"IN VITRO SELECTION OF SINGLE STRANDED OLIGONUCLEIOTIDE LIGANDS (APTAMERS) THAT INHIBIT THE ACTIVITY OF RECOMBINANT JAK2 KINASE DOMAIN"

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COMMITTEE DECISSION

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تعتمد كلية الدراسات العليا
هذه النسخة من الرسالة
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DEDECATION

To my mother for her love and support during my life;

To my father for his advice and support;

To my brother Wa'el for his encouragement;

To my beautiful sisters Mohyna, Domo, Eftikar, Asia;

To the beautiful flowers, my nieces Wajd and Sadeen

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LIST OF ABBREVIATIONS

SELEX	**Systematic Evolution of Ligands by Exponential Enrichment**
JAK	**Janus kinase**
JH	**Janus Homology**
HIV	**Human Immunodeficiency Virus**
VEGF	**Vascular Endothelial Factor**
LAN	**Locked Nucleic Acid**
MHC	**Major Histocompatibility complex**
INF	**Interferon**
ALL	**Acute Lymphoplastic Leukemia**
LIF	**Laser Induced Fluorescence**
CE	**Capillary Electrophoresis**
AMV	**Avian Myeloblastosis Virus**
RT	**Reverse Transcriptase**
MMLV	**Molonel Murine Leukemia Virus**
PDGF	**Platelet Derived Growth Factor**
AMD	**Age-related Macular Degeneration**
ETDR	**Early Treatment for Diabetic Retinopathy**
PSMA	**Prostate-specific Membrane Antigen**
HTS	**High Throughput Screening**
IC50	**Inhibitory Concentration 50**
Tyk2	**Tyrosine Kinase 2**
FDA	**Food and Drug Administration**
CML	**Chronic Myelocytic Leukemia**
CLL	**Chronic Lymphocytic Leukemia**
AML	**Acute Myelocytic Leukemia**
MPD	**Myeloproliferative Disorders**
CDK	**Cycline Dependent Kinase**
MALDI-MS	**Matrix-Assisted Laser Desorption/Ionization Mass Spectrometer**
TE	**Tris-EDTA**
TBE	**Tris-Borate-EDTA**

"IN VITRO SELECTION OF SINGLE STRANDED OLIGONUCLEIOTIDE LIGANDS (APTAMERS) THAT INHIBIT THE ACTIVITY OF RECOMBINANT JAK2 KINASE DOMAIN"

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ABSTRACT

Aptamers are nucleic acid ligands which are isolated from combinatorial oligonucleotide libraries by an in vitro selection method called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Aptamers exhibit highly complex and sophisticated molecular recognition properties and are capable of binding tightly and specifically to targets ranging from small molecules to complex multimeric structures.

Besides their promising application as molecular sensors, many aptamers targeted against proteins are also able to interfere with the biological function of proteins. One of these proteins is Janus kinase 2 (JAK 2) which is the non-receptor tyrosine that plays a key role in inducing many hematological malignancies such as in Myeloproliferative Disorders (MPD) when acquired genetic abnormality lead to constructive activation of JAK 2. This study discloses nucleic acid ligands (Aptamers) capable of binding with the kinase domain of Janus Kinase 2 (JAK2) and blocking its function by inhibition the kinase activity. The disclosed nucleic acid ligands are selected by a SELEX method.

1. Introduction.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is a screening technique that involves the progressive selection of highly specific ligands by repeated rounds of partition and amplification from a large combinatorial nucleic acid library (Sampson, 2003; Klug and Famulok, 1994). The products of the selection are called aptamers, which are short single stranded DNA, dsDNA, or RNA molecules, which bind with high affinity, attributed to their specific three-dimensional shapes, to a large variety of targets, ranging from small molecules to complex mixtures (Wilson and Szostak, 1999). It was initially introduced by two groups and it is based on general nucleic acid and protein separation techniques (Tuerk and Gold, 1990; Ellington and Szostak, 1990). Numerous aptamers have been developed during the past 18 years that can inhibit the activity of many pathogenic proteins. One of this is Macugen (Pegaptanib sodium) which is an antivascular endothelial growth factor aptamer approved in 2004 by the Food and Drug Administration of the USA for the treatment of age-related macular degeneration disease (Eugen et al., 2006).

Janus kinase 2 is a member of non-receptor tyrosine kinases that is characterized by the presence of three specific domains: a pseudo-kinase domain known as Janus homology domain 2 (JH2), a kinase domain (JH1), and regions of homology (JH3-7). Since many types I and II cytokine receptors lack protein kinase activity, they depend on JAK2 as signal transducer and activator of many transcription pathways to initiate cytoplasmic signal pathways (Yamaoka et al, 2004). Because of the crucial role of jak2 tyrosine kinase in the mediation of growth-promoting activities of cytokines, any abnormality in this protein that will lead to constitutive activation of JAK2 may have

pathological consequences. These abnormalities include chromosomal translocations and point mutations (Smith and Fan, 2008).

With the assertion of the therapeutic properties of aptamers to work in a way similar to monoclonal antibodies and the development of technologies to efficiently deliver them intracellularly, selection of anti-JAK2 inhibitor aptamers will hopefully be effective in the treatment of many disorders caused by constutive activation of JAK2.

2. Literature review.

2.1. Nucleic acids.

Establishing the structure of deoxyribonucleic acid (DNA) has been one of the major achievements of the twentieth century. Not only it yielded a myriad of practical benefits, but also it gave scientists the philosophical pride of understanding how heredity works. Although the structure of DNA was unknown until 1953 when Watson and Crick described the double helix structure, its chemical components had been known for thirty years. In the 1920s, the basic chemistry of nucleic acids was determined by Phoebus A. T. Levene, when he isolated from yeast cells and thymus tissue two types of nucleic acids: ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (Watson and Crick, 1953).

Since the discovery of DNA as the genetic material, nucleic acids (DNA or RNA) it was established that they do not only have a templating function, but they can also fold into complex three dimensional structures allowing them to perform other biological functions. For example, the tetrahymena thermophila group 1 introns are naturally occurring RNAs that can catalyze trans-esterification reactions (Grabowski et al., 1981; Kruger et al., 1982; Been et al., 1988). Other examples emerged from studies on Human Immunodeficiency Virus (HIV) and Adenoviruses which showed the ability of these viruses to encode small structural nucleic acid ligands that bind to viral or cellular proteins with high affinity and specificity and modulate the activity of these proteins that are essential for the replication of those viruses or inhibit the activity of cellular proteins that work as anti-viral agents (Cullen et al., 1989; Marciniak et al., 1990; Brugert et al., 2002).

2.2. Combinatorial chemistry.

Combinatorial chemistry is a new technology used to discover new functional molecules from large libraries of related but structurally different molecules that are then used for therapeutic, catalytic, or diagnostic purposes. Nucleic acid ligands are one kind of these molecules used in combinatorial libraries selected by a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Kopylov and Spiridionova, 2000; Osborne and Ellington, 1997).

2.3. Aptamers and SELEX.

2.3.1 The Concept of Aptamer.

Aptamers come from the Latin word "*aptus*" which means “to fit” and the Greek suffix "-*meros*" which means “portion”. They are non-naturally occurring ssDNA, dsDNA, or ssRNA that range in size from approximately 6 to 40 kDa and are selected from a pool of random sequences that have the ability to fold into complex three dimensional structures produced by a combination of Watson-crick base baring and non-canonical intermolecular interactions. They are capable of binding to a desired target with high affinity ranging from picomolar to nanomolar Kd, and with high specificity enabling it to distinguish between very closely related targets. Actually aptamers can be as sensitive and as specific as monoclonal antibodies if not better (Bunka et al., 2006; James, 2007; Famulok et al., 2007).

The first aptamer was actually created almost 40 years ago. In the late 1960s, Sol Spigelman realized that three fundamental processes of evolution could be applied to a population of RNA molecules in vitro. He used RNA-dependent RNA polymerase, the replicase protein of Qß bacteriophage, to copy complimentary RNAs that have mutations resulting from the intrinsic error rate of the polymerase in copying variants of Qß genomic RNA. The selection was based on the ability of particular RNAs to serve as efficient templates for the production of additional copies of themselves. The result, after multiple rounds of selective amplification and mutation, was a population of evolved RNA molecules that were amplified much more efficiently by the replicase compared with their original RNA molecules (Joyce, 2002).

Ellington and Szostak (1990) used a method to select RNA nucleic acid ligands that bind in high affinity and specificity to small molecules such as organic dyes and it was them who named the selected ligands as “Aptamers”. In the same year, Turek and

Gold were selecting nucleic acid ligands to bacteriophage T4 DNA polymerase (gp43) and they were the ones who gave the process its name; Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Tuerk and Gold, 1990).

The widespread success in the selection of functional RNAs molecules raised the issue of whether related nucleic acids could also give rise to functional sequences. Single stranded DNA aptamers have been selected for many different targets and so clearly oligonucleotides such as RNA and DNA can perform many simple biochemical tasks (Ellington and Szostak, 1992).

2.3.2 Structure of aptamers.

All nucleic acids share a universal force of structure formation which is the staking of hydrogen-bonded base pairs. Single- and double-stranded RNA and DNA molecules are shaped likewise by base pairing and base pair staking. Single-stranded molecules have coarse-grained structures called secondary structures, which are physically meaningful as folding intermediates. This contrasts the situation in protein structure formation, where secondary structures play a less important role. The secondary structure of single-stranded nucleic acids is built from substructures that are assumed to contribute additively to the free energy of the molecules (Santalucia, 1998). DNA and RNA molecules resemble in many algorithms and parameters used in the prediction of secondary structures. However DNA as a molecule in solution is more stable than RNA and less easily degraded. Moreover, DNA secondary structures are less stable because of weaker staking energies (Santalucia and Hicks, 2004; Flamm et al., 2000).

2.3.3 SELEX.

SELEX is a method used for in-vitro selection and evolution of nucleic acid ligands under specific conditions from large combinatorial nucleic acid libraries ranging in size from 10^{13} to 10^{15} different sequence that have the potential to bind in high affinity and specificity to a desired target (James, 2000; Gopenath, 2006). It involves three processes, namely: (I) screening of ligand sequences that bind to a target; (II) the oligonucleotide complexes with the target are separated from non-bound oligonucleotides via affinity methods; (III) and amplification of bound aptamers (Figure 1). Many modifications developed in conventional SELEX method since it was discovered to get more efficient selection for specific applications (Famulok et al., 2000; Djordjevic, 2007; Joyce, 1994; Eaton, 1997).

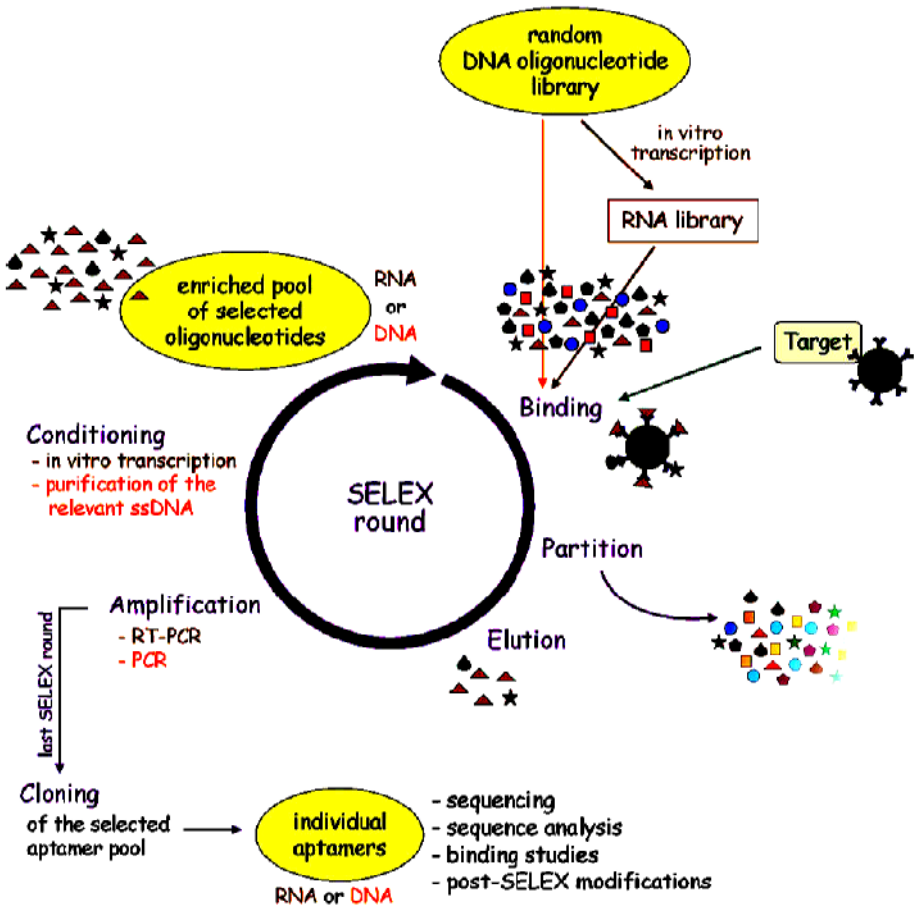


Figure 1. SELEX Processes. (Stoltenburg et al., 2007)

2.3.4 Aptamers library and complexity.

To construct a combinatorial library, the initial template is obtained via chemical synthesis of DNA fragments that contain a random sequence of 30-60 nucleotide flanked with defined sequences. For example, when the random sequence has 35n, there will be $N_{max}=4^n=4^{35}$ which is equal to $1.18*10^{21}$ individual sequence obtained. The actual degeneracy of a library depends on the amount of DNA used for selection rather than the maximum number of possible sequences. That means 10^{13} to 10^{15} of different sequences are needed for each selection process (Gold et al., 1995; Famulok and Jenne, 1998).

2.3.5 Aptamers characteristics

Aptamers have many properties and advantages that make them suitable for in wide applications in biotechnology.

2.3.5.1 Control of selection.

Aptamers are in-vitro selected which means that conditions such as pH, ionic strength, temperature and the process of selection is easily controlled to achieve efficient selection (Hianik et al., 2006; Musheev and Krylov, 2006).

2.3.5.2 Range of targets.

Several aptamers have been developed that can bind to a wide range of different targets. These include whole organisms (Trypanosome brucei) (Homann and Goringer, 1999), cells (cancer cells and bacterial cells) (Cerchial et al., 2005; Hamula et al., 2008), proteases (thrombin) (Bock et al., 1992), transcription factors (transcription factor E2F) (Ishizaki et al., 1996), growth factors (vascular endothelial growth factor) (Ruckman et al., 1998), viral proteins (HIV reverse transcriptase)

(Tuerk et al., 1992), bacterial spores (anthrax spores) (Bruno and Kiel, 1999),vitamins (B12 vitamin) (Lorsh and Szostak, 1994), peptides (neuropeptide nocicepttin/orphanin FQ) (Faulhammer and Eschgfaller, 2004), small molecules (Chloramphenecol) (Burke and Hoffman, 1997), amino acids (argenin) (Famulok, 1994),nucleic acids (ATP) (Sassonfar and Szostak, 1993) and even ions (zink) (Ciesiolka et al., 1995).

2.3.5.3 High affinity.

Aptamers bind to the target with high affinity comparable to those observed with monoclonal antibodies. The aptamer-target binding has an equilibrium dissociation constant (kd) ranging from picomolar to nanomolar concentrations without obvious correlation between the physical and chemical properties of the target and the affinity of binding of aptamer to the target. For example, the anti-l-selectin aptamer has 60 picomolar affinities, while L-selectin has a negative net charge at physiological pH (Jenison et al., 1998; Watson et al., 2000).

2.3.5.4 High specificity.

Aptamers are highly differential binders, as they are capable of binding to a member of a family of molecules in high specificity versus other related members of the same family (Eaton et al, 1995). For example, anti-L-selectin aptamers are 8,000-15,000 fold and 200-500 fold more specific to their target as opposed to P-selectin and E-selectin, respectively (O'Connell et al., 1996).

2.3.5.5 Toxicity.

Aptamers exhibit no or little toxicity when administered to the host as therapeutic agents. For example, a single-dose and sub-chronic doses with the aptamer macugen/EyE001 were administered to rats, rabbits, and rhesus monkeys showed only minimal toxic potency (Drolet et al., 2000; Eyetech study group, 2002, 2003).

2.3.5.6 Immunogenicity.

As for immunogenicity, studies on humans and primates showed no immunogenic responses. For example, no antigenic response was detected when single and multiple doses of intraviteral injection of anti-VEGF aptamer (macugen/EyE001) were administered in rhesus monkeys (Eyetech study group, 2002; Vater and Klussmann, 2003).

2.3.5.7 Cost effectiveness.

Aptamer production is very cost effective. Aptamers are produced by chemical synthesis rather than biological expression, as in the case of proteins, which makes their production much less expensive compared to the production of antibodies and other therapeutic proteins. The overall expected cost for the production of aptamers will be < 100/g US$ in the next few years.

2.3.5.8 Modification.

One of the major problems effecting the application of aptamers as diagnostic and therapeutic tools is their degradation by nucleases in a relatively short time. However, such a problem is answered by pre and post modifications of nucleic acids with chemical modifications or by using locked nucleic acid (LNA) technology that will increase the stability by increasing the resistance of aptamers to nucleases (Jellinek et al., 1995; Kubik et al., 1997; Lin et al., 1996., Pagratis et al., 1997; Schmidt et al., 2004).

2.3.5.9 Antidot availability.

An Antidote is available which can control the pharmacokinetic activity of aptamers. Rusconi and colleagues developed such a concept and design, utilizing Watson-Crick base pairing between the aptamer and an antidote oligonucleotide to alter the shape of

aptamer and then inhibit the binding of aptamer to its target (Figure 2) (Rusconi and Roberts, 2004; Dyke et al., 2006).

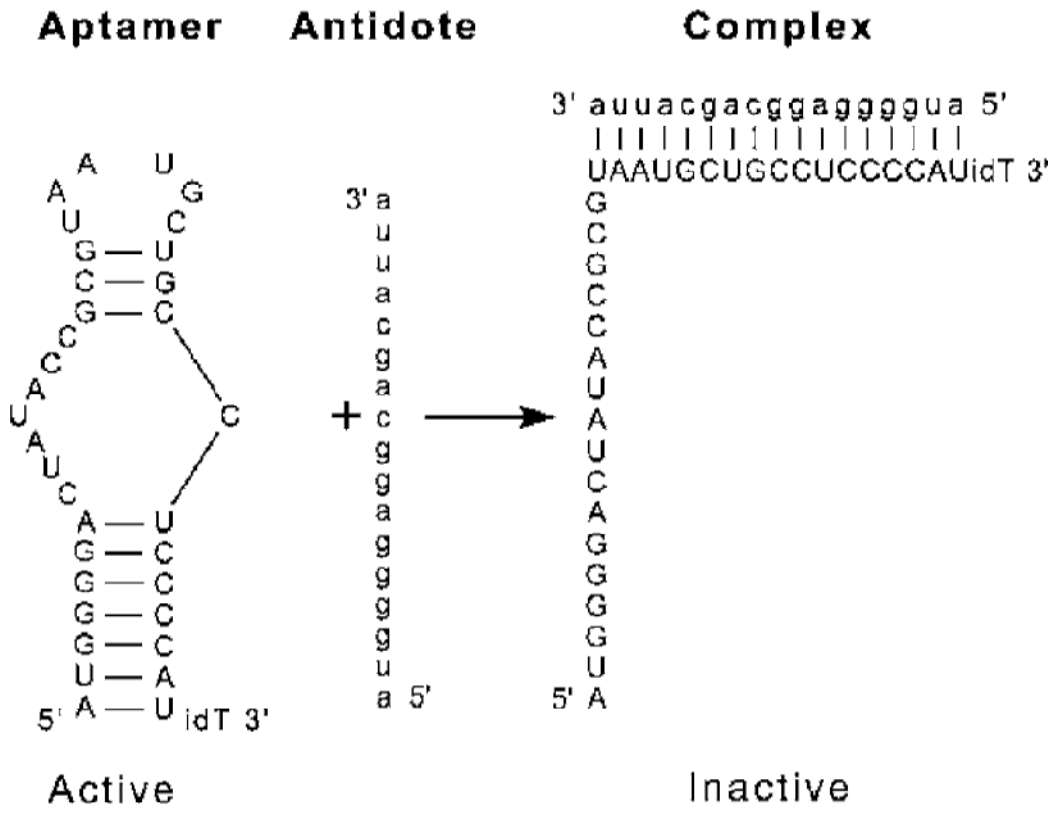


Figure 2. Mechanism of Antidote action against Aptamer (Rusconi and Robrts, 2004).

2.4 Aptamers and the targets.

2.4.1 Aptamers to proteins.

The collected experience of both academic and industry research suggests that aptamers are capable of binding to targets from any protein class. In nature, nucleic acid-based ligands or aptamers have been utilized by viruses and cells to bind to target proteins for millions of years. However, the concept of utilizing aptamers to bind and inhibit the activity of pathogenic proteins in the laboratory or the clinic has only been known since 17 years. The properties of aptamers make them an attractive class of molecules that meet and exceed the properties of antibodies, a predominant class of therapeutic compounds. In other words, aptamers now rival antibodies in many applications including diagnosis and therapy (Table 1) (Nimjee et al., 2005; Jayasena, 1999; Pendergrast et al., 2005).

2.4.1.1 Aptamers to enzymes.

Bergan et al (1994) described a new class of tyrosine kinase inhibitor, oligodeoxynucleiotides (ODNs) that directly inhibit autophosphorylation of Bcr-Abl p210 fusion transcripts in vitro by aptameric action of antisense oligonucleotides that targets Bcr-Abl mRNA with ki of 0.5 μM. These findings were supported by reduction in the growth of chronic myelogenous leukemia cells when DNA aptamers were introduced into these cells by electroporation (Schwartz et al., 1998).

Table 1. Properties of Aptamers viruses Antibodies (Nimjee et al., 2005)

Aptamers	Antibodies
Binding affinity in low nanomolar to picomolar range	Binding affinity in low nanomolar to picomolar range
Entire selection is a chemical process carried out in vitro and can therefore target any protein	Selection requires a biological system, therefore difficult to raise antibodies to toxins (not tolerated by animal) or non-immunogenic targets
Can select for ligands under a variety of conditions for in vitro diagnostics	Limited to physiologic conditions for optimizing antibodies for diagnostics
Iterative rounds against known target limits screening processes	Screening monoclonal antibodies time consuming and expensive
Uniform activity regardless of batch synthesis	Activity of antibodies vary from batch to batch
PK parameters can be changed on demand	Difficult to modify PK parameters
Investigator determines target site of protein	Immune system determines target site of protein
Wide variety of chemical modifications to molecule for diverse functions	Limited modifications of molecule
Return to original conformation after temperature insult	Temperature sensitive and undergo irreversible denaturation
Unlimited shelf-life	Limited shelf-life
No evidence of immunogenicity	Significant immunogenicity
Cross-reactive compounds can be isolated utilizing toggle strategy to facilitate pre-clinical studies	No method for isolating cross-reactive compound
Aptamer-specific antidote can be developed to reverse the inhibitory activity of the drug	No rational method to reverse molecules

Bock et al (1992) and Coughlin (2000), isolated single stranded DNA aptamers to Thrombin which is a serine protease enzyme that plays key role in the coagulation cascades. The selected ssDNA aptamers prolonged the clotting time from 25 seconds to 169 seconds in the purified protein and from 25 seconds to 43 seconds in human plasma (Griffin et al., 1993; Denanda et al., 1994).

An obvious target for SELEX in HIV therapy is reverse transcriptase (RT). Tuerk et al (1992) used RT as a target to isolate RNA ligands that inhibited HIV replication. Through selection they isolated a ligand that specifically bound to HIV RT and

inhibited its activity. Cellular experiments showed a 90%–99% reduction in HIV-1 replication in vitro and in cell culture (Joshi and Prasad, 2002).

2.4.1.2 Aptamers to receptors and adhesion molecules.

In 1998, two aptamers were described that were specific to CD4 receptors in the human and rat homologies that bind the receptor with high affinity (Kraus et al., 1998). CD4 is a cell surface receptor expressed on a variety of immunological cells, most notably helper T cells. This costimulatory receptor acts to enhance the primary immune response conveyed by the T cell receptor. The ligand targeting the rat receptor was shown to inhibit CD4 function in vitro, whereas the ligand specific to the human receptor is used as a targeting moiety to fluorescently label CD4 cells (Davis et al., 1998) and to deliver cargo (including siRNA) to cells expressing the receptor (Guo et al., 2005).

The selectins, which include L-, E-, and P-selectin, are a family of cell adhesion molecules that are expressed on leukocytes, endothelial cells, and platelets. They are implicated in a number of inflammatory diseases as well as tissue injury and infection. A 2`-fluropyramidine-modified RNA library was screened by SELEX against P-selectin/IgG (PS-Rg) fusion protein to isolate anti-inflammatory aptamers (Bevilaqua and Nelson, 1993). After twelve rounds of selection, an RNA aptamer with kd 18 pmol/L binds to PS-Rg protein on activated human platelets and inhibits both cellular adhesion of PS-Rg to neutrophil and completely blocks neutrophil rolling on activated platelets in vitro (Jenison et al., 1998).

2.4.1.3 Aptamers to cytokines and growth factors.

An endothelial receptor tyrosine kinase, Tie 2, plays an important role in vascular stability. Angiopoitin-2 is an antagonist to Tie 2 that appears to be expressed only during active angiogenesis seen in tumors (Halash et al., 1999). White et al (2003), developed RNA aptamers that inhibit angiopoitin-2 function in cell culture and in a rat corneal angiogenesis model, where the aptamer inhibited neovascularization by >40%.

The function of major histocompatibility complex (MHC) class I and II genes as well as intracellular adhesion molecules 1 are upregulated by interferon γ (IFN- γ) in numerous cell types. Secretion of IFN- γ can result in inflammatory and autoimmune diseases (Van Seventer and Shimizu, 1990). RNA selections using 2`-fluropyramidines, and 2`-aminopyramidines modified RNA and a mixture of the two modifications were screened for aptamers that bound to and inhibited IFN- γ. The selected aptamers inhibited IFN- γ binding to its receptors on A549 human lung carcinoma cells with Ki of 10 nmol/L. These aptamers also inhibited IFN- γ-mediated induction of MHC class 1 antigen and ICAM-1 expression, exhibiting IC50 values of 700nmol/L and 200nmol/L respectively (Kubik et al., 1997).

2.4.1.4 Aptamers to antibodies and immunological molecules.

Autoimmunity usually entails aberrant recognition of self-antigens by antibodies, which lead to a variety of pathologies. Eleven rounds of RNA selection were completed against anti-insulin receptor antibodies, and the elected RNA aptamers bind to anti-insulin receptor antibodies and completely inhibit the monoclonal antibodies from binding to insulin receptor (Dunda et al., 2995). These results suggested that RNA aptamers can structurally mimic protein epitopes. However, the

problem was in the stability of the selected RNA aptamers against nucleases. This problem was answered by selection of 2`-aminopyrimidine-modified RNA aptamers. Its capacity to resist degradation by nucleases was measured by incubation of modified and nonmodified RNA aptamers in 10% human serum. While the nonmodified degraded completely in 15s, most amino-modified RNA aptamers were still present as a full-length ligand after 24 hours (Lee and Sullenger, 1996).

Complement C5 is a serum glycoprotein that is cleaved to vasoactive C5a and C5b during complement activation, and as a central component of inflammation, it subsequently stimulates neutrophil-endothelial adhesion, cytokine and lipid mediator release and oxidant formation, which are associated with numerous inflammatory states and injury (Morgan, 1995; Mulligan et al., 1997). An RNA library containing 2`-fluropyramidines were screened by SELEX to isolate aptamers that bind to C5. Aptamers were identified after 12 rounds of selection process that bound to C5 with a Kd of 20 to 40 nM and inhibited its activity in human serum hemolytic activity assay (Biesecker et al., 1999).

2.4.1.5 Aptamers to viral particles.

New therapeutics are urgently needed for the treatment of pandemic influenza caused by H5N1 influenza virus. Recently, Cheng et al (2008), screened DNA aptamers by SELEX process to target HA1 proteins of the H5N1 influenza virus, and after 11 rounds of selection, DNA aptamers that specifically bind to the HA1 protein of H5N1 virus were shown to have antiviral activity in in-vitro studies. These aptamers would be expected to disrupt virus entry, and thus slow the infection process so the host immune system has time to respond (Cheng et al., 2008).

2.4.2 Aptamers to whole organisms and cells.

Parasitic diseases are among the most devastating illnesses in the world. They cause the suffering of hundreds of millions of people and the problem is amplified by the fact that the number of drugs available for treating parasitic infections is very small. Most of the available therapeutics were discovered decades ago and are not very effective. Homann and Goringer (1999) were the first to apply the SELEX technology to Trypanosoma brucei which is the causative agent of sleeping sickness in humans. The SELEX experiment successfully identified an RNA aptamer family that recognized an invariant surface component of bloodstream stage trypanosomes. The experiment confirmed that SELEX technique can be performed with live cells and it further verified that even without knowledge of all elements of a target's surface, specific ligands can be selected and subsequently analyzed (Homann and Goringer, 1999).

Identifying cells associated with specific malignant states is critically important for the early detection and diagnosis of cancer. To facilitate this task, molecular probes, which bind biomarkers that are either specifically or differentially expressed in diseased cells relative to healthy cells, provide a simple and effective method. SELEX method provides the opportunity to select aptamers that recognize unique molecular signatures of cancer cells by a process named cell-SELEX. In 2006, Shangguan et al. isolated DNA aptamers that bind to T cell acute lymphoblastic leukemia (ALL) cell lines. Ramos cell lines were used as a negative control to reduce the collection of DNA sequences that could bind to common surface molecules present on both types of cells (Figure 3) (Shangguan et al., 2006;Shangguan et al., 2007; Phillips et al., 2008) .

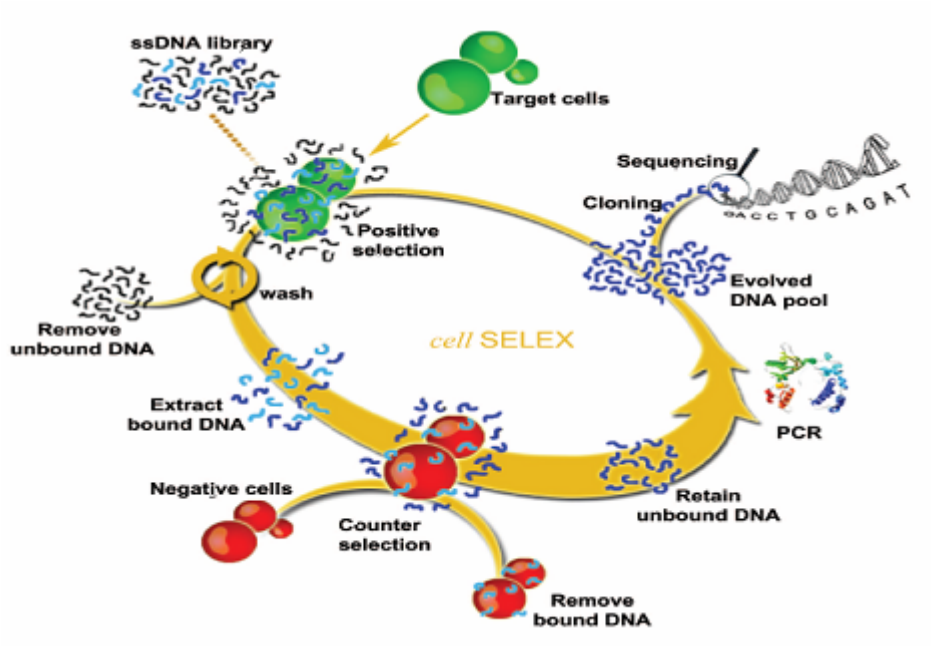
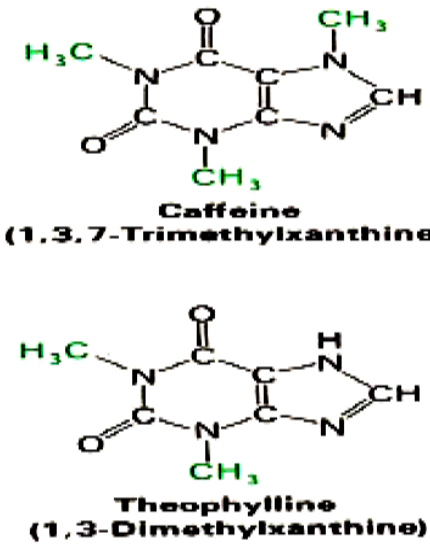


Figure 3. Cell-SELEX (Shangguan et al., 2006)

2.4.3 Aptamers to small molecules.

The RNA world hypothesis led to the belief that aptamers are capable of binding small molecules (Maurel and Hanni, 2005). According to this theory, RNA molecules with catalytic properties, so called ribozymes, should also be able to bind cofactors. Moreover, ribozymes might be regulated allosterically by small cellular components. Therefore, aptamers were shown to have the capability to bind small molecules such as ATP (Sassonfar and Szostak, 1993), coenzyme A (Burk and Hoffman, 1998), amino acids (Famulok, 1994), carbohydrates (Yang et al., 1998), organic dyes (Ellington and Szostak, 1992), antibiotics (Wallis and Schroeder, 1997), toxins (Hirao et al., 1997), or ions (Ciesiolka et al., 1995). In addition to the ability of aptamers to efficiently interact with small molecules they bind with high specificity and differentiate between very closed small molecules. For example, aptamers are able to differentiate between caffeine and theophylline which bind 10 000 times to theophylline better than caffeine (Fuger 4) (Jenison et al., 1994).



Figuer 4. Chemical structures of Theophylline and Caffeine (Jenison et al., 1994).

2.5 Applications of aptamers.

The applications of aptamers are so numerous that studies which described their use appear in the literature on a weekly basis. In comparison with the field of monoclonal antibodies, aptamers suffer from modest range of downstream technology which would enable their use routinely in diagnosis, therapy, delivery vehicle for other molecules, regulation of gene expression, and as reagents for high-throughput screening and drug discovery. The following paragraphs describe some applications of aptamers.

2.5.1 Analytical application of aptamers.

So far, several bio-analytical methods have used nucleic acid probes to detect specific sequences of RNA or DNA targets through hybridisation. More recently, specific nucleic acids, aptamers, selected from random sequence pools, have been shown to bind non-nucleic acid targets, such as small molecules or proteins. The development of in vitro selection and amplification techniques has allowed the identification of

specific aptamers, which bind to the target molecules with high affinity. Moreover, several aptamers were selected against difficult target haptens such as toxins and prions. This makes aptamers suitable for use in many analytical applications.

2.5.1.1 Aptamers in chromatography.

Aptamers represent a great promise as molecular recognition tools for their incorporation into analytical devices and they can be used as immobilized ligands in separation technologies as in the field of affinity chromatography (Clark and Remcho, 2002). For example, an aptamer specific for l-selectin has been used to purify human l-selectin receptor globulin (LS-Rg) fusion protein produced in Chinese hamster ovary cells transfected to express the protein. The 5`-biotinylated aptamer was fixed to a streptavidin-modified resin and then the resin was packed into a chromatographic column. In the first purification step, the aptamer-based column yielded a 1500-fold purification with 83% recovery. As a negative control, a column was prepared with a DNA having a scramble sequence with respect to the specific aptamer and purified LS-Rg was applied to this column. The new sequence failed to bind detectable levels of LS-Rg demonstrating the high selectivity of the column (Roming et al, 1999).

2.5.1.2 Capillary electrophoresis.

The separation and detection advantages of aptamers make them a very attractive affinity probe in capillary electrophoresis- based quantitative assays of proteins (German et al., 1998; Kotia et al., 2000). Electrophoresis can be used to efficiently separate free aptamers from the aptamer–target complex due to the changes in electrophoretic properties which can arise from structural transitions or changes in size when the aptamer interacts with the target molecule. Moreover, aptamers can be

easily fluorescently labelled allowing a sensitive detection in capillary electrophoresis with laser-induced fluorescence (LIF). Using an aptamer specific for the reverse transcriptase of the type 1 human deficiency virus (HIV-1 RT), a non-competitive capillary electrophoresis/LIF (CE/LIF) affinity assay has been developed. The method was based on the use of a fluorescently labelled single-stranded DNA aptamer and could quantify HIV-1 RT up to 50 nM, without any interference from RTs from other viruses such as avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV). The assay was not presented for the direct determination of HIV-1 RT in whole blood or serum, but it was proposed to determine RT activity in cell cultures of the HIV-1 virus. Several advantages of this aptamer-based assay have been evident, such as high specificity, decreased analysis time, simpler chemical procedure and the avoidance of radiolabeled materials (Pavski and Le, 2001).

2.5.1.3 Mass spectrometry.

Aptamers as substrates for protein capture and analysis in affinity matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) have been recently described. A DNA thrombin-binding aptamer has been used for direct detection of thrombin, after covalent binding to a fused-silica glass surface. A comparison with a scrambled oligonucleotide that cannot form the characteristic G-quartet demonstrated that thrombin is captured only by the specific aptamer. Different amounts of thrombin, from 5 to 50 pmol, were incubated with the aptamer spots. The results showed optimal detection at 10 pmol of thrombin with a detectability varying among aptamers spots, but reaching 5 pmol in the best cases. The cycle of capture, release and analysis could be repeated on the same spot by simply unfolding the aptamer upon application of low pH matrix. The aptamer-modified surface was able to

selectively capture thrombin also from a complex matrix such as human plasma (Dick and McGown, 2004).

2.5.1.4 Biosensors.

The application of aptamers as biocomponents in biosensors offers, over classical affinity sensing methods mainly based on antibodies, a multitude of advantages, such as the possibility to easily regenerate the function of immobilized aptamers, their homogeneous preparation and the possibility of using different detection methods due to easy labeling (Jayasena, 1999; O'Sullivan, 2002; Luzi et al., 2003; You et al., 2003).

The most recent growth factor aptamer-based detection method appeared in literature is centered on the use of a platelet derived growth factor (PDGF) DNA aptamer. This approach has been presented as well suited for point of care diagnostics, due to the high sensitivity and selectivity. The assay was based on the use of the PDGF aptamer modified with methylene blue (MB) immobilized onto a gold electrode which exploits the capability of the aptamer to fold in its characteristic structure when in contact with the target molecule in the unfolded structure. Hence in the absence of PDGF, the aptamer has only one of the three characteristic structural stems and MB, fixed at the aptamer end, is far from the electrode surface. In the presence PDGF, the aptamer adopts the three stems structure and the distance between MB and the electrode decreases improving the electron-transfer activity, with an increase of current (Figure 5). Exceptional sensitivity was presented for this method with a detection limit of 50 pM for PDGF, with the sensitivity examined in buffer and 50% diluted serum (Lai et al., 2007).

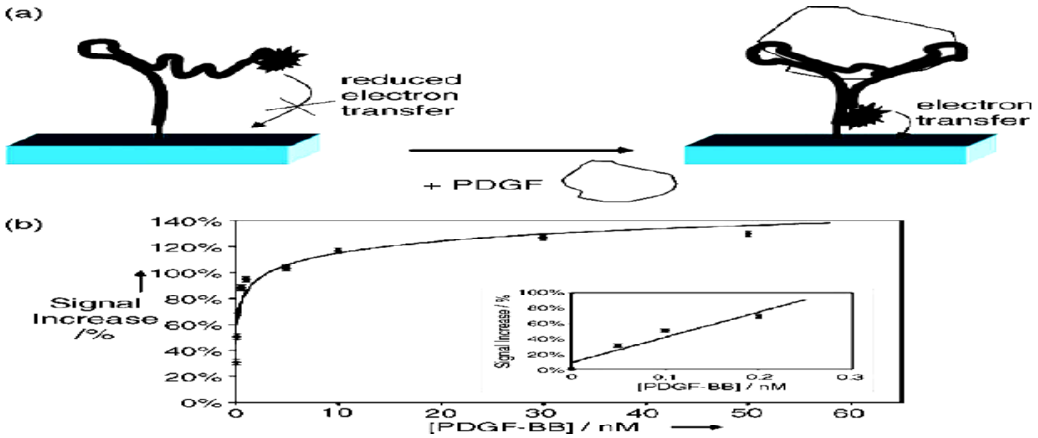


Figure 5.(a) Scheme of the aptamer-based electrochemical detection of PDGF. (b) Dose–response curve of the sensor for increasing concentrations of PDGF(Lai et al., 2007).

Moreover, several schemes have been adopted to transform aptamers into "signaling aptamers", which are mainly based on the report of the target presence by real-time fluorescence signaling "aptamer beacons" (Li et al., 2002). One of the strategies to modify aptamers with fluorophores and to design a real-time signalling aptamer is the attachment of a fluorophore at a specific position of an aptamer which can undergo a conformational change induced by the binding of the target (Figure 6) (Jhaveri et al., 2000).

Stojanovic and Landry (2002) used an aptamer specific for cocaine to develop a colorimetric probe for this molecule. A pool of 35 different dyes was first screened to examine their binding to the aptamer in the presence or absence of cocaine. One of these dyes, diethylthiotricarbocyanine iodide, was chosen to construct the colorimetric sensor: after incubation of the dye with the aptamer, cocaine addition causes a displacement of the dye with an attenuation of absorbance proportional to the concentration of the added cocaine (Figure 7) (Stojanovic and Landry,2002).

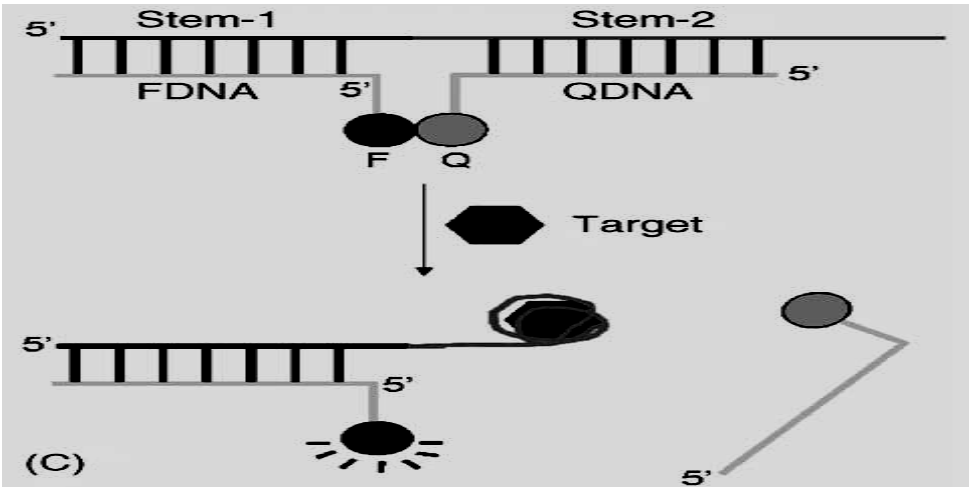


Figure 6. Fluorophore-labelled DNA aptamer with a small complementary oligonucleotide (QDNA) modified with a quencher. Without the target molecule the aptamer hybridises with the QDNA, bringing the fluorophore and quencher into proximity for fluorescence quenching. In the presence of the target, the formation of the aptamer–target complex is favorites with respect to that of the aptamer–QDNA duplex and the QDNA is released from the fluorophore-labelled aptamer causing an increase in the fluorescence intensity (Jhaveri et al., 2000).

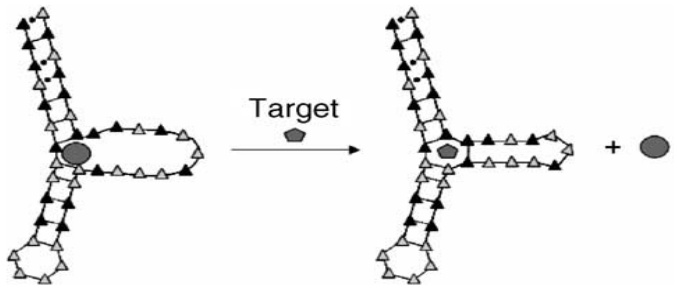


Figure 7. Colorimetric probe for detection of cocaine (Stojanovic and Landry, 2002).

Aptamer based assay has been recently presented by Liu et al., 2006, with a ‘‘dipstick’’ format with detection results which can be seen by the naked eye and do not need sophisticated instruments. Colorimetric sensors could represent an improvement in this direction. To overcome drawbacks, lateral-flow technology has been used for the development of an aptamer-based dipstick assay for cocaine. Nanoparticles were functionalized with two different DNA sequences, partially

complementary and containing the cocaine aptamer. The nanoparticles carrying the aptamer were further functionalized with biotin. In the absence of the aptamer target molecule, the particles aggregate and, due to the large size, they cannot migrate along a streptavidin-modified lateral flow device on which they are deposited .If the membranes are dipped into a solution containing cocaine, the aptamer binds to its target, the particles disassemble and in this form they can migrate along the pad. If streptavidin was applied on the membrane as a thin line, the protein can capture the aptamer-coated nanoparticles with biotin and a red line appears (Figure 8). The intensity of the red bands increased with increasing concentrations of cocaine and a detection limit of 10 µM could be reached. The dipstick device was also successfully applied to the detection of cocaine in serum (Liu et al., 2006).

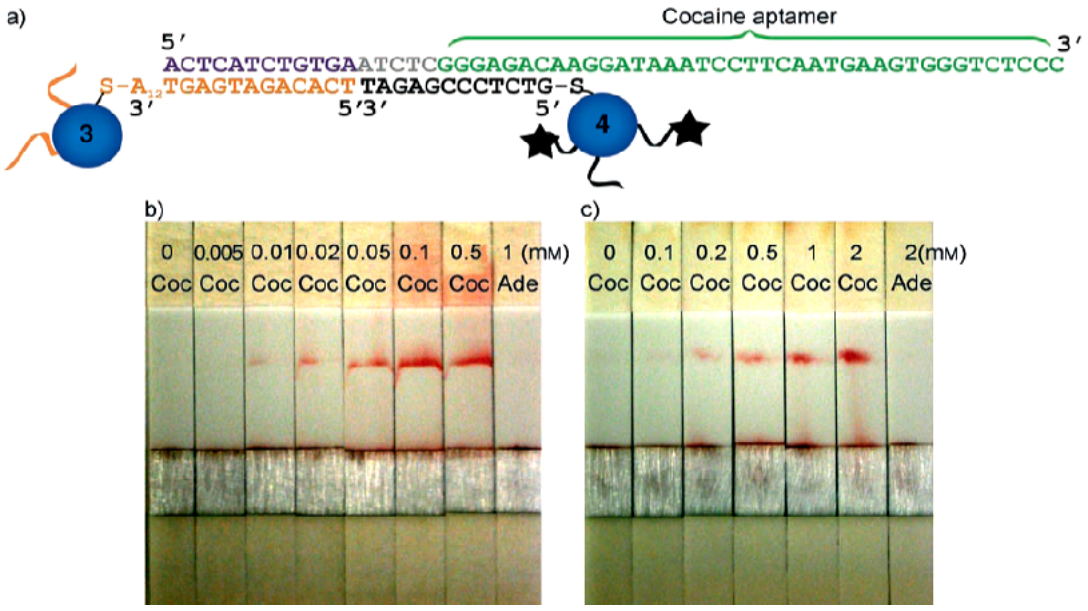


Figure 8. Dipstick assay for the detection of cocaine. (a) DNA sequences and linkages in cocaine aptamer-linked nanoparticle aggregates. Test of varying concentrations of cocaine in buffer solution (b) and in undiluted human blood serum (c). Coc, cocaine; Ade, adenosine (Liu et al., 2006).

2.5.2 Therapeutic applications of aptamers.

Aptamers have many properties as described previously that make them work as drug like molecules, keeping in mind the behavior of aptamers in vivo. In other words, the pharmacokinetics and pharmacodynamics of aptamers in vivo. The year 2004 was a milestone year for the therapeutic application of aptamers when the US food and drug administration (FDA) approved pegaptanib sodium (Macugene) which is a chemically modified RNA aptamer directed against vascular endothelial growth factor (VEGF-165) to treat age-related macular degeneration (ADM) disease. VEGF-165 isoform is primarily responsible for pathological ocular neovasculaization and vascular permeability (Figure 9). In Phase I clinical trials 80% of treated patients exhibited stable or improved vision three months after treatment, and 27% demonstrated a three-line or greater improvement on the Early Treatment for Diabetic Retinopathy Study (ETDRS). A phase II study reported no serious side effects resulting from multiple intravitreal injections of Macugen with or without photodynamic therapy, and 87.5% of the treated patients exhibited stabilized or improved vision three months after treatment and 25% demonstrated a three-line or greater improvement in vision on ETDRS. These results are the first to show that in vitro selected aptamers can be clinically efficacious drugs in humans (Eugene et al, 2006).

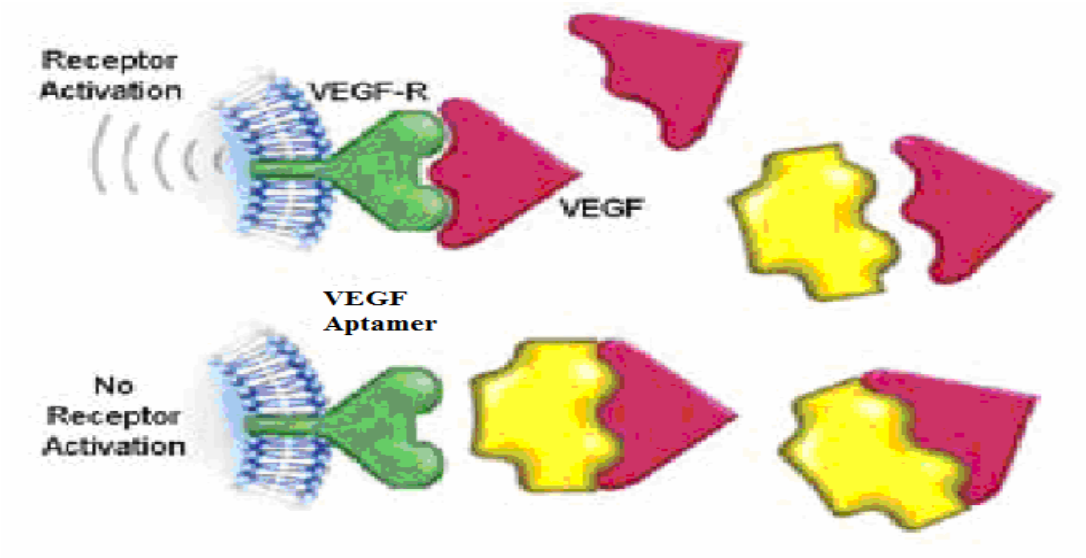


Figure 9. Mechanism of action of anti-VEGF aptamer (Nimjee et al., 2005).

2.5.3 Aptamers as delivery vehicles for other molecules.

Aptamers are frequently used as non-protein based alternatives to antibodies and are thus also potential targeting agents for the delivery of molecules such as small interfering RNA (siRNA). Aptamers targeting antigens as well as whole cells have previously been selected (Blank et al, 2001; Cerchia at al, 2005; Daniels et al, 2003). RNA aptamer were selected against prostate specific membrain antigen (PSMA) that is over expressed by prostate cancer cells (Lupold et al, 2002). Anti-PSMA aptamers were found to internalize themselves into the cell and to be capable of carrying nanoparticles into the cells expressing this antigen (Farokzad et al, 2004). The same aptamers were used to deliver functional siRNA molecules to cells that express PSMA antigen by generating aptamer: streptavidin: siRNA conjugate. The delivery using these aptamer conjugates was found to be efficient and specific for the cells expressing the PSMA antigen (Chu et al, 2006).

2.5.4 Aptamers that regulate gene expression.

The strong and highly selective ligands identified by SELEX are of potential interest for artificial regulation of gene expression. For example HIV-1 has 57 nucleotide long hairpin (TAR element) transcribed from the R region of the long terminal repeat of the HIV-1 genome (karn et al, 1999). The TAR hairpin located at the very 5` end of the retroviral mRNA serves as the binding site for the HIV protein Tat and for two cellular proteins, cyclin T1 and Cycline dependent kinase 9 (CDK-9) (karn et al, 1999; Rana and Jeang, 1999). This Tat-associated kinase phosphorelates the C-terminal domain of RNA polymerase and consequently trans-activates the transcription of the viral genome. DNA aptamers selected against the HIV-1 TAR RNA element form RNA/DNA kissing complex with TAR loop that compete the binding of proteins with TAR loop and consequently transfer with the trans-activation of transcription (boiziau et al, 1999; collin et al, 2000).

2.5.5 Aptamers as reagents for high-throughput screening.

Biochemical competition assays enable the screening of vast compound libraries under high-throughput screening (HTS) conditions. These assays usually require natural or synthetic compound partners. In other words, if a library compound competes with the interaction partner it suggests that this compound has a potential biological activity. Aptamers have advantages that make them proper reagents in HTS methods for efficient new drug discovery. Green and co-workers provided the proof of concept for aptamer-based screening in 2001 when they use anti-PDGF aptamer and anti-wheat germ agglutinin aptamer to screen a different set of small molecules. Both screening processes yielded inhibitors capable of inhibiting target activity in cell culture with low micromolar and in some cases nanomolar IC50 values (Green et al, 2001).

2.6 Kinases.

Kinases are enzymes that catalyze the transfer of phosphate groups from adenosine triphosphate (ATP) to determined target proteins (Drevs et al., 2003). Protein kinases constitute one of the largest recognized protein families represented in the human genome that control many different cellular functions by phosphorelating proteins involved in gene expression, metabolic pathways, cell growth and differentiation, membrane transport, and apoptosis (Cowan-jacob et al., 2006; Hunter, 1995; Kostich et al., 2002). There are 518 kinase sequences encoded in the human genome representing about (1.7 %) of all human genes of which 430 are expected to be catalytically active (Schijen and Griffin, 2002).

2.6.1 Tyrosine kinases.

Tyrosine kinases, based on the sequence homology, are the largest subgroup of human protein kinases with over 90 members. Tyrosine kinases are enzymes that phosphorylate tyrosine residues on protein substrates by transforming γ phosphate of ATP to protein substrates, on the kinase itself (autophosphorylation), or downstream signaling proteins (Till and HubBard, 2000).

The tyrosine kinase family is divided into receptor tyrosine kinases (58 members) such as VEGFR, PDGFR, C-KIT, and non-receptor tyrosine kinases (32 members) such as c-ABL, and JAK2 (Cowan-jacob et al., 2006). In general, tyrosine kinases play a key role in signal transduction pathways that control cell proliferation, differentiation, metabolism and death. One of the normal functions of tyrosine kinases is hematopoiesis and hematopoietic cell division. Subsequently, many tyrosine kinases may become oncogenic as a result of genetic alterations that induce

constitutive kinase activity (Schijen and Griffin, 2002; Paul et al., 2004). Mutated tyrosine kinases have a significant role in inducing many malignances, especially hematological malignances. Examples include BCR-ABL fusion transcripts seen in the vast majority of CML patients, as well as the constitutive activation of other oncogenes such as FLT3 in acute mylocetic leukemia (AML) pateints, c-Kit in systemic mastocytosis and gastrointestinal stromal tumers, platelets derived growth factor receptor (PDGFR) in chronic myelomonocytic leukemia patients, and Janus Kinase 2 in chronic myeloproliferative disorders (Schijen and Griffin, 2002; Paul et al., 2004;Gilliand and Levine, 2007; Campbell et al. 2006, Chalandon et al., 2005). Accordingly, tyrosine kinases, including JAK2, are considered as potential targets for many newly developed anti-cancer drugs and therapies. These therapeutic approaches included gene therapy using ribozymes and anti-sense nucleic acids, chemical inhibitors as well as monoclonal antibodies, all of which can specifically be targeted against a particular cancer related tyrosine kinase, whether at the RNA or protein level. Recently, aptamers have been described as another class of such inhibitory molecules (Ferrajoli et al., 2006; Wadieigh et al., 2005; Krause et al., 2005; Singer et al., 2004).

2.6.2 Janus kinases.

The JAK kinases were originally given the undistinguished name "just another kinase" (Wilks, 1989). However, because of the importance of JAKs as signal transducers and activators of many transcription pathways, they were then renamed "Janus kinases" in honor of the Roman God of Gates and doorways (Kaushansky, 2005).

Janus kinases are a family of non-receptor tyrosine kinases with four members including JAK1, JAK2, JAK3, and Tyrosine kinase 2 (Tyk2). In humans, JAK1 gene is located on chromosome 1p31.1, JAK2 is on 9p24, JAK3 is on 19p13.1, and Tyk2 is on 19p13.2. Since many types I and II cytokine receptors lack protein kinase activity, they depend on JAKs to initiate cytoplasmic signal pathways (Ihle, 1995). Jaks are relatively large kinases of approximately 1150 amino acids with apparent molecular weights of about 120–130 kDa. Their mRNA transcripts range from 4.4 to 5.4 kb in length (Leonard and O`Shea, 1998).

2.6.3 Janus kinase 2 (JAK2).

JAK2 (130 KD) is characterized by the presence of three specific domains: a pseudo-kinase domain known as Janus homology domain 2 (JH2), a kinase domain (JH1), and regions of homology (JH3-7) (Figure 10) (Giordanetto and Kroemer, 1998).

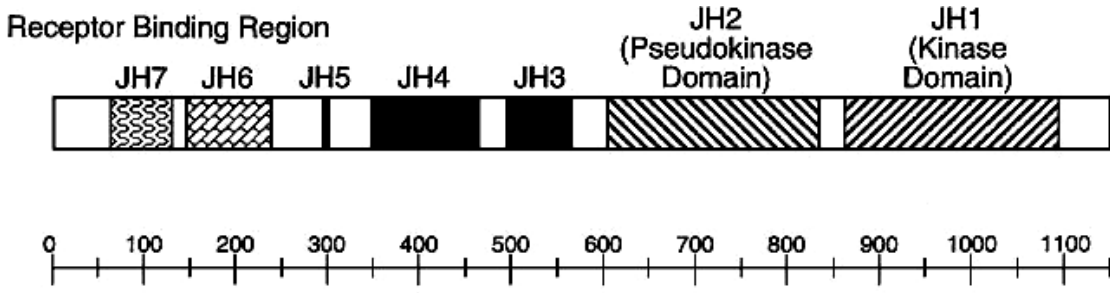


Figure 10. Structure of JAK 2 (Leonard and O`Shea, 1998).

2.6.4 JAK2 activation and signal transduction.

Activation of JAK2 requires phosphorylation of activation loop in kinase domain (JH1). Such activation occurs at three levels:

First, a transphosphorylation event of activation loop resulting from aggregation of kinase molecules of ligand-receptor-mediated regulation of activation. The second level of regulation involves psudokinase domain (JH2). Molecular modulation of JH1 and JH2 has two faces of interaction. One involves the activation loop in the kinase domain, and so phosphorylation of the activation loop requires the disruption of the interaction between the JH2 and JH1 that consequently relieve JH2 domain inhibition of kinase activity. The third level of regulation of JAK2 involves the FERM domain (four-point-one, ezrin, radixin, meosin) found in the N-terminal binding domain of JAK2 which contains the sequences necessary to promote association of the kinase with specific cytokine receptors. Recent studies show that autophosphorylation of Y119 residues in the FERM domain is a crucial event in down regulation of receptor-mediated kinase activation. Such phosphorylation results from aggregation of JAK2 binding receptor that will lead to loss of receptor binding (Figure 11) (Wilks et al., 1991; Saharinen et al., 2000; Funakoshi et al., 2006; Feng et al., 1997; Khwaja et al., 2006).

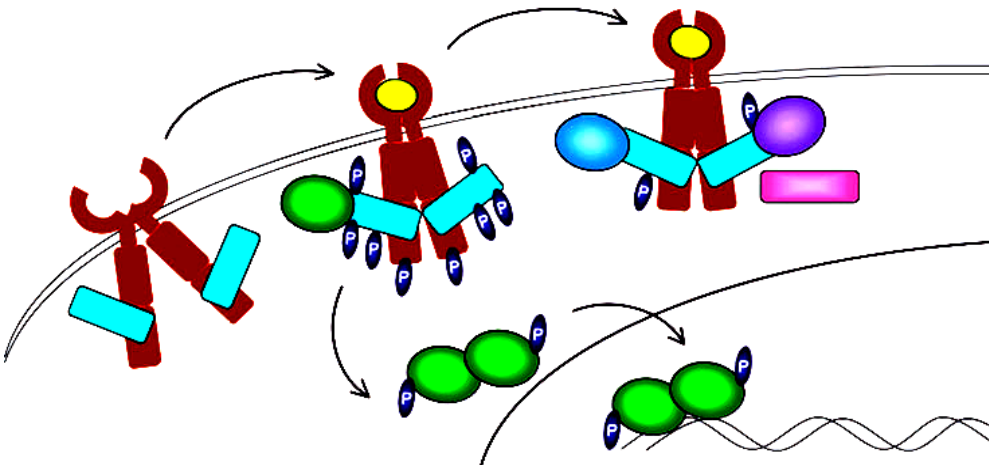


Figure 11. Signaling pathway of JAK2 (Morgan and Gilliland, 2008)

2.6.5 JAK2 genetic abnormalities.

Because of the crucial role of jak2 tyrosine kinase in the mediation of growth-promoting activities of cytokines, any abnormality in this protein that will lead to constitutive activation of JAK2 may have pathological consequences. These abnormalities include chromosomal translocations and point mutations (Figure 13).

2.6.5.1 Chromosomal translocations.

Chromosomal translocations have been identified in some hematological malignances such as fusion of BCR/JAK2 (t(9;2)(p24;q11)) present in CML and atypical CML patients, fusion of TEL/JAK2 (t(9;12)(p24;p13)) present in ALL, AML, and atypical CML patients, and fusion of PCM1/JAK2 (t(8;9)(p21-23;p23-24)) present in ALL, AML, and atypical CML patients (Reiter et al., 2005; Lacronique et al., 1997; Steelman et al., 2004).

2.6.5.2 Point mutations.

Point mutations have been recognized such as the recently described point mutation in JH2 domain which changes Valine at position 617 to Phenylalanine leading to the conistutive activation in JAK2. This mutation is expected to be present in approximately 99% polycythemia vera patients, and 50% of essential thrombocytosis and myelofibrosis patients (James et al., 2005; Levine et al., 2005; Baxter et al., 2005; Klarovics et al., 2005; Tefferial and Gilliand, 2005).

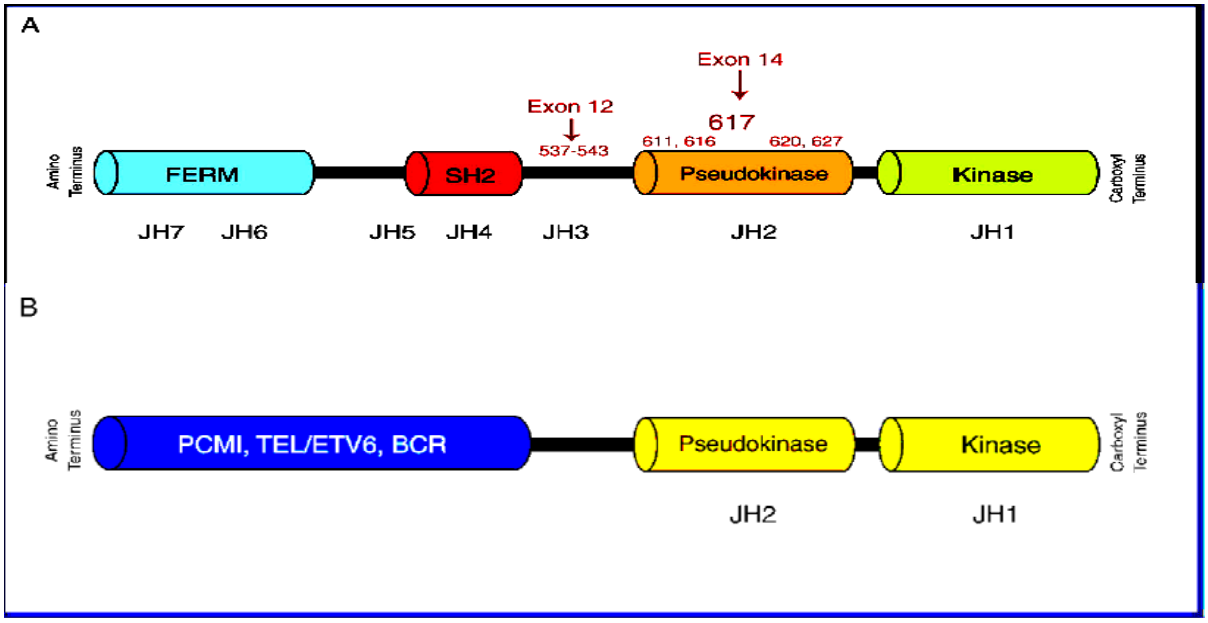


Figure 12. Genetic abnormalities in JAK2, A) Point mutaions, B) Chromosomal translocations (Smith and Fan, 2008).

2.7 Aims of the study.

1- In vitro selection of ssDNA aptamers that work as inhibitors for JAK2 kinase activity.

2- Establishment of theoretical and practical experiences in SELEX technology in our laboratory.

3. Materials and methods.

3.1 Instruments and equipments.

- Thermal cycler (MJ Research, USA)
- Gel documentation system (UVP, USA)
- Spectrophotometer (Smart Spec TM plus, BIORAD, USA)
- Electrophoresis chambers (Clever Scientific, UK)
- Electrophoresis power supply (Power Pac Basic TM, BIORAD, USA).
- Ultra-viol transilluminator (UVP, USA)
- Autoclave (Selecta, Spain)
- Incubator (flii Galli, Italy)
- Centrifuge (Biofuge, Heraeus, Germany)
- Digital balance (Denver, UK)
- Vortex mixer (HARRIS, UK)
- ELIZA reader (Tipo, UK)
- Pipettes (Witopet, Germany)
- PCR tubes (Bio basic inc, Canada).

3.2 Reagents.

- Active JAK2 kinase domain (poly-His tagged) (Upstate, USA).
- Tyrosine kinase activity assay kit (Chemicon, USA).
- ssDNA library (Medland certified company, USA).
- Synthesis of aptamers (Medland certified company, USA).
- Primers (Medland certified company, USA).
- Plasmid sequencing primer (Fermantas, France).
- Magna-His protein purification system (Promega, USA)
- Taq DAN polyemerase (Bio basic inc, Canada).
- $MgSO_4$ (Bio basic inc, Canada).
- PCR buffer 10x (Bio basic inc, Canada).
- Deoxy nucleiotides triphosphate (dNTPs) (Bio basic inc, Canada).
- Free nuclease water (Promega, USA).
- DNA ladder (Promega, USA)
- Hind III restriction enzyme (Fermantas, France)
- Hind III restriction enzyme buffer (Fermantas, France)
- pUC-19 plasmid (Fermantas, France).
- E-coli starin MJ107 (Fermantas, France).
- LE-agarose powder (Promega, USA).
- Low melting point agarose (Promega, USA).
- TE-saturated phenol (Promega, USA).
- Plasmid extraction kit (Bio basic inc, Canada).
- DNA plasmid transformation kit (Fermantas, France).
- Ligase enzyme (Fermantas, France).

- Ligase buffer 2X (Fermantas, France).
- Binding buffer (Promega, USA).
- Elution buffer (Promega, USA).
- Tris-Borate-EDTA 10X (Bio Basic Inc, Canada).
- TE buffer (Promega, USA).
- Loading dye (Promega, USA).
- Ethidium bromide (Promega, USA).
- Absolute ethanol (Reidel-de Haen, Germany).
- NaCl (Promega, USA).
- Sodium acetate (Reidel-de Haen, Germany)
- HEPES (Promega, USA).
- LB media (agar) (Invitrogen. USA).
- LB media (broth) (Invitrogen. USA).
- Ampicillin powder
- 5-Bromo-4-chloro-3-indolyl-β-D galactoside (Xgal) (Bio Basic Inc, Canada).
- Isopropyl-1-thio-β-D-Galactoside (IPTG) (Bio Basic Inc, Canada).
- $MgCl_2$ (Promega, USA)
- EDTA (Invitrogen. USA).
- Di-Methyl Sulphoxide (DMSO) (Gibco, USA)

3.3 Methods.

3.3.1 ssDNA library and primers design.

The initial template is obtained via chemical synthesis using cyanoethyl phosphoroamitide chemistry and purified by trityl-selective perfusion HPLC (TSPgrade) (Medland certified company, USA). DNA fragments were 79 base length containing random sequence of 35 nucleotide flanked with defined sequences for forward and reverse primers annealing and Hind III restriction site on the two defined sequences (Table 2).

5`-GGGC AAGCTT CTGAATTCGCAG-**35N**-GTGGATCCGT AAGCTT CGACTG-3`

Hind III Hind III

Where N is one of four deoxynucleiotides; A, T, C or G. The designed template will give theoretically 4^{35} of different sequence which is equal to 1.18 x 10^{21} individual sequences.

Table 2. Sequences of Forward and Reverse primers.

Primer	Direction	Sequence
F3	forward	5`-GGGCAAGCTCTGAATTCGCAG-3`
F1	reverse	5`-CAGTCGAAGCTTACGGATCCAC-3`

3.3.2 Small-Scale amplification.

The chemically synthesized oligonucleiotide pool should be first amplified enzymitically by PCR before initiating a selection experiment in order to eliminate damaged templates and provide multiple copies of the original templates. Optimal amplification conditions can be determined before large-scale amplification by carrying out small-scale trials. The optimal conditions were as follows:

The small-scale amplification for optimization to prepare the initial pool of dsDNA mixture was prepared by amplifying 0.33 ng of synthesized template which represents approximately 4 x 10^{12} different sequences in 100 µl of total volume PCR reaction in PCR mix containing 10 mM KCl, 10 mM $(NH4)_2SO_4$, 20mM Tris HCl (pH 8.75), 0.1% Triton-x 100, 0.1 mg/ml BSA, 200 µM of each dNTPs mix (Bio basic inc, Canada), 1 µM of the forward primer F3 5`-GGGCAAGCTCTGAATTCGCAG-3` and 1 µM the reverse primer F1 5`-CAGTCGAAGCTTACGGATCCAC-3 (Medland certified company, USA), 2 mM $MgSO_4$, 8 U of Taq DNA polymerase (Bio basic inc, Canada). PCR program was as follows :5 min at 95ºC for first denaturation, then 15 cycles of 1 min at 95ºC, 1 min at 50ºC, 1 min at 72ºC, followed by 10 min at 72ºC for final extension.

3.3.3 Large-Scale amplification.

The initial pool of dsDNA mixture was prepared by amplifying 10 µg of synthesized template which represent approximately 1.2 x 10^{14} different sequences in 3000 µl of total volume PCR reaction mix divided into 30 PCR tubes, each containing 100 µl of PCR reaction mix with same conditions optimized in small-scale PCR as described above. The average PCR efficiency identified by measuring the average number of

doubling for 15 cycle of PCR which is equal to approximately 7331 copy of each sequence of the original synthetic ssDNA template.

3.3.4 Small-Scale asymmetric PCR.

To produce ssDNA library from dsDNA we use the asymmetric PCR method which is a method that uses different primer ratios, one over the other, to yield one strand more than the other when amplified by PCR.

The small-scale amplification by asymmetric PCR was used to optimize the preparation of initial pool of ssDNA. It was performed by amplifying 3 µl of dsDNA PCR product of large-scale amplification in 50 µl of total volume PCR reaction in PCR mix containing 10 mM KCl, 10 mM $(NH4)_2SO_4$, 20mM Tris HCl (pH 8.75), 0.1% Triton-x 100, 0.1 mg/ml BSA, of each primer, 200 µM of each dNTPs mix (Bio basic inc, Canada), 2 mM $MgSO_4$, 4 U of Taq DNA polymerase (Bio basic inc, Canada).

PCR program was as follows :5 min at 95ºC for first denaturation, then 25 cycles of 1 min at 95ºC ,1 min at 50ºC, 1.5 min at 72ºC, followed by 10 min at 72ºC for final extension.

Fore asymmetric PCR, different forward to reverse primers ratios were used including 100:1, 50:1, 25:1, and 10:1. Then, the results for ratio10:1 was the ratio that gave the best yield of ssDNA.

3.3.5 Large-Scale asymmetric PCR.

Small-scale amplification by asymmetric PCR was performed by amplifying 10% of dsDNA PCR product of large-scale amplification in 5000 µl of total volume PCR reaction mix divided into 100 PCR tubes each containing 50 µl total volume in the same PCR conditions described in small-scale asymmetric PCR.

3.3.6 Agarose gel electrophoresis

PCR products from all amplification reactions were detected by electrophoresis on 4% agarose gel.

3.3.6.1 Preparation of 4% agarose gel

An adequate volume of electrophoresis 1X TBE buffer was prepared by dilution of one volume of 10X TBE buffer (Promega, USA) in nine volumes of distilled water. Then 2 grams of agarose powder were added (Promega,USA) into 50 mL of 1X TBE buffer and then the mix was melted in a microwave with swirling to ensure even mixing and clear appearance. Then 5 µl of 2.5mg/ml ethidium bromide was added to the mix and poured in the gel into the mini gel casting platform and allowed to harden.

3.3.6.2 Loading the samples

The gel casting platform containing the set gel was placed in the electrophoresis tank with sufficient electrophoresis buffer to cover the gel. About 10 µl of PCR product were mixed with 3 µl of loading dye (Promega, USA) and loaded into the well. Each run has 4 µl of DNA ladder mixed with 2 µl of loading dye to determine the size of the bands of the PCR product. To begin the electrophoresis the leads were connected with the power supply and the gel was run at 100 volt for 20 minutes. The DNA was visualized by placing on a UV light source and it was photographed directly.

3.3.6.3 Preparation of 4% low melting point agarose.

Preparation of 4% low melting point agarose (Promega, USA) is similar to preparation of 4% agarose gel described above.

3.3.7 Isolation of ssDNA fragments from low melting point agarose gel.

Asymmetric PCR produces ssDNA and dsDNA and so a proper method must be used to extract and purify the ssDNA product only with highest yields.

The method of extraction of ssDNA was from low melting point agarose was as follows:

After gel electrophoresis of asymmetric PCR product, the ssDNA desired band is cut out with clean scalpel and transferred it into microcenterfuge tube followed by addition of 100 µl of TE buffer, pH 8.0, (Promega, USA) to decrease the agarose percentage. After that, the gel slice is melt at 65°C for 10 minutes until the gel is dissolved. Equal volume of TE-saturated phenol, pH 8.2, (Promega, USA), is then added and mixed on a vortex for 2 minutes and then centrifuged for 15 minutes at 13,000 rpm. After centrifugation, the aqueous layer is collected into a new clean microcenterfuge tube and 2.5 volume of absolute ethanol and 0.1 volume of 3 M sodium acetate was added. This was followed by mixing well and incubation at -20˚C overnight to increase precipitation of ssDNA. Finally, mixture was centrifuged for 15 min at 13,000 rpm. Which produced a very small transparent pellet that appeared on the bottom of the tube. The supernatant was discarded and ssDNA was washed pellet with 70% ethanol to remove residual phenol and salts followed by centrifugation for 10 minutes at 13,000 rpm and the pellet was then rehydrated with binding buffer.

3.3.8 In vitro selection method.

3.3.8.1 Folding of ssDNA library.

Folding of ssDNA library into 3D structures was performed by heating the candidate mixture at 95ºC for 10 minutes in 200µl of binding buffer containing 100 mM HEPES (pH 7.5), 10 mM imidazole, 5 mM $MgCl_2$, and 150 mM NaCl (Promega, USA). Then the mix was rapidly cooled into 4ºC for 5 minutes and then incubated on ice for 15 minutes to insure proper folding of ssDNA ligands and then equallyberized at room temperature for 10 minutes.

3.3.8.2 Incubation of ssDNA library with His-Taged JAK2 kinase domain.

Selection for high affinity ssDNA ligands was done by incubation of candidate mixture of ssDNA with magne His Ni-particles in the first three rounds to eliminate false background selection of high affinity ligands to Ni particles. Then, the remaining ssDNA ligands were incubated with the kinase domain of JAK2 (200-400 ng, 400 ng in the first 7 rounds of selection and 200 ng for the next 7 rounds) for 1 hr at 37°C in a 200 µl binding buffer. Then, separation of the protein-ssDNA complexes from non-bound ssDNA was performed by addition of 5 µl of magne His Ni-particles to capture the JAK2-Aptamer complex for 10 minutes and washing Ni-JAK2-ssDNA complex three times with 200 µl of binding buffer for each washing in the first 7 rounds of selection and 5 washing times in next 7 rounds.

3.3.8.3 Elution of ssDNA from Ni-JAK2-ssDNA complex.

Separation of JAK2-ssDNA complex from Ni-JAK2-ssDNA complex was performed by addition of 100 µl of elution buffer containing 100 mM HEPES (pH 7.5), 500 mM imidazole, and 0.5 M NaCl (Promega, USA) for 5 minutes at room temperature. Then the separation of ssDNA from JAK2-ssDNA complex was performed by heating the complex at 80˚C for 5 minutes and extraction by TE-saturated phenol (pH 8.2) and precipitated with 2.5 volume of absolute ethanol and 0.1 volume of 3M sodium acetate, and washed with 70% ethanol. Then, ssDNA was amplified by PCR using the same conditions described above except the number of cycles which was 25 cycle in the first 7 rounds and 15 cycle for next 7 rounds because of the increase in the amount of eluted ssDNA after round 7 and then performing asymmetric PCR in the same conditions described above to produce ssDNA candidate mixture for the next round of selection.

3.3.8.4 Negative control PCR.

After each round, a negative control of PCR was performed to ensure that there is no contamination that interferes with the enrichment process of aptamers which give false selected sequences later in cloning and sequencing.

3.3.8.5 Rounds of selection

Fourteen rounds of SELEX were performed to select anti-JAK2 aptamers with conditions summarized in Table 3.

Table 3. Rounds of SELEX Conditions

Round	Number of PCR cycles*	Quantity of ssDNA	Quantity of JAK2	Incubation temperature	Number of washing	Incubation time
1	25	-	400 ng	37c°	3	1hr
2	25	1 µg	400 ng	37c°	3	1hr
3	25	1 µg	400 ng	37c°	3	1hr
4	25	1 µg	400 ng	37c°	3	1hr
5	25	1 µg	400 ng	37c°	3	1hr
6	25	1 µg	400 ng	37c°	3	1hr
7	25	1 µg	400 ng	37c°	3	1hr
8	15	0.5 µg	200 ng	37c°	5	1hr
9	15	0.5 µg	200 ng	37c°	5	1hr
10	15	0.5 µg	200 ng	37c°	5	1hr
11	15	0.5 µg	200 ng	37c°	5	1hr
12	15	0.5 µg	200 ng	37c°	5	1hr
13	15	0.5 µg	200 ng	37c°	5	1hr
14	15	0.5 µg	200 ng	37c°	5	1hr

* Number of PCR cycles for eluted ssDNA after incubation with JAK2.

3.3.9 Cloning.

After fourteen rounds of selection, the PCR product of round number fourteen was cloned into pUC-19 plasmid vector to determine the sequence of each individual aptamer as follows:

3.3.9.1 X-Gal preparation.

Preparation of 3% X-Gal was performed by dissolving 0.1gram of X-Gal powder (Bio Basic Inc, Canada) in 3 mL of DMSO (Gibco, USA) to final concentration 33 mg/ml.

3.3.9.2 IPTG preparation.

Preparation of IPTG was performed by dissolving 1gram of IPTG powder (Bio Basic Inc, Canada) in 50 ml of autoclaved distilled water to final concentration 10 mM.

3.3.9.3 LB solid media preparation.

Preparation of LB solid media was performed by dissolving 16 grams of LB powder in 500 ml of distilled water followed by heating the media until completely dissolved the powder. Then, the media was autoclaved for 15 min at 121˚C and after cooling the media to 50˚C about 25 mg of ampicillin powder to final concentration 50 µg/ml was dissolved in the media. Then 5ml of 10mM IPTG to a final concentration 0.1 mM and about 0.3 ml of X-Gal to a final concentration 20 µg/ml was added to the media. Then after well mixing, the media was poured into Petri dishes and stored at 4˚C.

3.3.9.4 LB broth media preparation.

Preparation of LB broth media was performed by dissolving 2 grams of LB broth powder in 100 ml of distilled water followed by heating the media until complete dissolving of powder. The media was then autoclaved for 15 min at 121˚C. After cooling the media to 50˚C, 5 mg of ampicillin powder was added to a final concentration 50 µg/ml and then poured into sterile 1.5 ml microcenterfuge tubes (1ml/tube) and stored at -20˚C.

3.3.9.5 Digestion of PCR product with Hind III restriction enzyme.

About 10 µl of PCR product of the round fourteen were digested by 20 units of Hind III restriction enzyme in 1X buffer containing 10mM Tris-HCl (pH 8.5), 10mM MgCl2, 100mM KCl, 0.1 mg/BSA. The reaction was completed with free nuclease

water to a final volume of 30µl and incubated at 37˚C for 4 hours. Then, Hind III restriction enzyme was inactivated by heating the reaction at 65c˚ for 20 minutes.

3.3.9.6 Digestion of pUC-19 plasmid with Hind III restriction enzyme.

About 0.5 µg of pUC-19 plasmid were digested by 20 units of Hind III restriction enzyme in 1X buffer containing 10mM Tris-HCl (pH 8.5), 10mM MgCl2, 100mM KCl, 0.1 mg/BSA. The reaction was completed with free nuclease water to a final volume of 50µl and incubated at 37˚C for 4 hours. Finally, Hind III restriction enzyme was inactivated by heating the reaction at 65c˚ for 20 minutes.

3.3.9.7 Preparation of E-coli competent cells.

Under aseptic conditions, 0.5 ml of liquid LB media without ampicillin was added to powder of freeze-dried and mixed well. Inoculating loop was used for streaking the mixture on nutrient agar plate which is then incubated overnight at 37˚C. Then, one colony of bacterial culture was moved from overnight nutrient plate using inoculating loop into 1 ml of transformaide C-Medium. The culture was suspended by gently mixing and incubation of the tube in a shaker at 37˚C for 2 hours.

3.3.9.8 Ligation.

After digestion of PCR product and pUC-19 plasmid by Hind III restriction enzyme, the ligation was performed by incubation of 20 ng of digested PCR product with 150 ng of digested plasmid (molar ratio of about 6 fold molar excess of foreign DNA to

the plasmid) in a ligation reaction containing 4 µl of 5X ligation buffer, 5U of ligase enzyme, and free nuclease water in up to 20 µl total reaction volume. This was followed by Overtaxing and spining down in a microceterfuge for 5 seconds and incubation the tube at 22˚C for 30 minutes.

3.3.9.9 Transformation and growth.

After preparation of E-coli competent cells from a fresh culture plate in C-Medium as described above, the tube was spun at 13 000 rpm for 1 minute at room temperature then the supernatant was discarded and the the pelleted cells were resuspend in 300 µl of T-solution and incubated on the ice for 5 minutes. The cells were spundown again for 1 minute at 13,000 rpm at room temperature and then the supernatant was removed and the cells were resuspended in 120 µl of T-solution and then incubated on ice for 5 minutes. During that, LB-ampicillin agar plates are pre-warmed at 37˚C for 20 minutes.

Preparations of DNA for transformation was performed by taking 5 µl of ligation mixture into new microcetrifuge tube and sit it on ice for 2 minutes. Then 50 µl of the resuspended cells were added to the tube that contains DNA and incubated on ice for 5 minutes. Then the cells were plated on pre-warmed LB-ampicillin agar plates and incubated overnight at 37 ˚C. After growing the transformed cells, white and blue colonies were screened to determine the successful cloning; the white colonies indicate successful cloning.

3.3.9.10 Preparation of plasmid for sequencing.

Plasmid extraction was performed by the plasmid extraction kit EZ-10 (Bio Basic Inc, Canada) by incubation of each white colony in 1 ml of LB-ampicillin broth media overnight at 37˚C on shaking platform. the tube was Centrifuged at 12,000 rpm for 2 minutes then the supernatant was decanted and solutions I, II and III were used as described by the manufacture to lyse bacterial cells and prevent contamination of genomic DNA with plasmid DNA. Centrifugation at 12,000 rpm for 5 minutes was carried out and then the supernatant was transfered into EZ-column and centerfuged at 10,000 rpm for 1minute then the column was washed twice with 500 µl of washing solutions containing 50% absolute ethanol. After washing, the EZ-10 column was transfered into new microcenterfuge tube and 50 µl of elution buffer were added into column which was incubated for 2 minutes at 50˚C, and centrifuged at 10,000 rpm for 2 minutes.

3.3.10 Sequencing.

Extracted plasmids were sent for sequencing with the forward universal sequencing primer M13/pUC. The sequencing reactions were performed by Macrogen Inc., South Korea.

After sequencing, nine aptamer sequences were obtained from 9 successful sequencing reactions (table 4).

3.3.11 Synthesis of aptamer sequences.

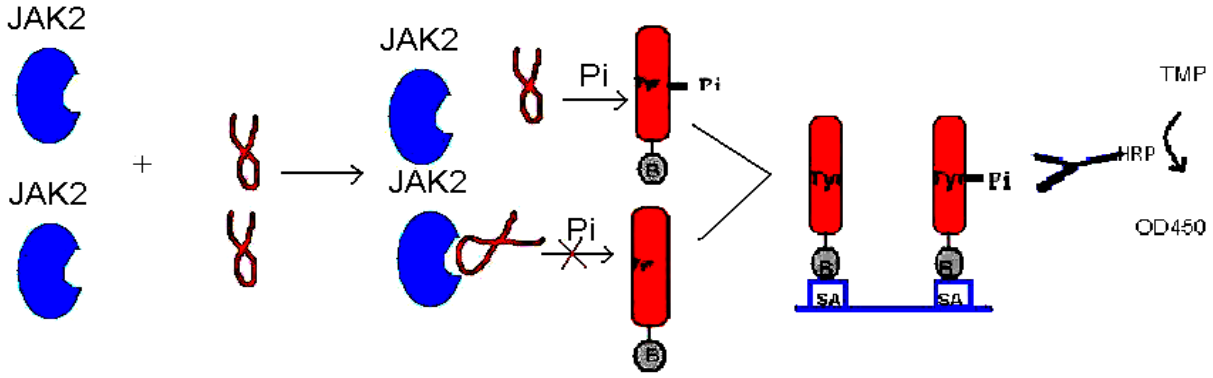
Each aptamer sequence was obtained via chemical synthesis using cyanoethyl phosphoroamitide chemistry (Medland certified company, USA) in 50 nanomole scale.

3.3.12 Inhibitory assay.

In vitro evaluation of the inhibitory effects of selected aptamers on the kinase activity of JAK2 was performed by a tyrosine kinase activity assay (Chemicon, USA) which is a non-radioactive tyrosine kinase assay consisting of a synthetic biotinylated peptide substrate, poly [Glu4:Tyr],:4:1 containing multiple tyrosine residues that can be phosphorylated by a wide range of PTKs . After incubation of 0.1 U of JAK2 enzyme with 2 µM of each selected anti Jak2 aptamers in 40 µl of binding buffer for 1 hr at 37°C, then after incubation, the reaction is started by the addition of 100 ng of peptide substrate, poly [Glu4: Tyr], 4:1 and 10 µl 100 µM ATP for 30 minutes at 30°C. Then the phosphorylation reaction is stopped by addition 10 µl of 120 mM EDTA. Both phosphorelated and dephosphorylated substrates are immobilized by binding to the streptavidine coated plate by transferring the reaction mix to strip wells and incubating at 37˚C for 30 minutes. Washing four times with 1X washing buffer was carried out to reduce background, and then 200 µl of blocking buffer were added solution to each well and incubated at 37˚C for 30 minutes. The blocking solution was discarded then 100 µl of 1:500 diluted mouse anti-PY20-HRP were added to each well and incubated at room temperature for 1 hr on a shaking platform. After incubation, washing four times with 1X washing buffer was followed by the addition of 100 µl of TMB substrate to each well incubation for 5 minutes. The enzyme reaction was stopped by addition of 100 µl of stop solution into each well the absorbance was read on a standard microplate reader using 450 nm as the primary wave length (figure 13).

Each assay contains positive control containing 0.1 U of JAK2 enzyme without aptamers, and negative control without enzyme to determine relative activity and

inhibition of each aptamer. Both controls were run in the same condition similar to reactions containing selected aptamers.



Figuer 13. Principle of aptameric inhibition to JAK2. as present in the figure the aptamer will binds to active site of JAK2 and then inhibit its ability to phosphorylate the substrate. Then the phosphorylated substrate detected by anti-phosphotyrosine antibodies labeled with HRP.

3.3.13 IC_{50} measurement

Inhibitory concentration 50 is the concentration of each aptamer required to inhibit 50% of 0.1U of JAK2 in vitro. Determination of IC_{50} was performed according to the method described in the inhibitory assay except the concentrations of each aptamer used and running a phosphopeptide standard to determine the quantity of phosphate incorporated into the tyrosine kinase substrate utilizing the phosphopeptide standard curve.

3.3.14 Predicting the secondary structure of aptamers.

For the prediction of the secondary structure of single stranded nucleic acids we used mfold software developed by Michael Zuker that available at the world web site http://www.bioinfo.rpi.edu/applications/mfold (Zuker, 2003). This software takes into consideration the folding conditions used in the selection process which includes 5 mM $MgCl_2$, 150 mM NaCl, and 37˚C as incubation temperature.

4. Results.

4.1 Results of small and large scale dsDNA amplification.

The chemically synthesized oligonucletides were amplified enzymitically by PCR in order to eliminate damaged templates and provide multiple copies of the original templates. Optimal amplification conditions were determined by small-scale PCR amplification prior to large-scale PCR amplification. The result of amplification was 79bp as seen in Figure 14.

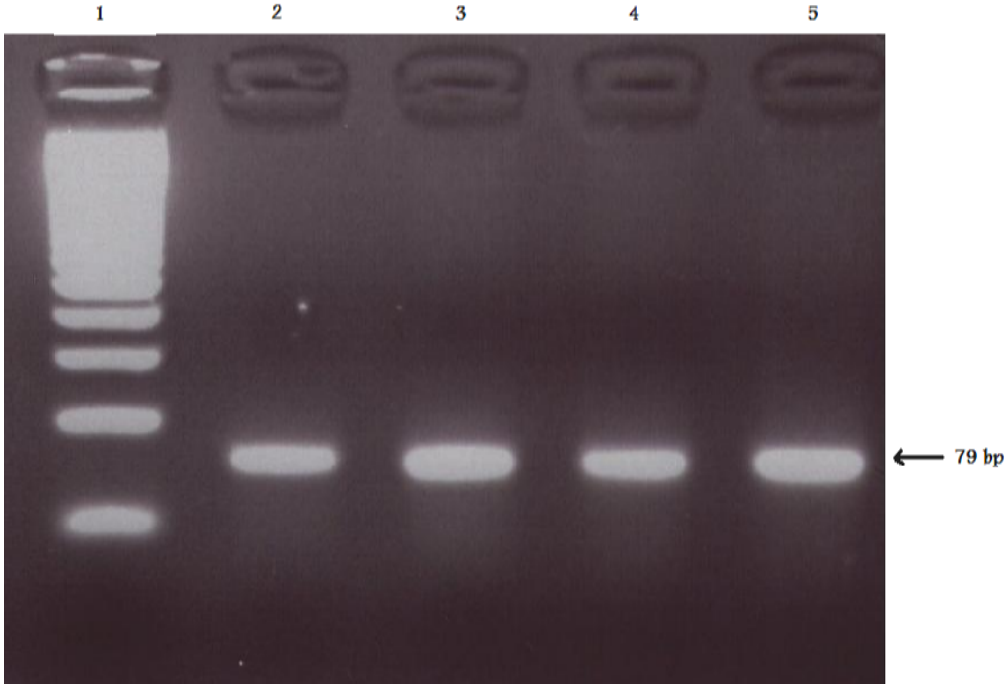


Figure 14. 79 bp PCR product of small-scale (lane 2 and 3) and large-scale (lane 4 and 5) amplification guided by 50bp ladder (lane 1).

4.2 Results of asymmetric PCR amplification.

Synthesis of the first ssDNA library was performed by trial extension reactions to optimize the optimal conditions and forward to reverse primers ratio. These trials showed that the best ratio of forward to reverse primers is 10:1 as seen in Figure 15.

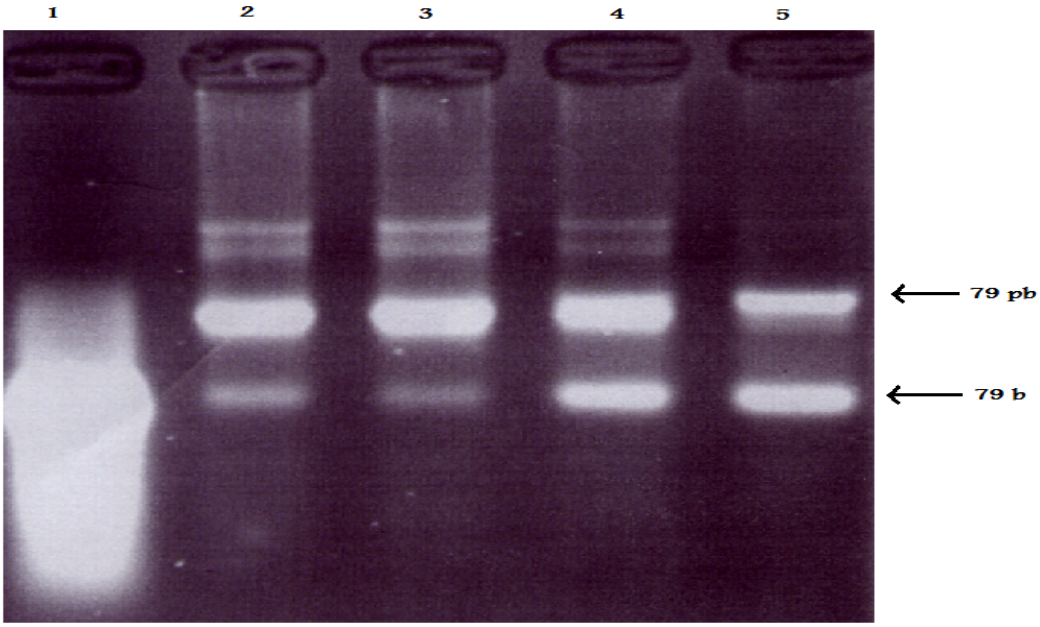


Figure 15. Asymmetric PCR product, 2µg of original library (lane 1), lanes from 2 to 5 asymmetric PCR product using forward to reverse ratio 100:1, 50:1, 25:1, 10:1 respectively.

4.3 PCR products of fourteen rounds of selection.

Fourteen rounds of SELEX processes were performed to select anti-JAK2 aptamers. After round number seven it was noted that there is an increase in the yield of PCR product which indicates an increase in the ssDNA eluted after incubation with the target. This means, an increase in the number of ssDNA molecules bound to JAK2 which is compatible with enrichment property of SELEX process. So the amount of ssDNA, JAK2, and number of PCR cycles was decreased in the next seven rounds of selection. About 10 µl of PCR product of each round loaded into 4% of agarose gel and the results were as present in figure 16.

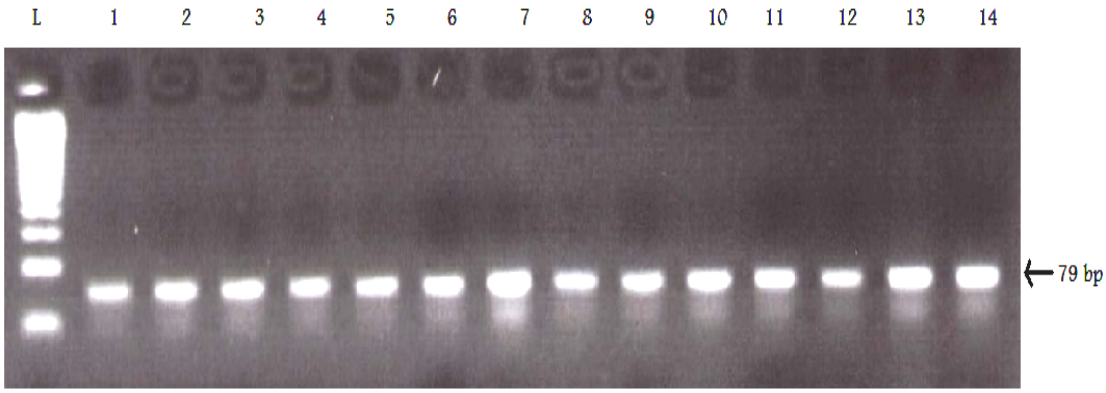


Figure 16. PCR product after each round of fourteen SELEX rounds guided by 50 bp ladder (lane L).

4.4 Sequencing results.

Nine sequences of aptamers were obtained from sequencing of nine successful cloned plasmids (Table 4). The sequencing results were highly accurate and monitored by precise quality control system (Figure 17).

Table 4. Full sequences of nine Selected Aptamers after cloning and sequencing.

NAME OF SEQUENCE	*SEQUENCE FROM 5` TO 3`*
C1	GGGCAAGCTTCTGAATTCGCAG**GTCTGTGTGTAGGGCGGTAATCACGTTGGCGGTAG**GTGGATCCGTAAGCTTCGACTG
C2	GGGCAAGCTTCTGAATTCGCAG**TAAGCAGCGCAATAACAAGTGGCGTAATGTGGGTC**GTGGATCCGTAAGCTTCGACTG
C3	GGGCAAGCTTCTGAATTCGCAG**AAGTAGGTGCGTGACAGGCGGCGATAGTTATGTTG**GTGGATCCGTAAGCTTCGACTG
C5	GGGCAAGCTTCTGAATTCGCAG**CAAGTGAGACGGGATACTACCAGAGCTGACCTGTT**GTGGATCCGTAAGCTTCGACTG
C7	GGGCAAGCTTCTGAATTCGCAG**TCGGTTCGTGTGATGGCTCTGTATCGATGTGCCGG**GTGGATCCGTAAGCTTCGACTG
C10	GGGCAAGCTTCTGAATTCGCAG**CTTGGGGGGGAGGGCGATTGTAGCACGGGTCGCGT**GTGGATCCGTAAGCTTCGACTG
C11	GGGCAAGCTTCTGAATTCGCAG**CCCGCGGATGCGAAAGCACAGAGCGTGTAGGGGTG**GTGGATCCGTAAGCTTCGACTG
C14	GGGCAAGCTTCTGAATTCGCAG**TTTGGGAGTAGGAGATGATGCACTGGTTGGTTTTG**GTGGATCCGTAAGCTTCGACTG
C15	GGGCAAGCTTCTGAATTCGCAG**CCGCACTCTGATCGCAGTAGGGGGGGATGGTCGTG**GTGGATCCGTAAGCTTCGACTG

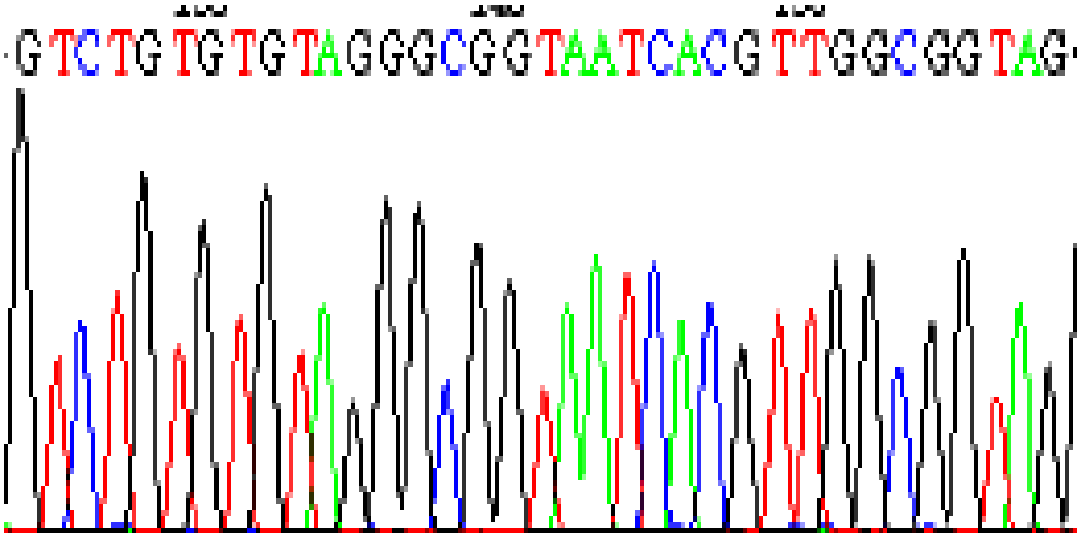


Figure 17. Electropherogram of the random region of C1 sequence.

4.5 Primary and secondary Structures of selected aptamers.

The secondary structures of anti-JAK2 aptamers were predicted by Mfold program described by Zuker which takes in consideration the factors that affect the folding of nucleic acid sequences, conditions of incubation, such as temperature of incubation and ionic strength. Each sequence showed different free energy (dG) which reflects the stability of folding structure of aptamer. The small free energy means the folded structure is more stable (Figures from 18 to 27).

5`GGGCAAGCTTCTGAATTCGCAGGTCTGTGTGTAGGGCGGTAATCACGTTGGCGGTAGGTGGATCCGTAAGCTTCGACTG-3`

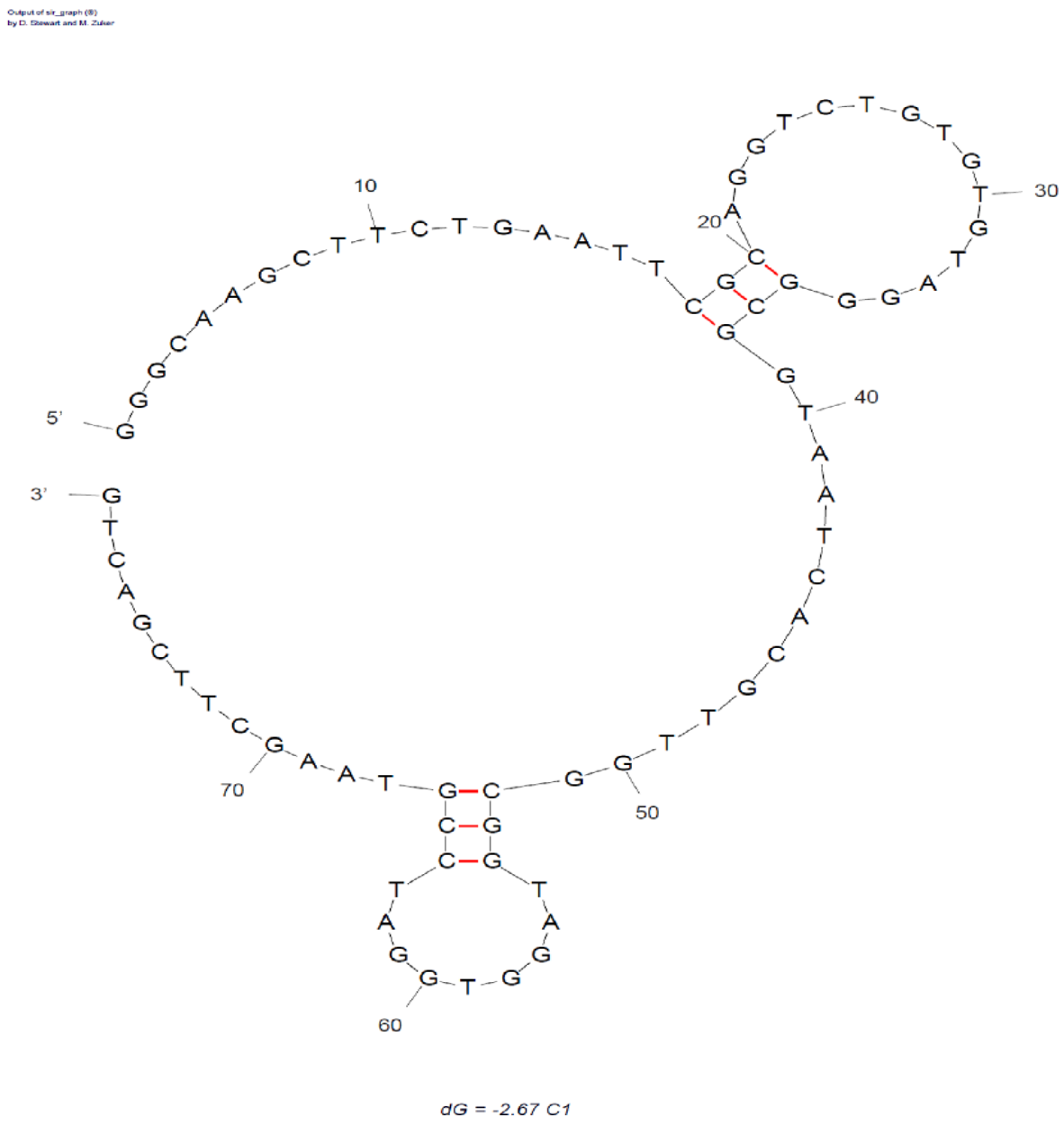


Figure 18. Primary and Secondary Structures of C1 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAGTAAGCAGCGCAATAACAAGTGGCGTAATGTGGGTCGTGGATCCGTAAGCTTCGACTG-3`

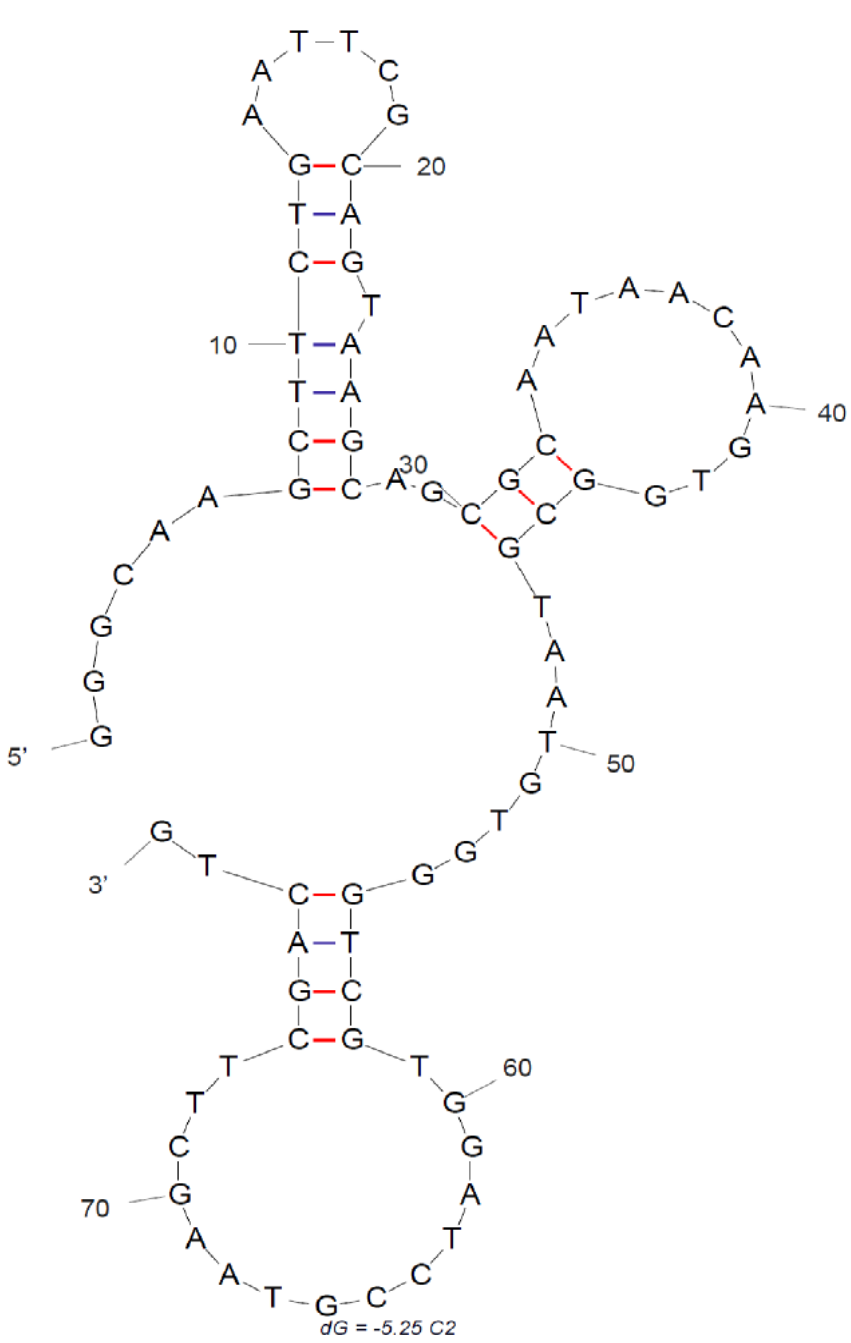


Figure 19. Primary and Secondary Structures of C2 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAGAAGTAGGTGCGTGACAGGCGGCGATAGTTATGTTGGTGGATCCGTAAGCTTCGACTG-3`

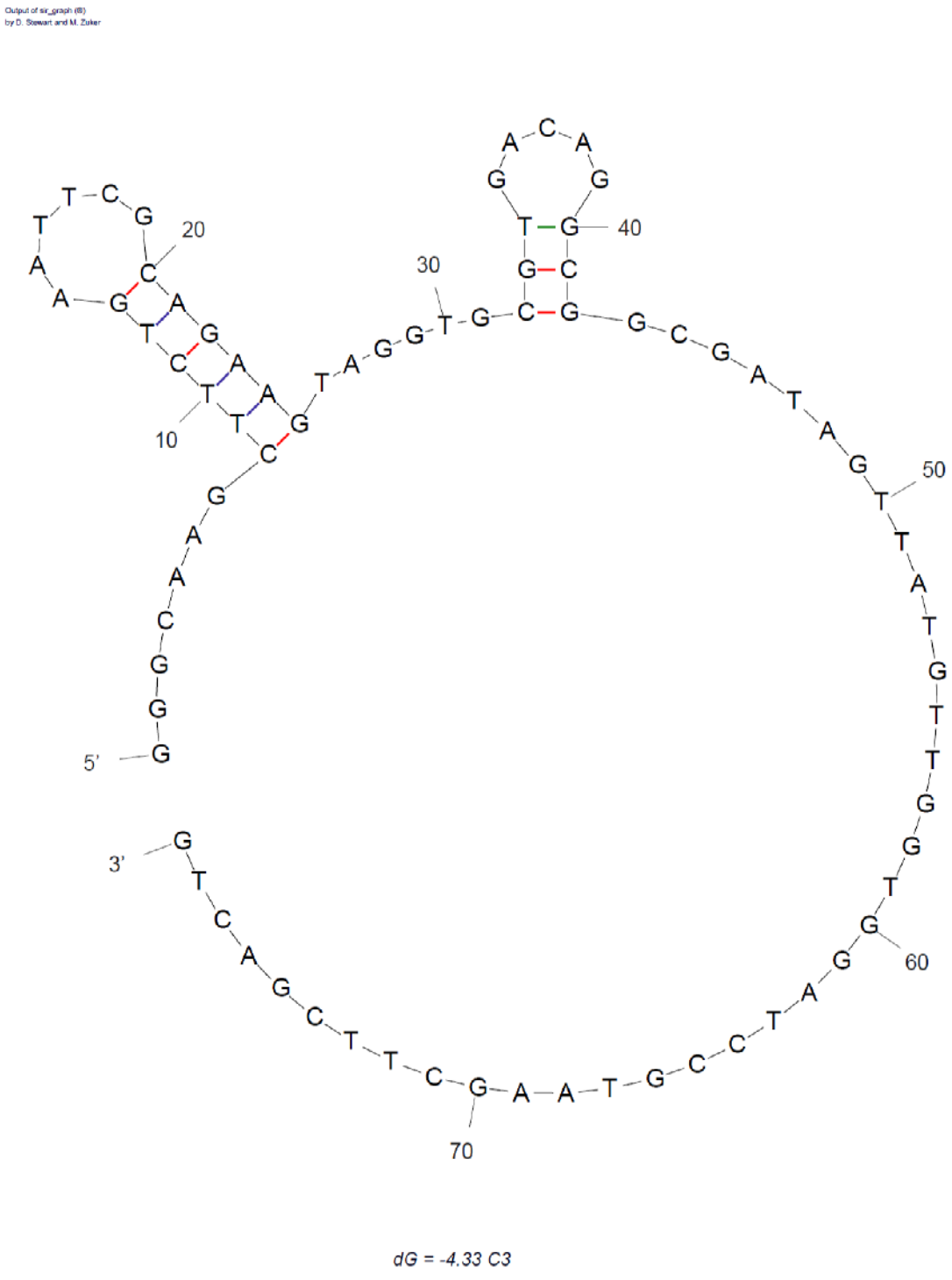


Figure 20. Primary and Secondary Structures of C3 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAGCAAGTGAGACGGGATACTACCAGAGCTGACCTGTTGTGGATCCGTAAGCTTCGACTG-3`

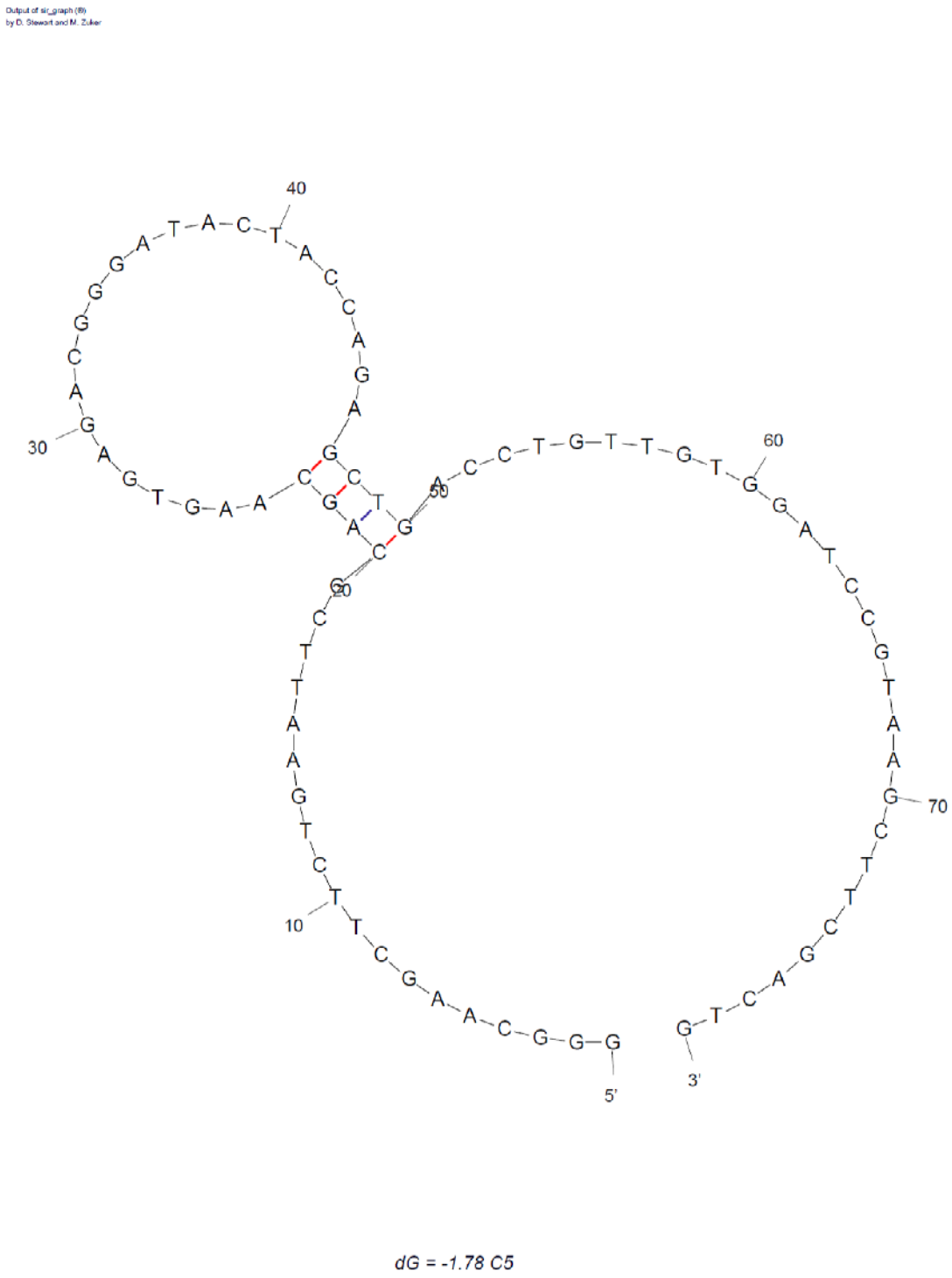


Figure 21. Primary and Secondary Structures of C5 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAG**TCGGTTCGTGTGATGGCTCTGTATCGATGTGCCGG**GTGGATCCGTAAGCTTCGACTG-3`

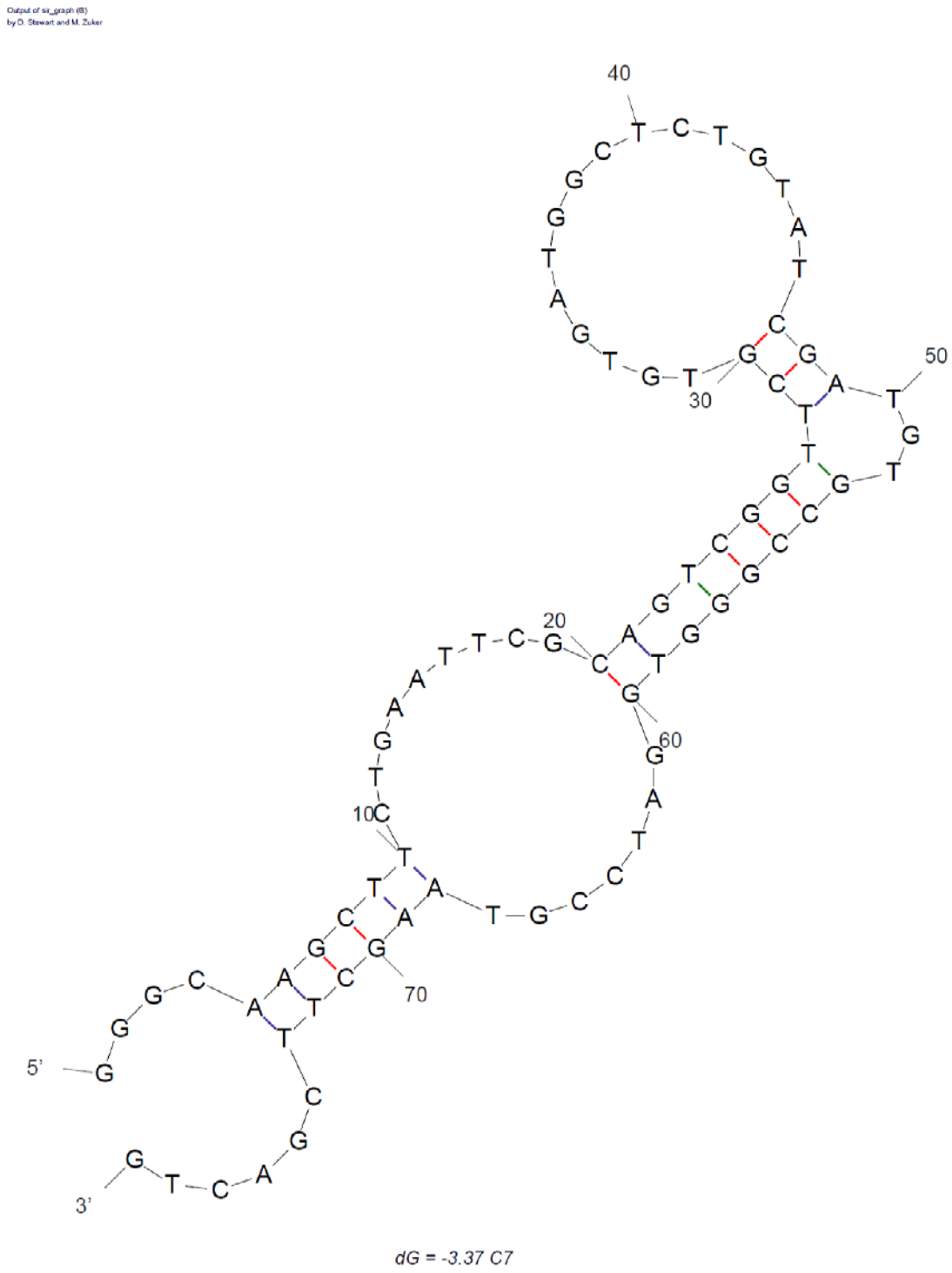


Figure 22. Primary and Secondary Structures of C7 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAGCTTGGGGGGGAGGGCGATTGTAGCACGGGTCGCGTGTGGATCCGTAAGCTTCGACTG-3`

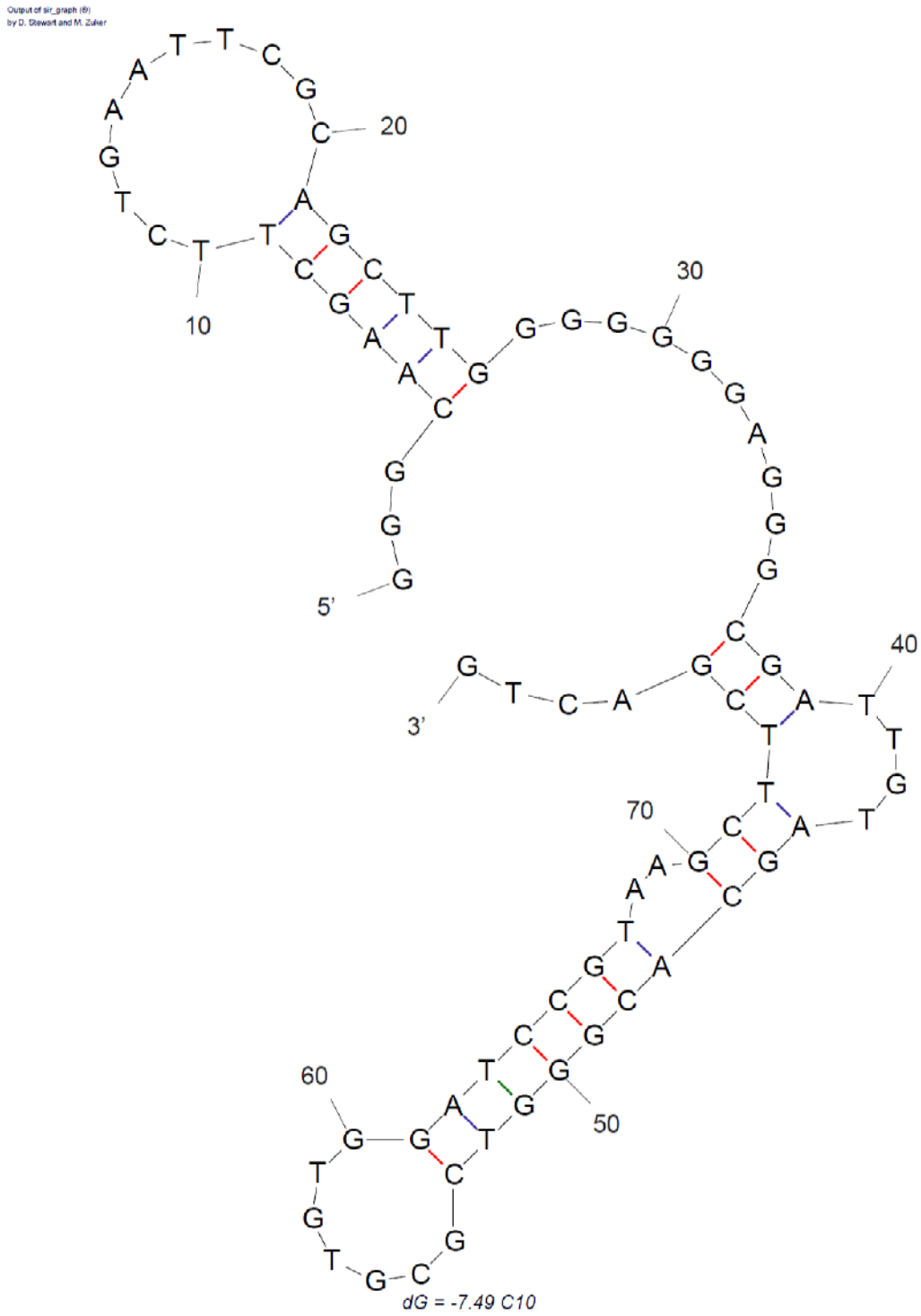


Figure 23. Primary and Secondary Structures of C10 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAGCCCGCGGATGCGAAAGCACAGAGCGTGTAGGGGTGGTGGATCCGTAAGCTTCGACTG-3`

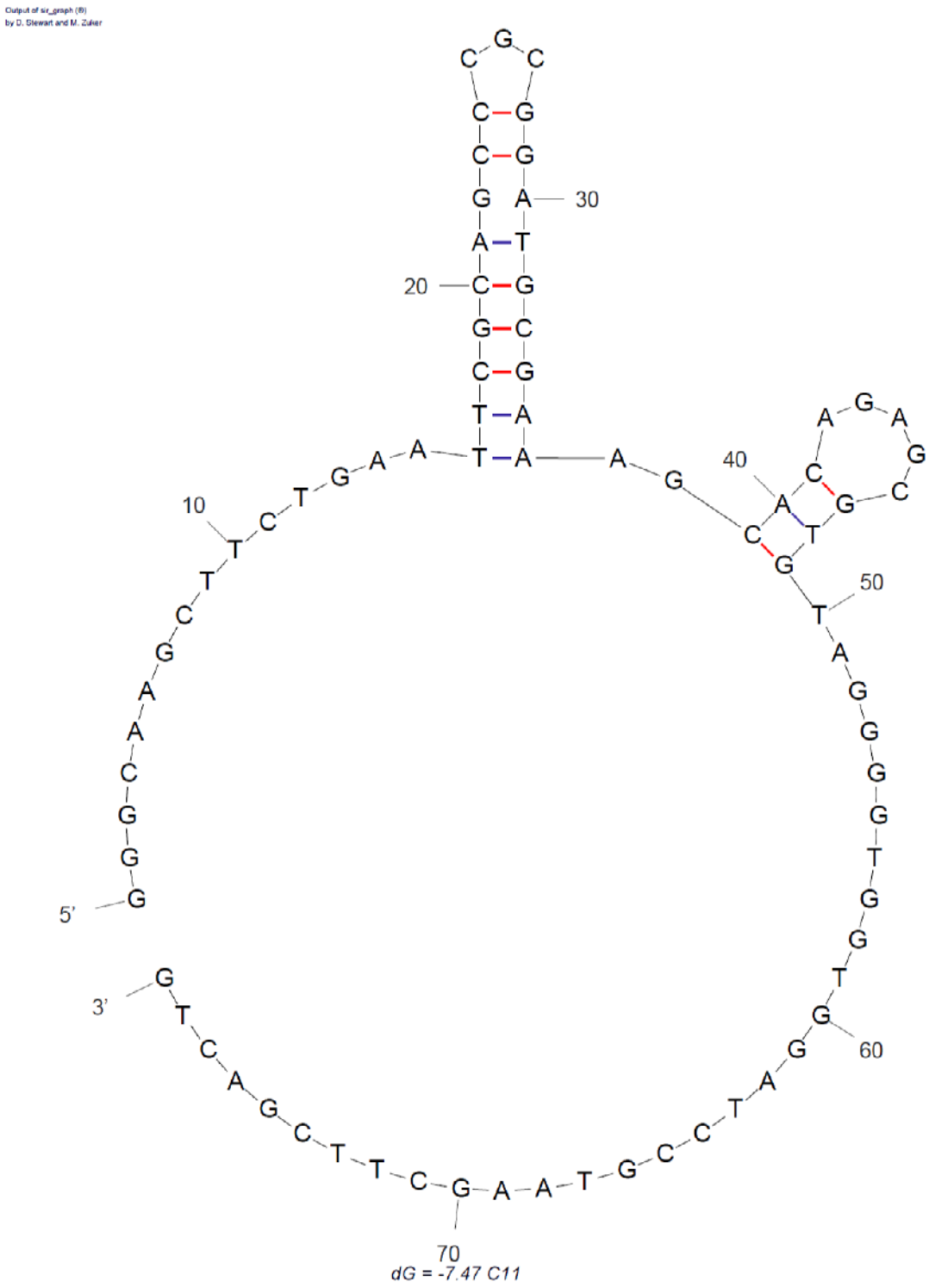


Figure 24. Primary and Secondary Structures of C11 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAGTTTGGGAGTAGGAGATGATGCACTGGTTGGTTTTGGTGGATCCGTAAGCTTCGACTG-3`

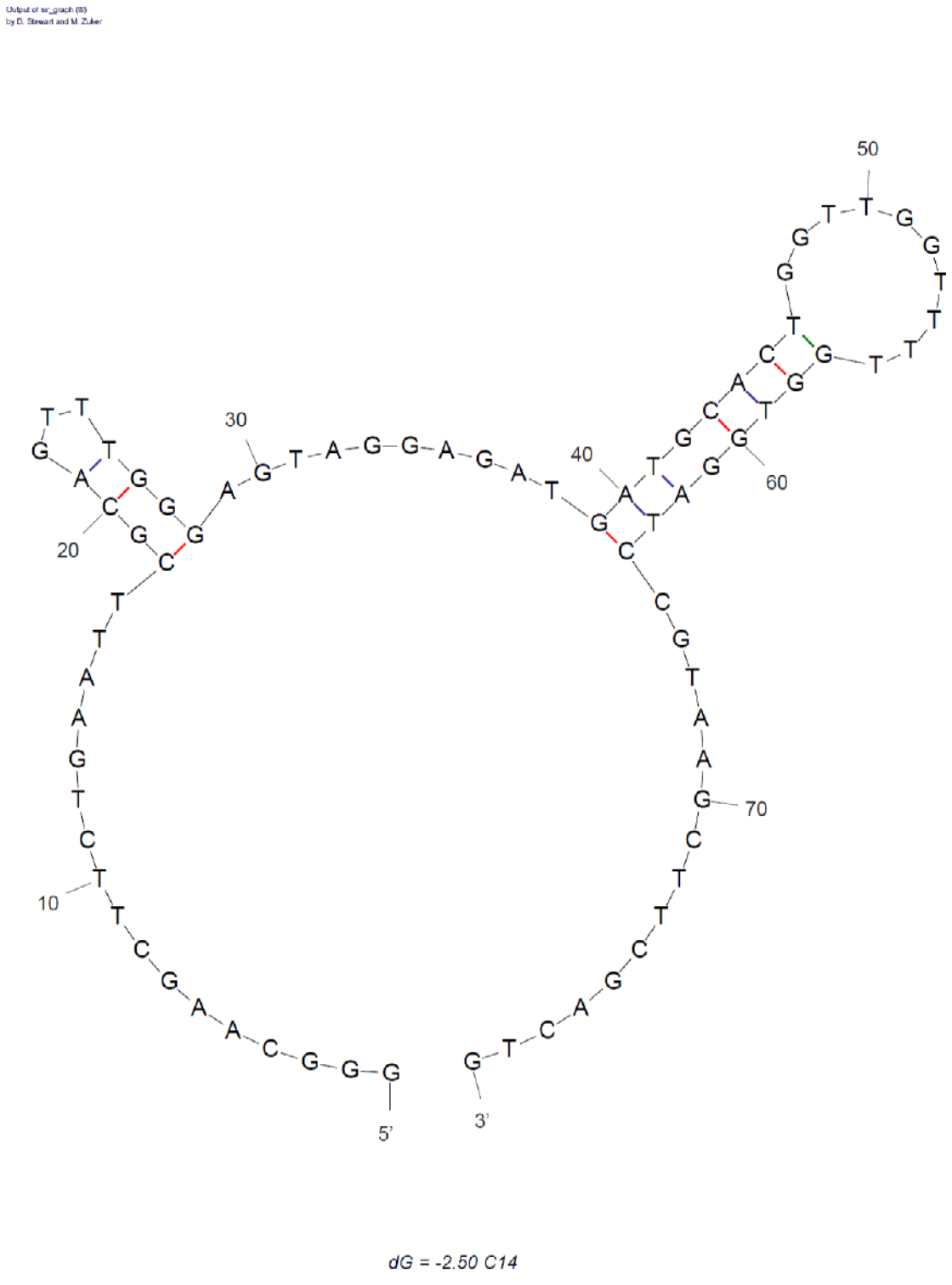


Figure 25. Primary and Secondary Structures of C14 Aptamer.

5`-GGGCAAGCTTCTGAATTCGCAGCCGCACTCTGATCGCAGTAGGGGGGGATGGTCGTGGTGGATCCGTAAGCTTCGACTG-3`

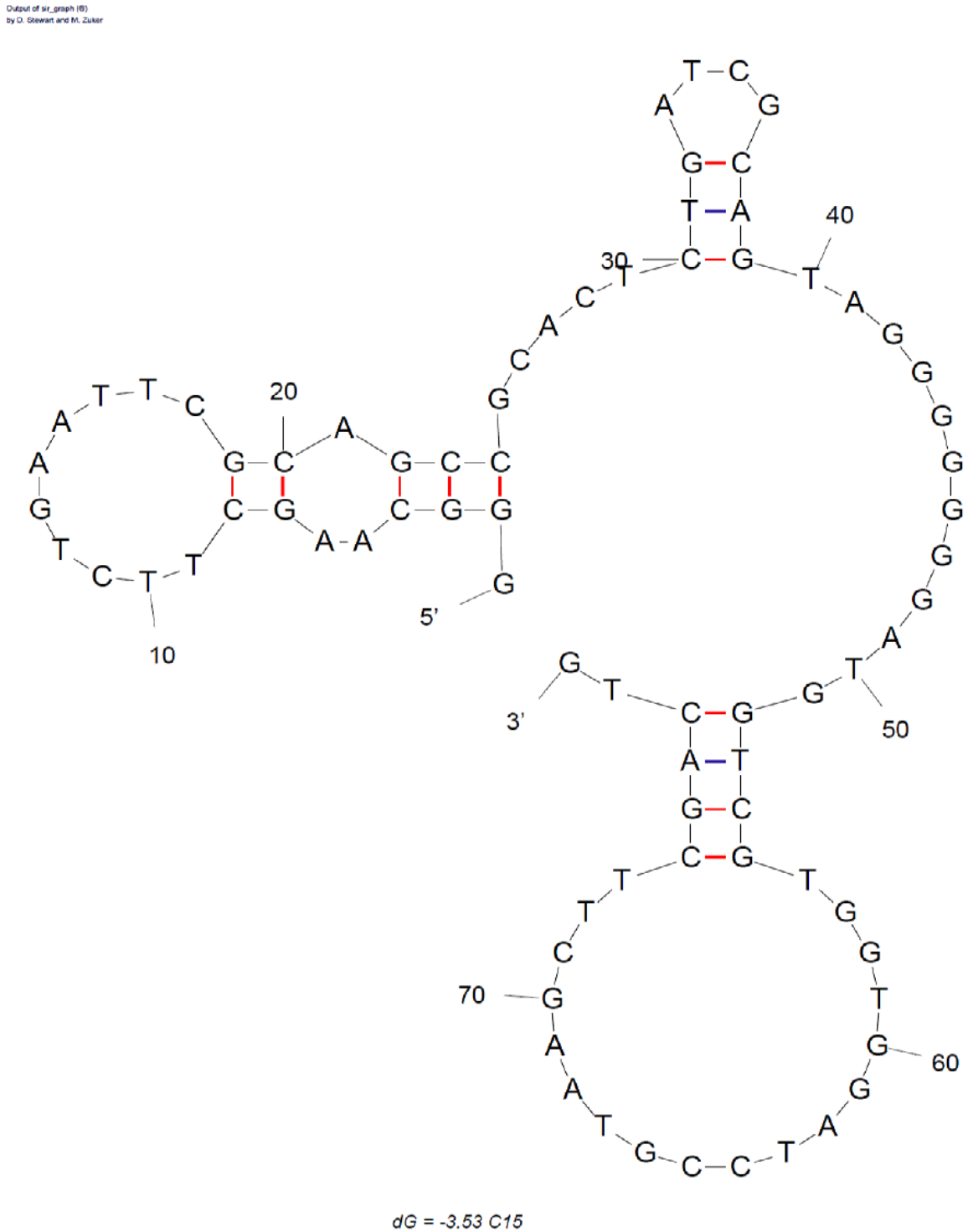


Figure 26. Primary and Secondary Structures of C15 Aptamer.

4.6 Results of inhibition and IC_{50}.

Evaluation of the inhibition efficiency for each selected aptamer on kinase activity of JAK2 was performed by tyrosine kinase assay after incubation of a fixed amount of each aptamer with a fixed amount of JAK2. The result of inhibition showed different ability of each aptamer to inhibit the kinase activity of JAK2 as shown in Table 5. More information about the efficiency of selected aptamers in the inhibition of kinase activity of JAK2 are obtained by measurements of each aptamer concentration required to inhibit 50% of JAK2 kinase activity which is indectaed by the number of phosphate molecules added to substrate in comparison with a phosphopeptide standard with known amount of phosphorylated substrate as summaraized in Table 6.

Table 5. Percent of inhibition for 0.1U of active JAK2 kinase domain at 2 µM concentration of each aptamer.

SEQUENCE	PERCENT OF INHIBITION ± SD
C1	69 ± 3.0
C2	44 ± 3.0
C3	48 ± 7.5
C5	28 ± 3.5
C7	80 ± 1.5
C10	87 ± 1.0
C11	68 ± 2.0
C14	37 ± 1.5
C15	82 ± 2.0

Table 6. IC_{50} value for each aptamer.

SEQUENCE	IC_{50} of each sequence in μM ± SD
C1	1.46 ± 0.07
C2	2.29 ± 0.16
C3	2.16 ± 0.34
C5	3.70 ± 0.47
C7	1.26 ± 0.03
C10	1.15 ± 0.01
C11	1.48 ± 0.05
C14	2.75 ± 0.12
C15	1.22 ± 0.03

4.7 Method of IC_{50} determination.

As an example, in one assay the OD for 0.1U of JAK2 control sample was 1.252 which indicates phosphorylation of tyrosine residues on the substrate equal to 7.62 ng of phosphorylated peptide standard. In the same assay 1.5 μM of C1 aptamer incubated with 0.1U of JAK2 gave OD of 0.721 which indicates phosphorylation of tyrosine residues on the substrate equal to 3.85 ng of phosphorylated peptide standard. Then by comparison of the two results (7.62 ng and 3.85 ng)it was found that the amount of phosphorylated tyrosine residues resulted from incubation of C1 and JAK2 to be equal to ~50% of the control sample (Figure 27).

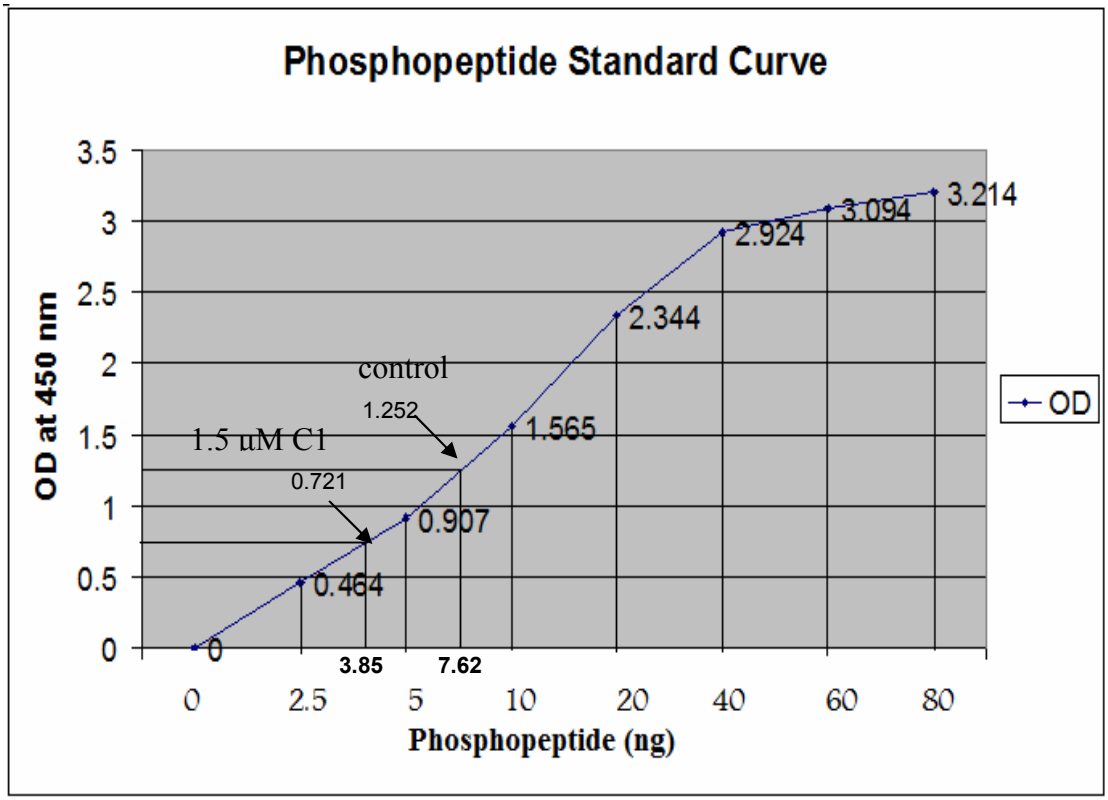


Figure 27. Phosphopeptide standerd curve for IC_{50} determination.

5. Discussion.

The initial template is obtained via chemical synthesis using cyanoethyl phosphoroamitide chemistry and purified by trityl-selective perfusion HPLC (TSPgrade). DNA fragments were 79 base length containing random sequence of 35 nucleotide flanked with defined sequences for forward and reverse primers annealing and Hind III restriction site on the two defined sequences.

The SELEX process is a versatile method for identifying nucleic acids that bind to a variety of molecular entities with high affinity and specificity. The aim of the SELEX experiment described here is to identify ssDNA aptamers that antagonize and inhibit the kinase activity of JAK2. This enzyme was selected in view of its importance of JAK2 in several proliferative disease states such as myeloproliferative disorders (Smith and Fan, 2008).

The nucleic acid pools used for in vitro selection experiments typically contain a randomized central core with 35 nucleotide length that can theoretically give up to 1.18×10^{21} individual sequences flanked by constant sequences that are required for enzymatic manipulations including PCR amplification and restriction digestion. The design of the sequence and the amplification by small-scale and large-scale PCR used in our selection process depends on the rules recommended in many selection protocols of aptamers (Kristin et al, 2000; Crameri and Stemmer, 1993).

Synthesis of the first ssDNA library was performed under the same conditions that were used during the trial extension reactions to ensure that the final pool has a complexity similar to the calculated one. In other words, when the small-scale PCR

reaction is optimized by using 0.33 ng (approximately 4 x 10^{12} different sequences) of synthesized templates in 100 µl total volume, it was posseple amplify 10µg of synthesized templates (approximately 1.2 x 10^{14} different sequences) in 3000 µl of reaction mix. Similar approaches were considered for asymmetric PCR.

Recovery of ssDNA from asymmetric PCR product was performed by extraction and purification from low melting point agarose gel. This method showed good yields of ssDNA after extraction, enough to recover the largest amount of amplified ssDNA with over 90% as described by Kurien and Scofield (2002).

While there are multiple possible configurations for in vitro selection experiments, this study describes a method for selection of ssDNA aptamers against Poly-His tag kinase domain of JAK2 using magna-His Ni particles as affinity separation method of aptamer-target complex.

The affinity separation method using a poly His-tag ligand-mediated magnetic system was described in many studies to isolate high affinity aptamers such as for prion protein and MUC1 tumor marker (Rhie et al., 2003; Ferriera et al., 2006).

After cloning and sequencing the successfully cloned plasmid, the sequences of nine aptamers were analyzed to determine the homology between the sequences of aptamers which showed conserved sequences such as GGC sequence which is present in C1, C2, C3, C4, C7, and C10 aptamers, and the sequence GGGGGGGA which is present in C10, and C15 aptamers.

The secondary structures of anti-JAK2 aptamers were predicted by Mfold program as described by Zuker (2003) which is the most commonly recommended in the literature because it takes into consideration the folding and binding conditions of aptamers such as temperature and ionic strength which affect the three dimensional structure of the aptamer. Each aptamer showed different free energy (ΔG) which is the difference between the energy content of the primary sequence and the energy content of the secondary structure. Free energy reflects the stability of the folding structure of the aptamer, the smallest free energy indicating that the folded structure is more stable (Zuker, 2003).

Evaluation of the inhibition efficiency for each selected aptamer on kinase activity of JAK2 was performed by tyrosine kinase assay after incubation of a fixed amount of each aptamer with a fixed amount of JAK2. The results of inhibition showed different ability of each aptamer to inhibit the kinase activity of JAK2 indicating that the route of inhibition is sequence dependent and dos not results from promiscuous inhibition. This is so because if the inhibition is promiscuous in origin, the percent of inhibition should be the same for all selected aptamers.

More information about the efficiency of selected aptamers in the inhibition of the kinase activity of JAK2 are obtained by the measurement of each aptamer concentration this is required to inhibit 50% of JAK2 kinase activity which is indicated by the number of phosphate molecules added to substrate in comparison with a phosphopeptide standard with known amount of phosphorylated substrate.

Ther is no study in the literature that describes the selection of aptamers against JAK2. However, Bergan et al (1994) described the aptameric inhibition of $p210^{bcr\text{-}abl}$

tyrosine kinase autophosphorylation by oligodeoxynucleiotides of defined sequence and backbone structure in vitro. This study described the ability of ODN to specifically inhibit $p210^{bcr\text{-}abl}$ tyrosine kinase autophosphorylation with a K_i of 0.5 μM. Moreover, they demonstrated that the presence of closely spaced GGC repeats contributes significantly to the ability of a particular ODN to inhibit $p210^{bcr\text{-}abl}$ activity. Because the tyrosine kinases have high homology among the kinase domains (AL-Obiedi et al., 1998), the findings of this study support the results obtained by Bergan , particularly with respect to the presence of GGC sequence in C1, C2, C3, C4, C7, and C10 aptamers.

Finally, this study demonstrates that ani-JAK2 aptamers can act as inhibitors with IC50s in the small micromolar range which indicates that anti-JAK2 aptamers may be potential therapeutic agents in the future.

6. Conclusion.

In view of the findings of this study, the ability of anti-JAK2 aptamers to potentially inhibit protein tyrosine kinase activity of JAK2 in an aptameric fasion makes these molecules appealing candidates for development as enzymatic inhibitors both for laboratory use and as potential therapeutic agents in the future.

7. Recommendations.

On the basis of the conclusions of this study, the following recommendations are needed.

1- Evaluation of the degree of specificity of anti-JAK2 aptamers on other kinases mainly tyrosine kinases.

2- Evaluation of the degree of affinity for each selected aptamer for the JAK2 kinase domain.

3- Evaluation of the efficiency of inhibition of anti-JAK2 aptamers in vivo.

4- Analysis of JAK2-aptamer 3D structure complex.

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تطويرجزيئات صغيرة من الحمض النووي وحيد السلسلة موجه لتثبيط نشاط الإنزيم "JAK2"

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ملخص

إن التطور الكبير و المتسارع في تكنولوجيا البيولوجيا الجزيئية مكن من فهم الكثير عن تراكيب واليّات عمل الكثير من الجزيئات الحيوية و دورها المباشر و غير المباشر في التسبب للعديد من الأمراض. كما ساهمة هذه التكنلوجيا في الكشف عن طرق عديدة لتشخيص و علاج الأمراض على عدة مستويات.

ففي عام 1990م قام العلماء بالكشف عن طريقة لعزل سلاسل من الحمض النووي تسمى "Aptamers" لديها القدرة على الإرتباط بألفة و تخاصيّة عالية جداً مع الجزيء المستهدف لاستخدامها في مجالات عديدة مثل التشخيص و العلاج.

بناءً على ذلك فإن هذا الدراسة تكشف عن سلاسل من الحمض النووي وحيدة السلسلة (Single Stranded DNA Aptamers) لديها القدرة للعمل كجزيئات رابطة موجهه لتثبيط نشاط الإنزيم "JAK2" وهو إنزيم يلعب دوراً حيوياً أساسياً في انقسام الخلايا و تكاثرها، خاصة في تكاثر الخلايا الدموية, ويعتبر هذا الإنزيم من البروتينات المسببة لعدة أنواع من السرطانات إذا ما حصل أي طفرات في الجين المسؤول عن إنتاج هذا الإنزيم. كما تكشف هذه الدراسة عن الطريقة التي تم بها الكشف عن هذة السلاسل من الحمض النووي و التي تسمى التطوير المنتظم لجزيئات رابطة بالتكثير المتضاعف (SELEX).