and result in similar anti-viral activity as G-quartets.

Another interesting area is general cancer research. Bates and co-workers (1999)
published a very interesting study in which they demonstrated that G-quartet
oligonucleotides inhibit proliferation of DU145, MDA-MB-231, MCF-7 and HeLa
carcinomas. They reported that these G-quartet oligonucleotides bind to a specific
cellular protein called nucleolin, a correlation between the biological activity of the
oligonucleotides and non-sequence specific binding to nucleolin was found. Nucleolin is
a multifunctional phosphoprotein that is suggested to be a major protein of exponentially
growing eukaryotic cells and is closely related to the rate of cell proliferation (reviewed
by Srivastava and Pollard, 1999). This opens up another potential therapeutic use for
FW, if the binding described between the G-quartet oligonucleotides and the nucleolins is
electrostatic or/and non-stereospecific then FW may also bind to these proteins and
inhibit proliferation of carcinomas in the same manner as G-quartet, thus resulting in the
same anti-proliferative effects. Similarly, FW may be able to replace a number of
previously studied antisense or other biologically active G-quartet oligonucleotides in
which their activities were suggested to be non-sequence specific (Pearson et al., 1993;
Lin et al., 1994; Burgess et al., 1995; Macaya et al., 1995; Bishop et al., 1996;
Benimetskaya et al., 1997a).

FW is a structure with great potential due its fascinating characteristics that may
find utility in numerous areas of biological research. The results of these initial uptake
studies are promising but much work needs to be done to optimize its biological usage and other areas.
CHAPTER 7: REFERENCES

7.1. References


