Selection and Characterization of Glutathione Binding DNA Aptamers

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The dissertation work embodied in this thesis entitled "Selection and Characterization of Glutathione Binding DNA aptamers" has been carried out at the School of Life sciences Jawaharlal Nehru University, New Delhi India. This work is original and has not been submitted so far, in part or full for the award of any other degree or diploma of this or any other university.

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CONTENTS

I	**INTRODUCTION AND REVIEW OF LITERATURE**	**1-22**
I.1	**Introduction**	**1-2**
I.2	**Aptamer**	**3-8**
I.2.1	Aptamers an emerging rival to antibodies	6
I.2.2	Advantages of aptamers	6-8
I.2.3	Limitations	8
I.3	**SELEX**	**9-15**
I.3.1	Basic principle	10-11
I.3.2	Starting random DNA oligonucleotide library	11-12
I.3.3	Selection	12-13
I.3.4	Amplification	13
I.3.5	Cloning and characterization	13
I.3.6	Modifications	13-15
I.4	**Application of aptamers**	**16-19**
I.4.1	Diagnosis	16-17
I.4.2	Therapeutics	17
I.4.3	Cancer	17-18
I.4.4	Drug delivery vehicle	18
I.4.4	Sensors	18-19
I.4.5	Aptamers as molecular switches	19
I.5	**Glutathione**	**20-22**
I.5.1	GSH as potential cancer treatment target	21-22
II.	**MATERIALS AND METHODS**	**23-33**
II.1	**Materials**	**23-26**
II.1.1	Materials used for routine work	23-25
II.1.2	Materials used for the generation of nucleic acid library	25
II.1.3	Primers used for PCR	26
II.1.4	Beads used for selection	26
II.1.5	Bacterial strains used	26
II.1.6	Plasmids used	26
II.2	**Methods**	**27-33**
II.2.1	Generation of oligonucleotide library	27-28
II.2.2	Freeze thaw method of purification of DNA from agarose gels	29

II.2.3	Asymmetric PCR	29
II.2.4	SELEX	30
II.2.5	Cloning of the selected aptamers	30
II.2.6	Competent cell preparation	31
II.2.7	Transformation of plasmid DNA in E.coli cells	31
II.2.8	Alkaline lysis Mini-prep	31-32
II.2.9	Digestion	32
II.2.10	Long term storage of bacterial cultures	32
II.2.11	Software and web servers used for sequence analysis	33
II.2.12	Kinetics	33-34
III	**RESULTS**	**35-56**
III.1	**Generation of oligonucleotide library**	**35-36**
III.2	**SELEX**	**37-38**
III.3	**Cloning**	**38**
III.4	**Sequence analysis**	**39-54**
III.4.1	Sequences of aptamer clones representing different cycles of SELEX	39-42
III.4.2	Phylogenetic prediction of different classes of glutathione binding DNA aptamer	43-44
III.4.3	Multiple Sequence alignment of glutathione binding DNA aptamers	44-47
III.4.4	Secondary structure prediction of glutathione binding DNA aptamers	48-53
III.5	**Kinetics**	**54-56**
IV	**DISCUSSION**	**57-62**
IV.1	In vitro selection of glutathione binding DNA aptamers	58-59
IV.2	SELEX	59
IV.3	Phylogenetic tree analysis of glutathione binding DNA aptamers	59-60
IV.4	Multiple sequence alignment of aptamers	60
IV.5	Secondary structure prediction of glutathione binding DNA aptamers	60-61
IV.6	Kinetics	61-62
IV.7	Prospects	62
V	**SUMMERY AND CONCLUSIONS**	**63-64**
V.1	Summary	63
V.2	conclusion	64
VI	**BIBLIOGRAPHY**	**65-72**

Introduction

I.1 Introduction

Before there were organized living systems, there were chemicals. Natural phenomenon transformed these chemicals and selected nucleic acid which pawed the way for evolution of modern life forms. Traditionally and truly the nucleic acids were thought to be there for storing the genetic information and their translation but as the knowledge about the structural complexity and flexibility of the nucleic acids increased, researchers began to think of functions conferred on them by these structures. Here evolves the concept of aptamer which is although prevalent in nature but recent advances made it a handy concept for in vitro selection.

Aptamers are relatively small biomolecules that bind their cognate ligand based on higher order structure. Biochemically aptamers could be nucleic acid or polypeptide while their ligands could be any other molecule. Nucleic acid aptamers are typically 15 to 70 nucleotides in length and can be composed of DNA, RNA, or nucleotides with a chemically modified sugar backbone (i.e., 2′-fluoro, 2′-O-methyl, phosphorothioate). Complementary base pairing defines aptamer secondary structure, consisting primarily of short helical arms and single-stranded loops. Stable tertiary structure, resulting from combinations of these secondary structures, allow aptamers to bind to targets via a variety of non covalent interactions like van der Waals interaction, hydrogen bonding, and electrostatic interactions etc. The huge number of possible tertiary structures afford good probability to select for aptamers to bind with most small molecules, peptide, or protein targets, with KD values typically ranging from 10 pM to 10 nM for proteins (Shannon et al, 2005; White et al, 2000).

The most efficient way to find aptamers is through a method utilizing nucleic acid libraries in conjunction with a selection scheme based on in vitro evolution principles. The process has been termed SELEX, standing for Systematic Evolution of Ligands by Exponential Enrichment. Since DNA and RNA molecules adopt stable and intricately folded three-dimensional shapes they are capable of providing a scaffold for the interaction with functional side groups of a bound ligand. Randomized sequence pools of oligonucleotides can be synthesized with sequence complexities in the range of 10^{15} different molecules. This represents a structural diversity not matched by any other combinatorial technique (Ellington et al, 1990; Tuerk et al, 1990).

Glutathione is a tripeptide thiol made up of the amino acids gamma-glutamic acid, cysteine and glycine and is also known as gamma-glutamylcysteinylglycine or GSH. It is found in vast majority of prokaryotic and eukaryotic cells and is the biologically most abundant low molecular weight intracellular thiol (Kunert et al, 1993). The thiol group confers on it the function of maintaining the redox state of the cell. Decrease in cellular reducing molecules results in increased ROS which induce apoptosis (Green et al, 1998; Marchetti et al, 1997; Coffey et al, 2000; Armstrong et al, 2001). Results of studies with Buthionine Sulphoximine (BSO), which is a known inhibitor of glutathione synthesis, has shown that reduction in cellular Glutathione induces apoptosis in cancer cells (Armstrong, et al, 2002) and make them more susceptible to anti-cancer drugs (Hayes 1999).

From the above research background here we undertook the task of selection of glutathione binding DNA aptamers. They can be good sequestering agents of cellular glutathione and hence an efficient tool for increasing the susceptibility of cancer cells to various anti-cancer drugs and to decrease the efficiency of cancer cells to become resistant to various drugs.

Review of Literature.

I.2 Aptamer

When we compared the composition of proteins and nucleic acids the latter, being composed of only four different nucleotides, seems to have very limited possibility of stable structure variants. But in reality, single stranded oligonucleotide sequences have an exceptional ability to assume a vast array of secondary and tertiary structures with different shape. In fact when we compare peptide and oligonucleotides of same length the latter have much higher number of possible thermodynamically stable structure variants. The credible character for this property is the ability of oligonucleotides to interact with each other through a number of non covalent interactions besides the covalent backbone (Gold et al, 1993; Gold et al, 1995). This structural flexibility bestows some very special characters to the molecule and finding a way to harvest them is of immense applicability.

The recent development of the in vitro selection and amplification technique and the ability of oligonucleotide sequences to assume a multitude of shapes within a random sequence library allowed us to harvest this structural property of nucleic acid to generate highly specific aptamers (Gold et al, 1993; Gold at al, 1995).

The conceptual framework and process of aptamer generation emerged from pioneering experiments by two independent groups, both of whom published their work in 1990. Tuerk and Gold described a process of in vitro selection, called "SELEX" (Systematic Evolution of Ligands by Exponential enrichment), to determine the sequence requirements of T4 DNA polymerase (Tuerk et al, 1990). An eight-base region of an RNA that interacts with T4 DNA polymerase was chosen and randomized. Two different sequences were selected from a calculated pool of 65,536 species. SELEX identified the natural bacteriophage recognition sequence of the polymerase as the primary high-affinity ligand, along with a single major sequence variant that showed similar affinity for the enzyme (Tuerk et al, 1990). Shortly thereafter, Ellington and Szostak published further groundbreaking experiments utilizing in vitro selection to isolate nucleic acids with specific ligand binding properties (Ellington et al, 1990). Affinity chromatography was used to isolate oligonucleotides that demonstrated highly specific binding to several organic dye molecules, none of which had been known previously to bind nucleic acid. Approximately 1 in 1010 random sequence oligonucleotides was found to fold in a manner that created specific dye binding sites. Ellington and Szostack introduced the term "aptamer" (derived from the Latin word "aptus", meaning "to fit") to describe these

nucleic acid molecules (Ellington et al, 1990). Since 1990, to date aptamers have been generated against hundreds of molecular targets. There has not been a restriction on the type of target for aptamer generation and aptamers have been generated against targets including, organic dyes (Ellington et al, 1990; Ellington et al, 1992), Metal ions (Ciesiolka et al, 1996; Hofmann et al, 1997), drugs (Jenison et al, 1994; Mannironi et al, 1997), amino acids (Geiger et al, 1996; Connell et al, 1993; Harada et al, 1995), co-factors (Lauhon et al, 1995; Burgstaller et al, 1994), aminoglycosides (Wang et al, 1995; Lato, et al 1995), antibiotics (Wallis et al, 1997; Wallace et al, 1998), nucleotide base analogs (Kiga et al, 1998), nucleotides (Huizenga et al, 1995) and peptides (Nieuwlandt et al, 1995; Williams et al, 1997) to numerous proteins of therapeutic interest like, growth factors (Jellinek et al, 1993; Green et al, 1996), enzymes (Tuerk et al, 1992; Bridonneau et al, 1998), immunoglobulins (Wiegand et al, 1996), gene regulatory factors (Jensen et al, 1994; Lochrie et al, 1997) and receptors. Beside all these, aptamers are also selected against intact viral particles (Pan et al, 1995), pathogenic bacteria (Camille et al, 2008) and whole cancer cell (Homann et al 1998).

The secondary structure of aptamers, consisting mainly of short helical arms and single stranded loops, is defined by complementary base pairing. Tertiary structure resulting from combinations of these secondary structures, are stable and in combination with the property of nucleic acids to form different non covalent bonds allow aptamers to bind to targets by any or mostly a combination of these forces viz. van der-Waals interactions, hydrogen bonding, structure compatibility, stacking of aromatic rings and electrostatic interactions (Thomas et al 2000).

Another area of intense research is to understand how the aptamers interact with their target. Many details have been revealed till date using wet lab as well as in slico techniques. Much of the molecular details in this area have been obtained by nuclear magnetic resonance spectroscopy. These studies revealed several insights. They showed that the finally folded conformation of aptamers form unique complex binding pockets to accommodate its target. Functional groups of aptamer are brought in close proximity to form a cluster of molecular forces that specify target interaction. NMR study of aptamer-Ligand complex revealed that small molecular targets are buried within the binding pockets of aptamers, leaving very little surface to interact with a second molecule. This may limit the possibilities of finding a second aptamer that could interact with a small

molecular target that is already bound to the first aptamer. Besides this impose strong specificity on the aptamer for its target (Feigon et al, 1996; Zimmermann et al, 1997; Jiang et al, 1996).

Aptamers binds to their target with high affinity with KD values typically ranging from 10 pM to 10 nM for proteins. Aptamers can recognize their targets with great specificity because surface area of interaction between an aptamer and its molecular target is relatively large, so even small changes in the target molecules can disrupt aptamers association. Thus aptamer can distinguish between closely related but non-identical members of a protein family or between different functional or conformational state of same protein (For instance, an aptamer to bFGF (FGF-2) binds with up to 20,000-fold greater affinity to bFGF than it does to its closely related fibroblast growth factor (FGF), while theofilin aptamer binds with 10,000 fold lower affinity to caffeine that differs only by a single methyl group) (Garret et al, 1997; Seiwert et al, 2000).

Aptamers are chemically stable as they can be boiled or frozen without loss of activity. They can be produced at microgram or kilogram scale. As synthetic molecules, they are amenable to a variety of modifications designed to optimize their properties for a specific application. They can be modified chemically as by base modification to reduce their sensitivity to degradation by enzymes in blood thus make them suitable for in-vivo application. This plasticity is a distinct advantage over other type of molecular ligands such as monoclonal antibodies where chemical modification is often variable, difficult to control and may harm the function of the molecule.

The above methodology of generating aptamers by selecting them from a large random sequence pool was formulated and established in the 1990s but natural aptamers also exist in riboswitches and is a very old concept in nature and diverse organisms use it as a mechanism for gene regulation (Michael et al, 2004). Cells use riboswitchs in a substrate dependent manner as all other switches like lac repressors. Riboswitch are cis-acting regulatory structures typically located in the non-coding 5"-UTRs portions of mRNAs, and are typically composed of two functional domains viz. the aptamer domain, which specifically bind the target molecule and serves as a molecular sensor and the second structure is the expression platform, which is typically located, immediately downstream from the aptamer domain, but in many instances the two domains overlap to some extent (Winkler et al, 2002). The role of the expression platform is to convey the signal from

metabolite-binding events into gene-control consequences by allosteric modulation of the structure of the 5'-UTR. The overall structure modulates gene expression by Controlling, the effectiveness of translation initiation (Winkler et al, 2002; Nahvi et al, 2002; Winkler et al, 2003), the transcription elongation of mRNA by the formation of rho independent transcription termination hairpins (Mironov et al, 2002; Winkler et al, 2002), folding in such a way as to sequester the ribosome-binding site, thereby blocking translation (Winkler et al 2002), self-cleavage and decreased stability (Sudarsan et al 2003) and folding in such a way as to affect the splicing of the pre-mRNA (Wachter et al, 2007).

I.2.1 Aptamers an emerging rival to antibodies

Starting from 1950s antibodies became widespread by 1970s when polyclonal sera from immunized animals were the popular choice for detecting analytes (Yallow et al, 1959) and with the discovery of monoclonal antibodies (Kohler et al, 1975), antibodies became indispensible in molecular and clinical diagnosis. By now antibodies have become the heart and soul of current molecular and clinical diagnosis beside their irreplaceable use in therapeutics. But of course it has its own limitations.

A better alternative to antibodies should not have the limitations posed by the antibodies but should match them in specificity and affinity. Recent research has shown this in aptamers and many comparative studies concluded with this point.

I.2.2 Advantages of aptamers

The intense research interest on aptamers and the enormous potential of it can be attributed to few of the basic properties of aptamers which makes them so handy to select and use.

One important point is that for the selection of an aptamer we need not to have thorough knowledge of the composition of the ligand which makes possible to select aptamers against targets whose function is known but composition is not well characterized.

High affinity and specificity aptamers having KD values in nanomolar to picomolar range can be selected by the simple SELEX procedure.

Comparison between aptamers and antibodies

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

(Nimjee et al, 2005)

It is easy and economical to select aptamers in vitro. Besides, we can design the selection conditions according to experimental need and hence it is applicable under non physiological conditions.

Aptamers can be generated against toxic and non-immunogenic molecules which is a limitation of antibodies. Thus aptamer approach offers the following advantages:

- The SELEX procedure can be modified as according to the need.
- Aptamers can be modified after selection to reduce size and enhance stability and specificity.
- Aptamers being nucleic acid molecules have very long shelf life.
- Aptamers are reproducible by chemical synthesis (Kawazoe et al, 1996).

- Aptamers can be expressed within cells (intramers). This permits the investigation of protein interaction in live cells (Famulok et al, 2001).
- Aptamers could be used for intracellular detection of target molecules or could function as intracellular inhibitors (Burgstaller et al, 2002).
- The small size of aptamers enables them to access protein epitopes that might otherwise be blocked or hidden (Lee et al, 2006).
- Aptamers display low to no immunogenicity, which is important for animal or human therapeutic applications (Nimjee et al, 2005).

I.2.3 Limitations

In spite of all the plasticity and applicability there are limitations of aptamers and the selection procedure, which cannot be ignored.

- All the aptamers cannot be selected through a standardized SELEX protocol and each time it has to be standardized according to the target, desired features of the aptamer and its application.
- The enrichment of unspecific binding species during aptamer selection process is often observed and one always needs to take steps to avoid them e.g., by negative selection steps in between.
- Many of the aptamers reported till date are manually selected. To manually accomplish the whole process from SELEX, followed by cloning to characterization and finally expression studies, is a time consuming process.
- One of the limitations of aptamers which limits its applicability as therapeutic agents is their low stability in biological fluids. Although many strategies e.g. chemical modifications, have been developed but still there is a need to do much.
- When applied in complex samples they are prone to non specific binding which makes it difficult to quantify the target by use of aptamer based assays, without sample pre-treatment. (Guthrie et al, 2006).
- Till now, high affinity aptamers are not as widely available as antibodies (Guthrie et al, 2006).

I .3 SELEX

The synthesis and screening of large libraries of related but structurally distinct compounds to identify and isolate the most appropriately functional molecules is known as combinatorial chemistry. The root of combinatorial chemistry can be traced back to 1960s when Bruce Merrifield at the Rockefeller University, investigated the solid phase synthesis of peptides using combinatorial chemistry based technique (Merrifield et al, 1963). In the 1980s H. Mario Geysen took this technique a step ahead and created library of different peptides on separate support. By 1990 industries began to use this technique and in its modern form, with the advances in robotics, it is an important technology for industry as well as biotechnological and pharmaceutical—research to discover new materials or molecules with desirable properties, new drugs, and catalysts.

Nucleic acids, although made up of only four different nucleotides have a great structural flexibility and are able to fold into defined secondary and tertiary structures. Besides, they can be amplified by PCR or in vitro transcription easily. Complex libraries of random sequence oligonucleotides with about 10^{15} variants can be synthesized chemically (James et al 2000). The above three properties make oligonucleotides very attractive compounds for combinatorial chemistry.

The combinatorial chemistry based screening of nucleic acid libraries to select oligonucleotides that binds very tightly and selectively to a certain non nucleic acid target was first performed by Tuerk and Gold to study the interaction between the bacteriophage T4 DNA polymerase (gp43) and the ribosome-binding site of the mRNA, which encodes the enzyme. They selected gp43 binding sequences from an RNA pool randomized at specific positions, and called this selection procedure SELEX (*Systematic Evolution of Ligands by Exponential enrichment)* (Tuerk et al, 1990).

At the same time Ellington and Szostak independently used a similar selection procedure to isolate RNA molecules from a random sequence RNA library with the ability to firmly and specifically bind small ligands, e.g. Cibacron Blue and Reactive Blue 4 (small organic dyes). They named these selected, individual RNA sequences 'aptamers'. They also studied the structure of these molecules and showed that these molecules form stable tertiary structures which form the binding site for the ligand (Ellington et al, 1990).

Two years later, Ellington and group showed that single stranded DNA molecules also have this property and ssDNA molecules binding ligand with high affinity and specificity can also be selected using the same SELEX method. These DNA aptamers were able to recognize and bind the ligands (Cibacron Blue and Reactive Green 19) in the same way like RNA aptamers (Ellington, et al, 1992).

In no time the SELEX technology became an important and widely used tool in molecular biology, pharmaceutical, and medical research. Additionally, SELEX is a flexible technique and can be modified to suit the need of different applications.

By now SELEX and numerous variants of the original SELEX process, have become well defined routine technique to select aptamers with high affinity and specificity for their targets. Many of the selected aptamers show affinities comparable to those observed for monoclonal antibodies. In addition, aptamers can distinguish between chiral molecules and are able to recognize a distinct epitope of a target molecule (Michaud et al, 2003).

I.3.1 Basic principle

The basic principle of SELEX is to minimize the pool size towards relatively few but high affinity binding motifs by repetitive cycles of enzymatic amplification and in vitro selection (Go¨ringer et al, 2003).

A typical SELEX process consists of five main steps. It starts with a chemically synthesized random oligonucleotide library consisting of about 10^{13} to 10^{15} different variants (James et al, 2000). For RNA aptamer the pool is converted to RNA by in vitro transcription. The random RNA or DNA pool is passed through column of target or incubated with target beads. The column or beads are then washed with appropriate buffer, eluted with elution buffer and then finally amplified by PCR in case of DNA SELEX or RT-PCR in case of RNA SELEX. This DNA is used as template for the next round of RT-PCR or PCR. The same cycle is repeated seven to ten or more times, depending on parameters like target features and concentration, design of the starting random DNA oligonucleotide library, selection conditions, ratio of target molecules to oligonucleotides, or the efficiency of the partitioning method, to get an enriched pool which specifically binds the ligand.

Modifications and additional steps can be introduced in the basic method to suit any special need of the ligand or to increase specificity, for example, negative selection steps and subtraction steps are strongly recommended to minimize enrichment of non-specifically binding oligonucleotides or to direct the selection to a specific epitope of the

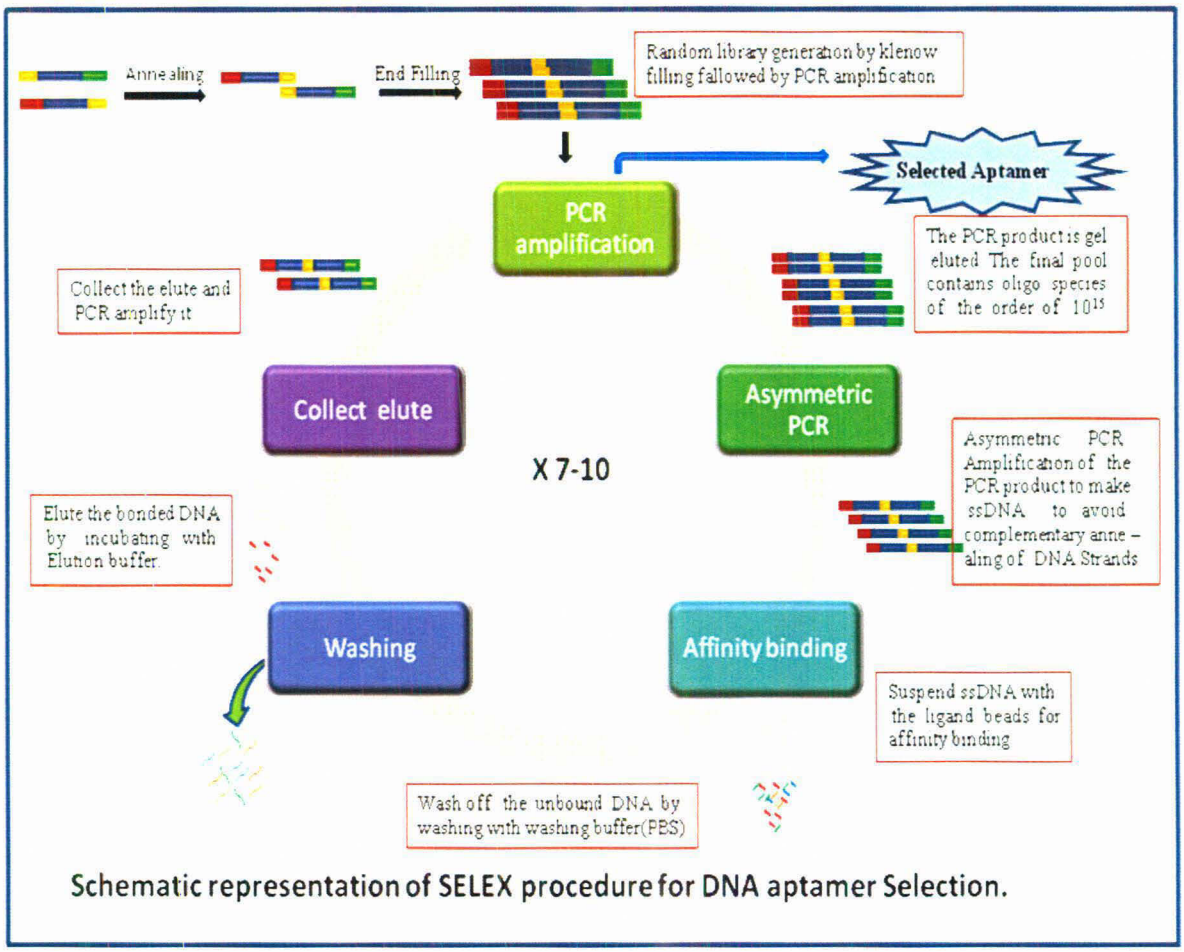


Fig-1: schematic summary of the basic SELEX method used for DNA aptamer selection.

target. Stringency of the selection procedure has gigantic effect on the quality of the selected aptamer in terms of specificity and affinity. Hence typically with the course of the cycle the stringency is progressively increased. This can be achieved by reducing the target concentration in later SELEX rounds or changing the binding and washing conditions (buffer composition, volume, time) (Marshall et al, 2000).

I.3.2 Starting random DNA oligonucleotide library

The SELEX process starts with a random oligonucleotide library. A random pool of about 80 nucleotide can be calculated to contain about 10^{48} variants. Typical design of a SELEX

library contains a central random region of 20-80 nucleotide flanked by two primer annealing regions of 15 to 20 nucleotide (Davis et al, 2002). It is preferable to amplify and purify the pool before initiating SELEX to eliminate fragments which cannot be amplified by PCR and to give enough representation to each species (Marshall et al, 2000). Different polymerases have different template preferences so it is possible to lose some of the sequences after the first cycle. For RNA aptamer the pool has to be converted to RNA by in-vitro transcription and for that the consensus region need to contain a 5'- T7 promoter beside the primer. Several of the properties of the finally selected aptamer depend on the pool so designing a good pool is key to the final successful selection of a good binding aptamer. After truncation or mutation the functional binding domains are only few nucleotides long (Bock et al, 1992). This with the benefit of easy handling, cost benefit and preference of short aptamers for various applications has set the trend of designing short oligo libraries of average 40 random nucleotide. But a longer random region gives batter structural complexity and may be preferred in case of ligands with special functional requirements (Marshall et al, 2000). Beside this, sometimes interaction between the ligand and the aptamer over an extended domain contribute to the high affinity binding of the aptamer (Klussmann et al, 2006). In few instances a partially structured library has been used and was found to be a superior source of high affinity aptamer (Davis et al, 2002; Nutiu et al, 2005). Using chemically modified bases enhance the affinity, conformational stability and nuclease resistance of aptamers. (Jayasena et al, 1999; Klussmann et al, 2006; Kusser et al, 2000).

I.3.3 Selection

A good selection is the key to get the best binding aptamer and the selection conditions can be made according to the application of the aptamer which is one of the main advantages of aptamers. In this step the oligonucleotide library is incubated with the ligand for affinity binding followed by partitioning of the unbound nucleotides. The ligand can be immobilized on the matrix of a column e.g. sepharose or agarose (Liu et al, 2005). But the column needs lots of pure ligand to load the column. Beads are a good alternative to columns when the amount of pure ligand is less or costly to make (Stoltenburg et al, 2005). By using magnetic beads we can automatize the procedure. During the past several years many groups have used different methods of separation like Capillary Electrophoresis (Mendonsa et al, 2004), Flow Cytometry (Davis et al, 1997),

Electrophoretic Mobility Shift Assay (Tsai et al, 1998), Surface Plasmon Resonance (Misono et al, 2005) and centrifugation (Rhie et al 2003).

The separation of the unbound nucleotides is followed by the elution of the bound nucleotides by competitive binding or by changing the physical conditions.

Radioactive or fluorescent labels are used to keep track of the nucleotides during the whole process (Ellington et al, 1992; Beinoraviciute-Kellner et al, 2005; Stoltenburg et al, 2005).

I.3.4 Amplification

The finally eluted oligos represent an extremely small fraction of the random pool and they need to be amplified before the next cycle, by simple PCR amplification in case of DNA aptamers or by RT-PCR in case of the RNA aptamers. The amplification can be used to add additional functional groups for immobilization, enlargement, detection etc.

I.3.5 Cloning and characterization

After the execution of the selection procedure the finally selected aptamers are cloned and sequenced for structural studies. Alignment studies are usually carried out using the web based program CLUSTAL-W (http:/www.ebi.ac.uk/clustalw/). By the alignment studies we can find the homologous regions between the aptamers. These sequences can be considered to be the ligand binding regions (Chenna et al, 2003). Secondary structure of the aptamers can be predicted using the program M-fold which provides information about relevant binding structures based on energy minimization method (http:/www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi).

I.3.6 Modifications

SELEX being an in-vitro process added with the plasticity of nucleotides, allows us to adopt conditions of further application already during the selection process. Researchers have explored this possibility and by now numerous variants of the originally established procedure, by Tuerk and Gold, are described. They are developed to increase the specificity or affinity of the aptamers or to optimize the procedure for specific needs. All of them make a long list but here I like to list a few of them.

I.3.6.1 Negative SELEX

It is a method to eliminate non-specifically binding species. The pool is incubated with unrelated molecule before starting the SELEX procedure as well as in between few cycles (Blank et al, 2001; Vater et al, 2003).

I.3.6.2 Counter-SELEX

It is designed to select high specificity aptamers which discriminate between closely related molecules. Pool is incubated with the related molecule to eliminate species which are not able to discriminate between the related molecule and the target (Jenison et al, 1994; White et al, 2003).

I.3.6.3 Chimeric SELEX

This facilitates selection of aptamers with more than one desired feature. Aptamers are selected from two different libraries. Each of them is selected for a distinct feature the final aptamers are then fused to get chimeric aptamers (Burke et al, 1998).

I.3.6.4 Cross-linking SELEX

It is designed to select aptamers containing reactive groups which are capable of covalent linking to the target molecule (Jensen et al, 1995; Kopylov et al, 2000).

I.3.6.5 Photo-SELEX

This refers to selection of aptamers bearing photo-reactive groups, which on excitation photo cross-link to the target (Brody et al, 1990; Johnson et al, 1999).

I.3.6.6 Cell –SELEX

This allows selection of aptamers against whole cell. (Shangguan et al, 2006; Chen et al, 2008; Shangguan et al, 2008; Tang et al, 2007).

I.3.6.7 Non-SELEX

This is a process that involves repetitive steps of partitioning with no amplification between them (Berezovski et al, 2006).

I.3.6.8 EMSA-SELEX

This is based on electrophoretic mobility shift assay (EMSA) for partitioning in every round (Tsai et al, 1998).

I.3.6.9 On-chip selection

This refers to the selection of DNA aptamers on chips allows parallel selections (Asai et al, 2004).

I.4 Application of aptamers

With the advancements in technologies novel approaches are discovered which challenge the traditional methods. Today, technological advancements have enabled researchers to engineer the properties of molecules quite contrary to what nature has designed them for and generates variants that suit our need. For example, the antibody molecule that evolved to recognize antigens with high affinity and specificity is now being modified to catalyze different reactions (Schultz et al, 1990). The development of the SELEX protocol (Tuerk et al, 1990; Ellington et al, 1990) has enabled to select aptamers against a broad range of target molecules.

The possibility of selecting aptamers to a broad range of ligands together with its high specificity and affinity makes aptamers useful for a vast array of diagnostic assays and therapeutic application besides being a powerful tool in molecular research.

I.4.1 Diagnosis

Detection and quantification of molecules lies at the heart of basic molecular as well as clinical research. This field has a long history complimenting its crucial role and evolved to beat the need of the time. Specificity and affinity for its target is the utmost criteria for a molecule to be useful for diagnostic application. Technologies that allow specific detection and precise quantification of molecules are evolving very fast but for the last few decade and till now antibodies have been the most popular class of molecules providing molecular recognition needed for a vast range of application and they contributed enormously towards the advancement of diagnostic assays and have become indispensible in most of the clinical diagnostic tests.

But in the face of the ever increasing demand for diagnostic assays to assist in the management of existing and emerging diseases we need to look for alternative methods. The development of the SELEX procedure (Tuerk et al, 1990) and its ability to select high specificity and high affinity aptamers opened a new window for devising new aptamers based methods which overcomes the earlier difficulties and fulfil molecular recognition needs in both in-vivo (Charlton et al, 1997) and in vitro diagnostic assays. Aptamers have been used in many different diagnostic assay formats and found to be promising.

Aptamer against VEGF were used in a two-site binding assay format as the detector ligand (Drolet et al, 1996). Another group demonstrated the simultaneous binding of an aptamer and an antibody to human CD4 expressed on cell surfaces (Davis et al, 1998). Human neutrophil elastase (HNE) binding DNA aptamers (Lin et al, 1995) was modified to attach fluorescein at different positions, away from the target binding site, using different linkers, and was used to stain HNE-coated beads for flow cytometry. (Davis et al, 1996). This aptamer was also labelled with fluorescein at the 59 end and used in a fluorescence polarization (FP) assay to detect HNE. A significant change in FP was observed when the labelled aptamer interacted with HNE. The addition of a nonspecific target, human serum albumin, produced a very small change in fluorescence polarization only at high concentrations. This demonstration suggests that fluorescein- labelled aptamers could be used to design non-competitive fluorescence polarization assays for target detection. Aptamers selected to recognize aminoglycoside antibiotics have been used in competitive fluorescence polarization assays to understand their binding characteristics to a family of aminoglycosides Cho et al, 1998; Wang et al, 1996).

I.4.2 Therapeutics

Antisense or antagonistic therapy is one of the most widely used and successful method. Development of antisense strategies in 1970 was the pioneering thought to use nucleic acid molecules as antisense therapeutic. From the initial phase of aptamer development they were expected to deliver on this front and now after about 19 years of intensive research we have got some convincing results. A stringent SELEX selection mostly yields a high affinity and specific antagonist of the targeted protein and with few post selection optimization the molecule can be tested for pharmacologic effect in animals or used in vivo in target-validation studies. The biggest reward to this is the recent approval of the first aptamer (anti VEGF aptamer) based drug called Macugen, by the U.S Food and Drug Administration for the treatment of age related muscular degradation (AMD) (Ng EW et al, 2006).

I.4.3 Cancer

Biomarker assays are key to cancer diagnosis. There is a vast array of biomarkers ranging from glycolipids to proteins; here arise the need of a method that can produce probes which can bind to any class of molecules. SELEX is the ideal method and aptamer is a

potent probe for this (Bock et al, 1992; Jhaveri et al, 2000; Daniels et al, 2003). Till date aptamers have been generated against many biomarkers; besides, the SELEX procedure has also been used for selecting aptamers using whole cells (Hamaguchi et al, 2001; Hicke et al, 2006; Wang et al, 2003). Known as cell SELEX, the method has been used to select aptamers that can bind specifically to cancer cell (Shangguan et al, 2006; Chen et al, 2008; Shangguan et al, 2008; Tang et al 2007). Aptamers selected by this method can be used to select molecular probes for cancer cells without knowing the detailed biochemical differences between cancer cells and healthy cells. Aptamers selected in this manner can be used to affinity purify their target proteins from the cell membrane (Mallikaratchy et al, 2007; Shangguan et al, 2008). Hence aptamers derived from the cell-SELEX procedure can be used to discover new biomarkers thus increasing the overall utility of aptamer technology.

Further, there are aptamers selected against molecules like VEGF, known to play role in cancer and angiogenesis. Hence aptamers can also be used as antagonistic therapeutic agents to limit cancer progression.

I.4.3 Drug delivery vehicle

Many of the currently used chemotherapeutic agents are toxic because of their systemic distribution. Aptamers targeted to surface markers of cell or other specific tags of diseased cell may serve as a highly targeted drug delivery vehicle.

I.4.4 Sensors

Rapid detection and quantification of analyte is crucial for many applications. Biosensors are devices for measuring the concentration or interaction of biological molecules. A biosensor converts the recognition interaction between the immobilized specific binder, like antibodies and aptamer and the passing protein or cell, into an electronic signal. Biosensor with antibody as the immobilized specific binder has been developed (Morgan et al 1996). But the obvious limitation is the non regenerate nature and mild condition requirement of the antibody. Aptamers have many different properties to be a good sensor. Firstly, it can be selected under many different conditions hence we can select aptamers under conditions mimicking the condition in which it has to be used. Aptamers could be modified for immobilization easily and attached to different surfaces in a

controlled and reproducible manner (Maskos et al, 1992; Fodor et al, 1991; Guo et al, 1994).

I.4.5 Aptamers as molecular switches

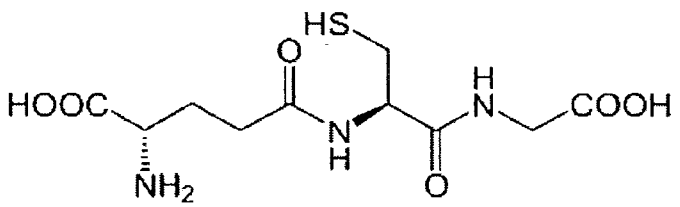
Aptamers get an edge over antibodies in most of the cases just due to one fundamental property, i.e. the renatureable nature of oligonucleotides. This property coupled with the ability to select them under desired condition makes them suitable as molecular switches. In fact we can make an aptamer sensitive to any one or a combination of physical factors like heat, pH, chelating agent etc (Dang et al, 1996; O'Connell et al, 1996; Bridonneau et al, 1999). Aptamers binding to specific ligand under specific condition can be developed and such aptamers can be used as a molecular switch i.e. "regulates one sensing the other". As an example DNA aptamer was selected which binds L-selectin in a metal ion dependant manner (O'Connell et al, 1996). Another fine example is the use of aptamer specifically binding to the active site of taq polymerase as a molecular switch for in situ hot start PCR reactions. The extra merit of aptamer based switch is that it can be selected to denature at 40 to 50^0C, required for reverse transcriptase. None of the other available alternatives, i.e. antibody neutralized (75^0C) and Ampli TaqGold (90^0C), can be modified for this (Kellogg et al, 1994; Birch et al, 1996).

I.5 Glutathione

In 1888 de Rey Pailhade discovered a molecule which he called "hydrogen 'nant le soufre," and hinted its involvement in sulphur metabolism (De Rey Pailhade, et al, 1888). After about 41 years this substance was found to be Glutathione (Hopkins et al, 1929). Later in 1960s Kosowers coined the word Glutathione (Kosower et al, 1969).

Now after 121 years, researchers have documented different aspects of its function and innumerable situations in which glutathione participate in maintaining essential aspects of cellular homeostasis.

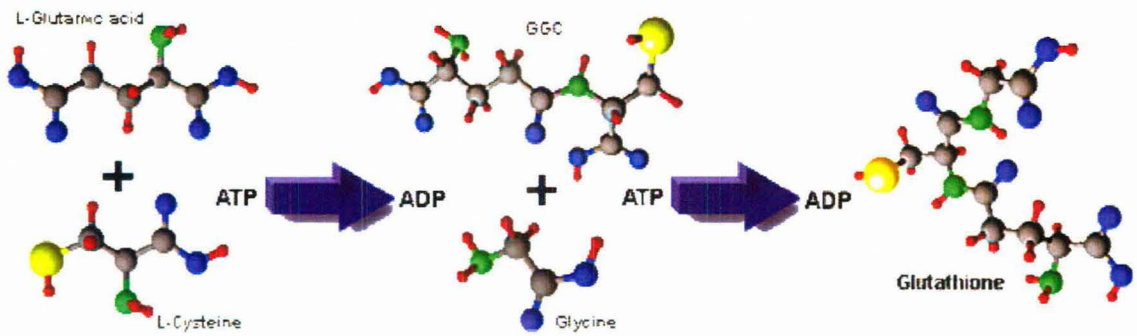
Glutathione is a tripeptide thiol made up of the amino acids gamma-glutamic acid, cysteine, and glycine and is also known as gamma-glutamylcysteinylglycine or GSH. It is found in vast majority of prokaryotic and eukaryotic cells and is the biologically most abundant low molecular weight intracellular thiol. (.5-8 mM) (Kunert et al, 1993). Glutathione lacks the toxicity associated with cysteine (Vina et al, 1983), making this compound suitable as a cellular thiol "redox buffer" to maintain cellular thiol/disulfide redox potential. In cells, glutathione can be free or bound to Proteins.



Glutathione has a molecular weight of 307 dalton and the molecular formula is $C_{10}H_{17}N_3O_6S$. It contains an unusual peptide linkage called gama linkage, between the amino group of cysteine and the carboxyl group of the glutamate side chain which is thought to protect it from degradation by aminopeptidases, while the backbone bonds are normal peptide bonds. The reduced form of glutathione is its standard form but it exists in cells in several additional forms. The oxidised form of glutathione is known as Glutathione disulfide (GSSG). Sometimes, this species is referred to as "oxidized glutathione," but further oxidation products are also formed from GSH, e.g., sulfonates.

Glutathione is synthesized from its constituent amino acids in two consecutive steps. The first step to join L- Glutamate and L-cysteine is catalyzed by the enzyme □-glutamyl-cysteine synthase which utilizes an ATP molecule to give gamma-glutamylcysteine

(GGC) and then another enzyme Glutathione synthase adds Glycine to GGC to give Glutathione. (Meister et al 1988).



Glutathione is degraded to its constituent amino acids via two routes viz. □-glutamyltranspeptidase (Taniguchi et al, 1998) and cysteinyl-glycine dipeptidase (Meister et al, 1984; Jo¨sch et al, 1998).

Glutathione is both a reductant and a neutrophile and hence can react with oxidising species. This property makes glutathione one of the most important water soluble antioxidant. This property is conferred on it by the thiol group and through this thiol group, it participates in redox reactions i.e. disulphate formation and thioesether and thiolester formation. These redox reactions are catalyzed by various kind of GSH peroxidise (Brigelius-Flohe´ et al, 1999) and GSSG reductases. The glutathione S-transferases (GST) catalyze thioether formation. This antioxidant have many extensively reviewed roles including detoxification of a variety of exogenous and endogenous compounds such as xenobiotics, carcinogens, free radicals, and lipid peroxides; maintenance of protein structure and function; regulation of protein synthesis and degradation; protection against oxidative damage; and maintenance of immune function.

I.5.1 GSH a potential cancer treatment target

Many of the presently available anti cancer therapies are limited in their application because of their low efficiency and the most efficient drug is still to come. On another front researchers are engaged in designing strategies to increase the susceptibility of tumor cells to apoptosis which can increase the efficiency of a variety of cancer treatment.

The redox state of the cell has an important role in the induction of apoptosis (Green et al, 1998; Marchetti et al, 1997; Coffey et al, 2000; Armstrong et al, 2001). Many agents

known to induce apoptosis either induce ROS formation or depletion of antioxidants (Buttke et al, 1994). Investigations have suggested that the latter is most effective apoptosis inducers (Hug et al, 1994).

As discussed earlier glutathione largely determine the redox state of the cell. Although most of the glutathione is found in the cytocol (Fernandez-Checa 1997), but a small but significant percentage (10-15) of glutathione is found in the mitochondria. This mitochondrial fraction of glutathione is responsible for scavenging most of the reactive oxygen species produced during the electron transport chain and oxidative phosphorylation (Fernandez-Checa 1997). Deficiency in mitochondrial fraction of Glutathione impairs the redox state of mitochondria and results in the release of pro apoptotic molecule cytochrome C (Ghibelli 1999). Cytochrome C can combine with Apaf-1 and procaspase-9 to form the apoptosome which activates caspase-9 and caspase-3 and ultimately results in apoptosis.

There are various mechanisms in the cell for the efflux of glutathione conjugates. The formation and efflux of glutathione conjugates of anti-cancer drugs pertain to drug resistance of tumor cells (Hayes 1999).

Glutathione is thus a good target for increasing the susceptibility of cancer cells to apoptosis as well as to decrease the resistance of the tumor to anti-cancer drugs.

Buthionine Sulphoximine (BSO) is a known inhibitor of gamma-glutamylcysteine synthetase and treatment of cancer cells with BSO has shown to induce apoptosis, commitment to cell cycle arrest, release of cytochrome c and high production of ROS (Armstrong et al, 2002). This study has enough to indicate that the efficient scavenging of glutathione can be a good tool in cancer therapy.

Materials and Methods

II. Material and Methods

II.1 Materials

II.1.1 Materials Used for Routine work

Acrylamide(40%) solution	38 gram of Acrylamide and 2 gram of bis-acrylamide dissolved in water, make up to 100ml and sterilized by either Watmann paper or by 0.45µ filter and stored in dark bottle at 4^0C.
Agarose gels	0.5%-2 %,(w/v), for DNA analysis; with 1x TAE buffer, Ethidium bromide (0.5 µg/ml).
Agar plates	1.5 % agar, 2%LB, autoclaved added antibiotic (ampicillin: 100µg/ml).
Antibiotics stock	Ampicillin sodium salt, stock concentration 100 mg/ml in water.
EDTA	0.5 M (in double distilled water, pH adjusted to 7.5 with 5 M NaOH), autoclave.
Ethidium bromide (1000x)	1 mg/ml in H2O [Sigma]. Stored in dark bottle at 4^0C.
Loading Dye (DNA)	0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 30% Glycerol and final volume adjusted with autoclaved MQ water.
Luria Broth	2.0 gram of Luria Broth medium (Hi Media) in 100ml of water, mixed and sterilized by autoclaving.
PBS(1X)	8 gram NaCl (137mM), 0.2 gram KCl (2.7 mM), 1.44 gram of Na_2HPO_4 (2 mM) in one liter of

	water and sterilized by autoclaving.
Phenol	Pre distilled phenol (Sigma) was equilibrated with 0.5M Tris (pH 8.0) by stirring with magnetic stirrer. The upper phase was removed and equal volume of 0.1M Tris was added and stirred. This step was repeated thrice until pH reaches to 7.8 and stored in light opaque bottle at 4^0C.
RNAse A	10mg/ml in 0.01M sodium Acetate (pH 5.2), heated for 10 minutes at 90^0C, allowed it to cool and adjusted pH by adding 0.1 volume of 1M tris (pH 7.4), stored at -20^0C.
Sodium acetate (3M, pH 5.2).	40.8 gram of Sodium acetate in 80 ml of water pH adjusted to 5.2 with Glacial acetic acid and final volume adjusted with water to 100 ml, autoclaved and stored at room temperature.
Sodium Chloride (5M).	29.2 gram of NaCl in 80 ml of water, volume adjusted with water and sterilized by autoclaving.
SDS (10%).	10gram of sodium dodecyl Sulfate dissolved in 90 ml of water and final volume adjusted to 100 ml (not to be autoclaved).
TAE (50X).	24.2 gram of tris base, 5.7 ml of glacial acetic acid, 10 ml of EDTA (pH 8), final volume adjusted with water, autoclaved and stored at room temperature.
TBE (10X).	10.8 gram of tris base, 5.5 gram of Boric acid, 4 ml of EDTA, final volume adjusted to 100 ml with

	water, autoclaved and stored at room temperature.
Tris-Cl (pH 8).	12 gram Tris Base in 80 ml of water and pH adjusted with HCl, volume adjusted with water to 100 ml.
TE	Tris-Cl (pH 8) 10µl (10mM) and EDTA 1 µl (1mM) in 989 µl of water. Final volume 1ml.
X-gal	(5 bromo, 4-Chloro 3-indolyl β-D galactoside) 20mg of X-gal dissolved in 1 ml of dimethyl formamide, tubes wrapped with aluminum foil and stored at -20^{0}C.
X-gal, IPTG plates	20µl of X-gal (20mg/ml) and 100 µl of IPTG was mixed together, spreaded on Pre-made agar plates and incubated at 37^{0}C for 30 minutes, used immediately for transformation.

II.1.2 Material used for generation of nucleic acid library

Material	
Oligo 1	5'-CGAATTGGAGCTCCACCGCG (N_{50}) GAATTCGGATCCAAGCTT-3'
Oligo 2	5'-AACAAAAGCTGGGTACCGGG(N_{50}) AAGCTTGGATCCGAATTC-3'
Oligo 3	5'-TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCG-3'
Oligo 4	5'AACAAAAGCTGGGTACCGGG3'
NaCl	50mM

Note: Oligo 1is also named Sac I Random

Oligo 2 is also named Kpn I Random

Oligo 3 contains T7 Promoter + 20 nucleotides (T7 + 20 mer)

Oligo 4 contains 20 nucleotides (20 mer)

II.1.3 Primers used for PCR

Forward Primer	5'TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCG3'(Primer-1)
Reverse Primer	5'AACAAAAGCTGGGTACCGGG3' (Primer 2)

II.1.4 Beads used for selection

Name:	Glutathione –CL Agarose. (GeNei^{TM})
Binding capacity:	14mg/ml (Determined for GST-fusion protein).
Ligand Density:	3.4mg/ml beads.
Binding buffer:	15mM Sodium phosphate (pH-7.4) and 0.15M NaCl.
Wash buffer:	15mM Sodium phosphate (pH-7.4) and 0.15M NaCl.
Elution Buffer:	50mM Tris, pH 8.0 containing 5mM reduced Glutathione.

II.1.5 Bacterial strains Used

Strains	**Description**
Escherichia coli DH5α	φ80 d*lac* Z Δ M15, *rec*A1, *end*A1, *gyr* A96, *thi*-1, *hsd*17 (r_k^-, mk_k^-), *sup* E44, *rel*A1, *deo*R, (*lac*ZY A- *arg*F) U169

II.1.6 Plasmids Used

pGEMT-easy	The pGEM-T Easy Vector Systems are convenient systems for the cloning of PCR products. The vector is prepared by cutting the pGEM®-T Easy Vectors, with EcoRV and adding a3′terminal thymidine to both ends. These single 3′-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases.

II.2 Methods

II.2.1 Generation of nucleic acid library

To construct a combinatorial DNA library with a degenerate sequence of n nucleotides, a library of $N_{max} = 4^n$ was obtained. But, the actual degeneracy of library depends upon the amount of DNA used for selection rather than on N_{max}. For selection we used 10µg of DNA and calculated the number of DNA molecules in it using the formula:

Amount of primer (ng) = pmoles of molecules x 0.33 x n

Where, n = Length of oligonucleotide sequence.

The length of oligo was 88mer.

Therefore, 10 µg of DNA was equivalent to 344.4 pmoles.

To determine the pool size the number of molecules used for selection round were calculated from the 344.4pmoles as:

1.0 pmole = 1.0×10^{-12} x Avogadro number of molecules

i.e., $344.4 \times 10^{-12} \times (6.02 \times 10^{23}) = 2073.288 \times 10^{11} = 2.073 \times 10^{14}$ molecules

We started the SELEX with a pool size of 2.073×10^{14} molecules.

Annealing reaction

Component	**Amount**
Oligo 1 (1 µg/µl)	10.0 µl
Oligo 2 (1 µg/µl)	10.0 µl
NaCl (50mM)	0.8 µl

The two oligos were mixed well and the reaction was incubated at 95°C for 2 minutes followed by room temperature for 5min. Finally kept on ice for 5 min.

Klenow end filling reaction

Component	Amount
10X Buffer	2.0 µl
dNTP mix (2.5mM)	2.0 µl
Klenow fragment of DNA pol I (10U/µl)	1.0 µl
DNA from annealing reaction	5.0 µl
Final volume adjusted with water	**20.0 µl**

The reaction was mixed well and incubated at 37 ^{0}C for 1 hour. After incubation the reaction mix was extracted by Phenol-chloroform extraction method.

PCR Reaction

PCR was performed to replicate the DNA species which is required for a better selection.

Material	Amount
10X Buffer	10.0µl
dNTP mix(25mM each)	1.0µl
Taq DNA polymerase (1U/µl)	2.0µl
$MgCl_2$(50mM)	2.5µl
Primer-1	5.0 µl
Primer-II	5.0µl
Template(2/10 diluted)	1.0µl
Final volume adjusted with water	**100.0 µl**

The PCR reaction was set under following reaction conditions in thermocycler:

Step I (initial denaturation)	94^{o}C	5 minutes	
Step II (Denaturation)	94^{o}C	30 seconds	15 cycles
Step III (Annealing)	60^{o}C	30 minute	
Step IV (Extension)	72^{o}C	30 seconds	
Step V (Final extension)	72^{o}C	5 minutes	

II.2.2 Freeze Thaw method of Purification of DNA from agarose gels

The band of interest from digest or PCR generated fragment was electrophoresed in low melting temperature agarose gel, and band of interest was cut with sterile blade and the gel was crushed properly using 1ml pipette tips. To this 100-200µl of 10mM Tris-EDTA buffer or water was added. This was followed by addition of equal volume of phenol and vortexed, kept at -70°C for 15 minutes. After which the same was kept at room temperature to thaw. This process was repeated for 3 times and centrifuged; the supernatant was extracted with chloroform. To this 0.1 volume of 3M sodium acetate and 2.5 volume of ethanol were added and kept for precipitation for 30 minutes to 2 hours at -20°C. DNA was pelleted by high-speed centrifugation. The pellet was washed with 70% ethanol and dissolved in MQ.

II.2.3 Asymmetric PCR

Asymmetric RCR was performed for the selective amplification of one strand to get single stranded DNA.

Material	**Amount**
10X Buffer	10.0µl
dNTP mix (25mM each)	1.0 µl
Taq DNA polymerase (1U/µl)	2.0 µl
$MgCl_2$(50mM)	2.5 µl
Primer-1	10.0µl
Template(1µg/µl)	1.0 µl
Final volume adjusted with water:	**100.0 µl**

The PCR reaction was set under following reaction conditions in thermocycler:

Step I (initial denaturation)	94°C	5 minutes	
Step II (Denaturation)	94°C	20 seconds	60 cycles
Step III (Annealing)	60°C	30 minute	
Step IV (Extension)	72°C	20 seconds	
Step V (Final extension)	72°C	5 minutes	

II.2.4 SELEX

To select Glutathione binding DNA aptamers, **Glutathione –CL Agarose (GeNei^{TM}) Beads** were used. The beads (100µl) were washed with 5-10 bed volumes of deionised water to remove all the preservatives (20%ethanol). The beads were equilibrated with binding buffer. Then the ssDNA sample (300µl) dissolved in the same buffer was added to the beads and incubated, with mixing in between, for 20 minutes. This was followed by a short spin (30s) at 2000 rpm. The supernatant was collected in an eppendroff tube. The beads were then washed with binding buffer to remove any nonspecifically bound ssDNA and then incubated with 300µl of elution buffer twice for ten minutes. The fractions collected were ethanol precipitated, centrifuged at 13000rpm for 10 minutes at 4^0C, washed with 70%ethanol, dried and finally dissolved in 10µl of MQ water. PCR was set to amplify the eluted ssDNA and the product was gel eluted by the phenol-freeze-fracture method and used for the next asymmetric PCR which was used for the next round of selection.

Elution scheme

To elute the bound ssDNA, reduced glutathione was used which competes with the glutathione on the beads to bind with the ssDNA.

II.2.5 Cloning of the selected aptamers

The selected aptamers were PCR amplified, gel eluted and ligated with the pGEM-T vector.

Ligation

Material	**Amount**
2X Buffer	5.0 µl
pGEM-T (50ng/µl)	1.0 µl
T4 ligase	1.0 µl
PCR product	3.0 µl
Final volume	**10.0µl**

Mixed by gentle pipeting spined down and kept at 4^0c over night.

II.2.6 Competent Cell Preparation of Bacteria cells

E. *coli* strains XL1 blue or DH5α of were used for cloning the constructs. XL1 blue and DH5α cells were invariably pre selected for the tetracycline and ampicilin resistance respectively. The single pre selected colony was grown by shaking overnight in the 2-10ml cultures. This culture was inoculated (1:100, v/v) in fresh LB (Tet) medium and shaken at 37° C to an OD at 550nm of 0.6-0.7. This OD corresponds to approximately $6x10^7$ cells /ml. The cells were pelleted at 3500 rpm for 5mins at 4° C, re suspended in half the original culture volume of pre cooled 50mM $CaCl_2$, and incubated for 10-20 minutes. The cells were centrifuged again as above and suspended in one tenth of the original volume of pre cooled 50mm $CaCl_2$ with 20% glycerol. The cells were aliquot in 200-600 µl volumes and stored at -80° C until use.

II.2.7 Transformation of plasmid DNA in E.coli cells

For transformation typically 10ng of plasmid DNA was pre treated with 2µl of ice cold TCM solution (100mM Tris and 300mM of Ca Cl_2, 300 mM of $MgCl_2$) in ice for 5 minutes. The frozen competent cells were kept in ice for thawing, and 150µl of cell suspension was added to the TCM pre-treated plasmid DNA, and incubated for 40 min in ice. After 40 min, the transformation mixture were given heat shock at 42°C for 90sec and immediately kept in ice for 5 mins. To this 850µl of LB was added and incubated under moderate agitation at 37°C for 60mins, to allow the expression of antibiotic resistance gene. An aliquot of transformed cells (100-125µl) was spreaded on agar plates containing selective antibiotic and incubated overnight for selection of the transformants.

Blue white screening

For blue- white selection of transformants, the transformed cells were spreaded in LB agar plates containing Xgal, IPTG (plates were initially spread with 50µl of IPTG (100mM in water), 20µl of X gal (50mg/ml in dimethyl formamide) before plating the cells) and incubated at 37°C for overnight.

II.2.8 Alkaline Lysis Mini-Prep

A single bacterial colony was transferred into 2 ml of LB medium containing the appropriate

antibiotic in a 15 ml tube. The culture was incubated overnight at 37 °C with vigorous shaking (220 rpm). The culture was poured into a microfuge tube, centrifuged at 5000 rpm for 5minutes at 4°C in a microfuge. The medium was removed, leaving the bacterial pellet as dry as possible. The pallet was resuspended the in 100 µl of ice-cold Solution I (50mM glucose, 25 mM Tris-Cl pH-8, 10mM EDTA pH-8) by vigorous vortexing. Added 200 µl of freshly prepared Solution II (0.2N NaOH, 10% (w/v) SDS). The tube was closed tightly, and contents mixed by inverting the tube rapidly few times. Now 150 µl of ice-cold Solution III (5M potassium acetate, glacial acetic acid 11.5% (v/v)) was added. The resulting solution is 3M with respect to potassium and 5M with respect to acetate. The tubes were closed and vortex. And kept on ice for 3-5 min. Finally tubes were centrifuged at 13000 for 10min. at 4 °C in a microfuge. The supernatant was transferred to fresh tube and centrifuged again at 13000 rpm for 5 minutes. The supernatant was taken in a fresh tube and precipitated with 2.5 volume of 100% ethanol for 1hour at -80°C. The tubes were centrifuged at 13000 rpm for 10 minutes, supernatant discarded and the pallet washed with 70 % ethanol. The pallet was dried and finally dissolved in 30 µl of RNAse (20 µg/ml) added water.

II.2.9 Digestion

Digestion with Not-I was performed at 37^{0}C for 1 hour to confirm the positive clones.

Material	**Amount**
2X Buffer	2.0 µl
BSA	0.2 µl
Not-I(10U/µl)	2.0 µl
DNA	10.0µl
Final volume adjusted with MQ	**20.0µl**

Incubated at 37^{0}C for 2 hrs. Then resolved on 2% agarose gel.

II.2.10 Long Term Storage of Bacterial Cultures

All the confirmed constructs were transfected into XL-1 blue competent cells, grown in LB medium containing appropriate antibiotics, harvested and stored at -80 ^{0}C as glycerol stock (20% glycerol, 80% fresh LB medium).

II.2.11 Software and web servers used for sequence analysis

Multalin: for multiple sequence alignment, used with default settings (Corpet. F, 1988) (http://bioinfo.genotoul.fr/multalin/)

M fold: For secondary structure prediction, used with default settings (Zuker. M, 2003; Mathews et al, 1999) (http://mfold.burnet.edu.au/dna_form).

Bioedit sequence alignment editor: Used with default settings (Hall 1999) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)

Gene Bee: For phylogenetic studies (http://www.genebee.msu.su/index.html).

Sequence Massager: For removing gaps and changing the orientation of sequences (http://www.attotron.com/cybertory/analysis/seqMassager.htm).

II.2.12 Kinetics

Kinetics studies of aptamers were performed according to the method given in the "Handbook of RNA Biochemistry" (page-795).

The clone was amplified by asymmetric PCR (50 cycles) in presence of α P^{32}-CTP (10 μCi/100 μl). Aliquot 1μl from each reaction. The PCR product was then precipitated with 2.5 vol. of 100% ethanol and 1/10 vol of Sodium acetate. The DNA was dissolved in 50 μl MQ and passed through 1ml Sephadex G50 column. The column was washed with 100 μl BSA twice. The frictions were collected in an eppendorf tube and the concentration was calculated. Took 100ng of DNA in five tubes and Kept at 94^0C for 5 minutes followed by room temperature for 10 minutes. Took 20μl of glutathione beads in five 0.5 ml eppendorf and washed them with 5 bed volumes of PBS. Added 100ng of DNA to each tube and incubated for 15 minutes with gentle tabbing in-between. Washed four tubes with 3 bed volume of PBS to remove unbound DNA. Eluted them with different concentration of reduced glutathione (0.5μl, 1.0μl, 2μl and 5μl) seven times. The fractions were collected and cpm count was taken using Liquid Scintillation Analyzer (Packard Biosciences). The counts were plotted using Sigmaplot-8.0. The Kds were calculated using the formula given below.

$$Kd = [L] \left\{ \frac{(Vel - Vn)}{(Ve - Vel)} \right\}$$

Vel = median elution volume of aptamer eluted in the presence of free ligand.

Ve = Median elution volume measured in absence of free ligand with the same column buffer.

Vn = Median volume at which aptamer population having no interaction with the column would be eluted.

L = Free ligand concentration.

Results

III. Results

III.1 Generation of oligonucleotide library

For the generation of the oligonucleotide library the following four chemically synthesized oligonucleotides were used.

Oligo 1 5'CGAATTGGAGCTCCACCGCG (N_{50}) GAATTCGGATCCAAGCTT3'
Oligo 2 5'AACAAAAGCTGGGTACCGGG(N_{50}) AAGCTTGGATCCGAATTC3'
Oligo 3 5'TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCG3'
Oligo 4 5'AACAAAAGCTGGGTACCGGG3'

First of all we checked the four oligos on 12% denaturing acrylamide gel by electrophorises (Fig: III.1).

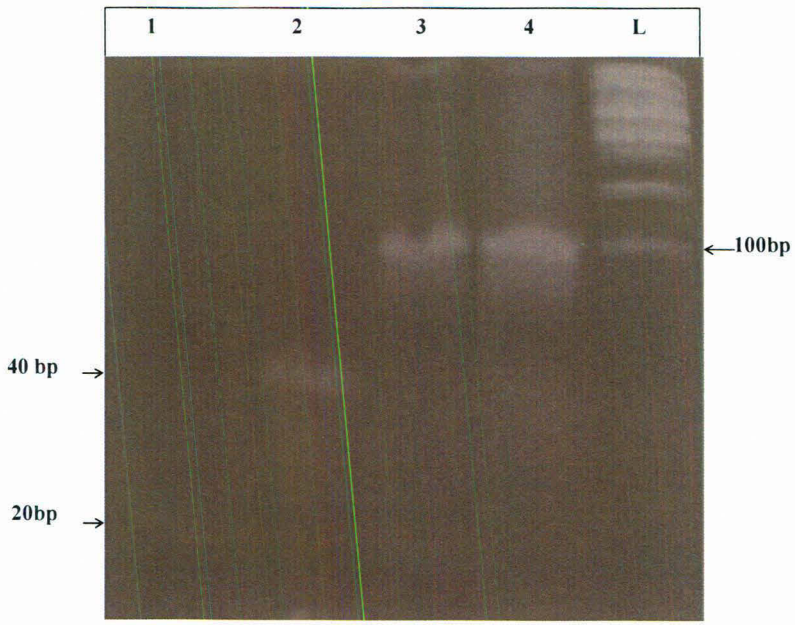


Fig: III.1. ***Oligonucleotides used,*** *lane 1,2,3 and 4 shows oligo 4,3,2 and 1 respectively. L shows 100bp ladder used as size marker.*

Oligo 1 and oligo 2 were then annealed together and end filled by klenow filling followed by PCR amplification using oligo 3 and 4 as primers

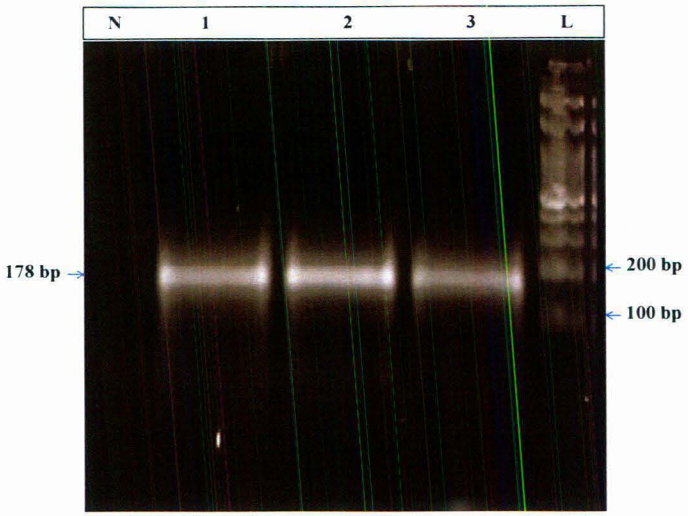


Fig III.2: ***PCR product resolved on 2% agarose gel*** *for gel elution to eliminate unwanted oligos. Lane 1, 2, and 3 shows the PCR product, L is 100 bp ladder.*

After resolving on agarose gel the gel area corresponding to 178 bp (Fig:III.2) was cut and eluted by the phenol freeze fracture method. The eluted product was then checked on 12% denaturing polyacrylamide gel (Fig:III.3).

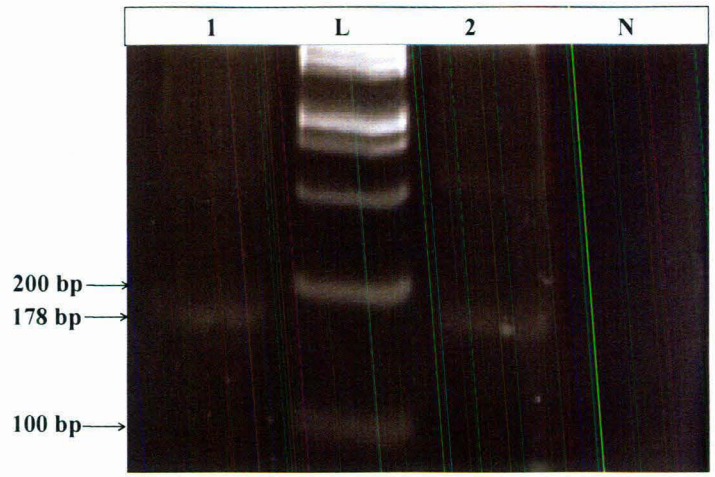


Fig III.3: ***Gel eluted 178 mer oligos*** *resolved on 12% denaturing polyacrylamide gel. Lane1 and 2 shows the gel eluted 178 mer oligos, L is ladder and N is the negative control.*

III.2 SELEX

After confirming the desired size of the oligos (178 mer), started the SELEX procedure for the selection of glutathione binding aptamers. Asymmetric and normal PCR products, resolved on 2% agarose gel, from representative cycles are shown below (Fig: III.4, 5, 6)

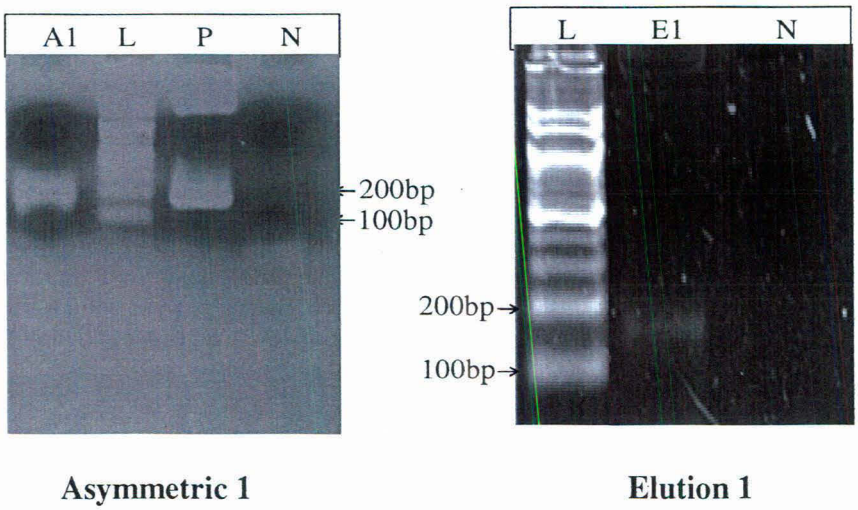


Fig: III.4. 1st cycle. *Asymmetric PCR and PCR from the elute of 1st cycle, A1 shows the asymmetric PCR product, E1 is the PCR product from elute, N is negative control and L is 100 bp ladder.*

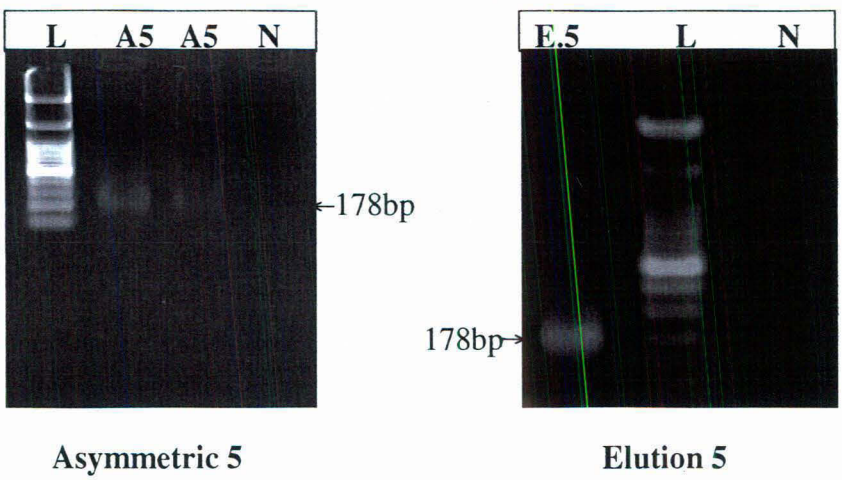
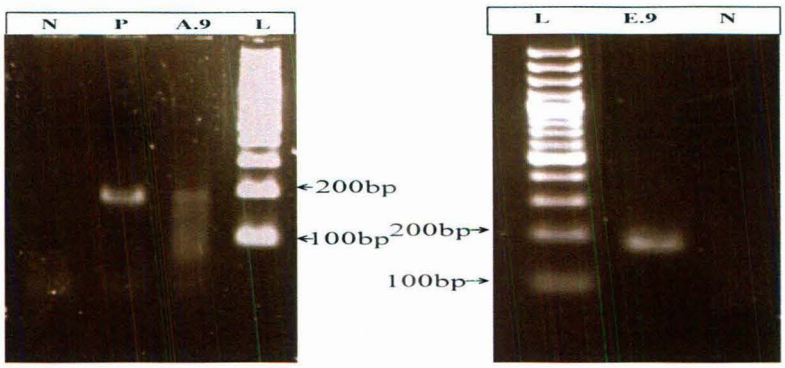


Fig: III.5. 5th cycle. *Asymmetric PCR and PCR from the elute of 5th cycle, A5 shows the asymmetric PCR product, E5 is the PCR product from elute, N and P are negative and positive control respectively and L is 100 bp ladder.*



Asymmetric 9 **Elution 9**

Fig: III.6. 9th cycle. *Asymmetric PCR and PCR from the elute of 9th cycle, A9 shows the asymmetric PCR product, E9 is the PCR product from elute, N and P are negative and positive control respectively and L is 100 bp ladder.*

III.3. Cloning

After selection, the aptamers were inserted in pGEMT-easy vector and cloned in DH5α cells. Cells carrying the vector with insert were identified by blue white screening. The positive colonies were inoculated in LB and plasmids isolated by miniprep. To confirm the size of the inserts the plasminds were digested by Not I and resolved on 2%agarose gel (Fig: III.7).

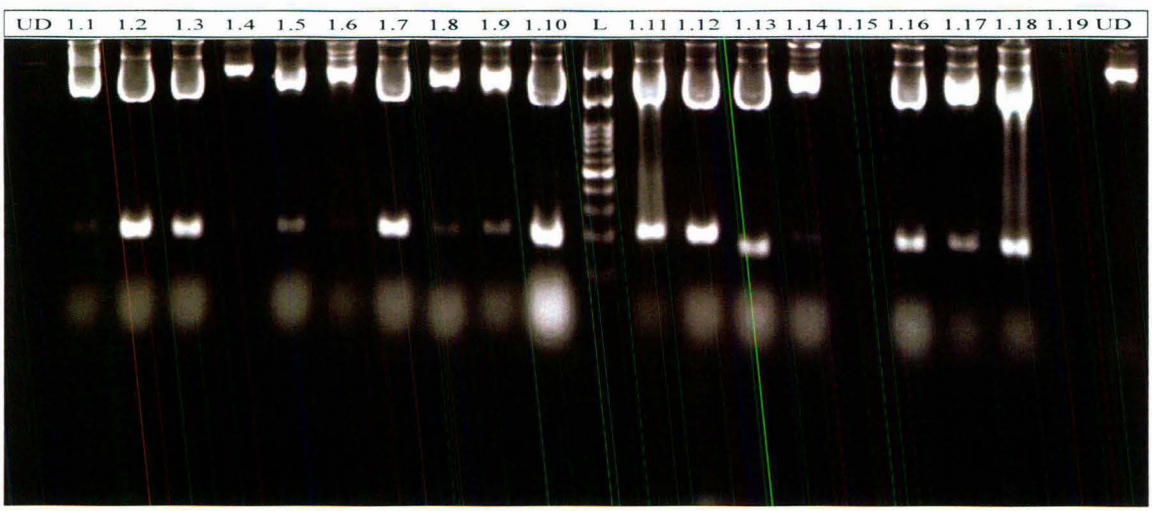


Fig: III.7. ***Not-I digested plasmids from first cycle,*** *resolved on 2% agarose gel. UD shows the undigested plasmids, L is 100 bp ladder. 1.1 to 1.19 shows the various clones from first cycle.*

III.4 Sequence analysis

III.4.1 Sequences of aptamer clones representing different cycles of SELEX

Five clones each from 1st, 3rd, 5th, 7th and 10th cycle were sequenced for phylogenetic and structural analysis. Sequences of clones from different cycles of SELEX are being presented below. The first number in the sequence name represents the SELEX cycle to which the sequence belongs and the second number represents the clone number. The red regions are the primer binding regions. The Blue regions are identical regions and the black regions are random regions.

Cycle-I

1.4
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGCGGAAAATTATTG
TGTTGGAACAACCGGGTAAAGTCCAGCTTGAGGATTGTGAATTCGGATCCAA
GCTTTACGTAGATCTACCACGGTAGCCCACGCATCCTAACCCTCGGCAGCCC
CCCCGGTACCCAGCTTTTGTT

1.14
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGGTGCTTTAAATG
TGTTATCCAGCTTGGTCACCATCCTCCCCTGAGGACTGAATTCGGATCCAAGC
TTGTCGCACAAAAGACGGACATACCTCGAGGACAGATATGCGTATGAACAGC
CCGGTACCCAGCTTTTGTT

1.15
TTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGCCAATGGTAG
GTGGGGTCGAATTCCTCGTCTGGGCATTTCTAACATGTTGAATTCGGATCCAA
GCTTCCAAAAGTGACACAGGTCCAACTTTATACAACAGAAGTACTACTCTTAA
CCCCGGTACCCAGCTTTTGTT

1.18
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTCTTCGCCTAGCA
GTGGAGGCGGGGTTACTTGTTCATGCTCAGCATATGTGAATTCGGATCCAAGC
TTTTGCTCACTGATGGGACCGCCGAATCGAAATAAAAATTATCGTCATCGGCC
CCGGTACCCAGCTTTTGTT

Cycle-III

3.1
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGAGGACACGAAAG
GGATGGGTCATGGATGTAACGTGGCGTACTAGTTAAGGGAATTCGGATCCAA
GCTTAACCGCCATCCGTTGTTTACAGTGAGATAAGAACGGCAAGTATTAGACC
TCCCGGTACCCAGCTTTTGTT

3.2
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGCCCAAAAGAGT
GGTCTCGGGTTTTATGTTAGTGTGGTGATCAGGTGTCTGAATTCGGACCAGTT
ATACAGTGGACGCGGCCAGCACGGAACACGGTCCACACACCGCACCCCCGGT
ACCCAGCTTTTGTT

3.3
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTTATGGAATGCTT
GAGATTGTGGCAGCATTTCCCCCTAAGTGTAGTGTTTGAATTCGGATCCAGCT
TTAAACCCGCGCAAATCGAAATTAGGCACTCCGTTAGTGGCGCGGACAAATC
CCGGTACCCAGCTTTTTTA

3.5
TAATACGACTCACTATAGGGCGAATTGGAGTCCACCGCGTTGAATGTTGTACA
GTCTGGTGGGGTAATCCCGGCCGTAATCAGCGGATAGAATTCGGATCCAAGCT
TTAGCTCCAATCTGACCAGGGGAAAGGGCCCTCCAAATAAAGCTTAGTGCCCC
CGGTACCCAGCTTTTGTT

3.7
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTCGAAGTTTTGA
GGTATCGTCTAAGTTTGTCTCAACGCCCTGTGTGTCTGAATTCGGATCCAAGCT
TACATGTACCAAATAGCCTTCTTACAGAGGTCTTCAACTCGCGATAGCCCGCC
CGGTACCCAGCTTTTGTT

Cycle-V

5.11
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTGGGTCGATAGGT
ATTTATCACTGCCACTAGTACGCAGAGTTTAAGACGTGAATTCGGATCCAAGC
TTTTCCCGCCGAATCTGTATCCAACTCAGAAGCCCCGCCCCGTAACCGGGCCC
CGGTACCCAGCTTTTGTT

5.12
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGAGTGATTCCGCAT
CTCCGTCATTTCCTCGTAAACGCTGCCTCTGCACAGTGAATTCGGATCCAAGC
TTTGGGGAGATACCCGGCAGACGTATCTCCTGCATTAATGCAGCCGATCGATC
CCGGTACCCAGCTTTTGTT

5.15
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTCTACAAACACA
AGGCGTCATTGGGAGTCAGCAATTACGACGGCGCAATTGAATTCGGATCCAA
GCTTCCCTCGTAGACCAAACACATGATCAAATATTAATCGAAAGTAAACATAG
ACCCGGTACCCAGCTTTTGTT

5.16
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTGTCCGCGGGCGC
ATACCTAGAGGTGGGTCGATATAGTGGATGATGACAAGAATTCGGATCAAGC
TTAGCTGTGGAAGTAGACGTGCAAAGCTCCCCAAACTACTTGTGAGCCGGATC
CCGGTACCCAGCTTTTGTT

5.17
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTCGGCTTCCAGGA
GTTTTTGCTGTAACTTTGACTTGGAGTCATCCACTTGGAATTCGGATCCAAGCT
TTCTTGACGCGAGGGGTTAACCCCAGTAAACTAATCGGTGCTATCAACAACCC
CGGTACCCAGCTTTTGTT

Cycle-VII

7.10
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTCTCTTTATACTC
ACGATCACCAAGGATTGTTCACCACGGTTGAGATGTGAATTCGGATCCAAGCT
TTTCTGAGTCGATATGCTTAAAGCACTCTGGCCGTCACTTCTGACTTAGGACCC
GGTACCCAGCTTTTGTT

7.11
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGGTGCTTTAAATG
TGTTATCCAGCTTGGTCACCATCCTCCCCTGAGGACTGAATTCGGATCCAAGC
TTGTCGCACAAAAGACGGACATACCTCGAGGACAGATATGCGTATGAACAGC
CCGGTACCCAGCTTTTGTT

7.13
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTTATGGAATGCTT
GAGATTGTGGCAGCATTTCCCCCTAAGTGTAGTGTTTGAATTCGGATCCAGCT
TTAAACCCGCGCAAATCGAAATTAGGCACTCCGTTAGTGGCGCGGACAAATC
CCGGTACCCAGCTTTTGTT

7.15
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTCATCCTGCATG
GTCGTTGTGTCCTCTTCCTCTAACAGACTCGACCGTAGAATTCGGATCCAAGC
TTACTCCCACCTGCCCCGGGACTCGGAAGCCCACTCATCCCCTACGCGATGCC
CCGGTACCCAGCTTTTGTT

7.16
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTCAGTCGCACGT
CTTGCTCACGTTGGTTGATCATTAACGTAGTGCCAGCGAATTCGGATCCAAGC
TTGGAACAGCTAAAGACCCTACACAGCTGCATTAGTAAGATGTCACACGGAC
CCGGTACCCAGCTTTTGTT

Cycle-X

10.1
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGGTGCTTTAAATG
TGTTATCCAGCTTGGTCACCATCCTCCCCTGAGGACTGAATTCGGATCCAAGC
TTGTCGCACAAAAGACGGACATACCTCGAGGACAGATATGCGTATGAACAGC
CCGGTACCCAGCTTTTGTT

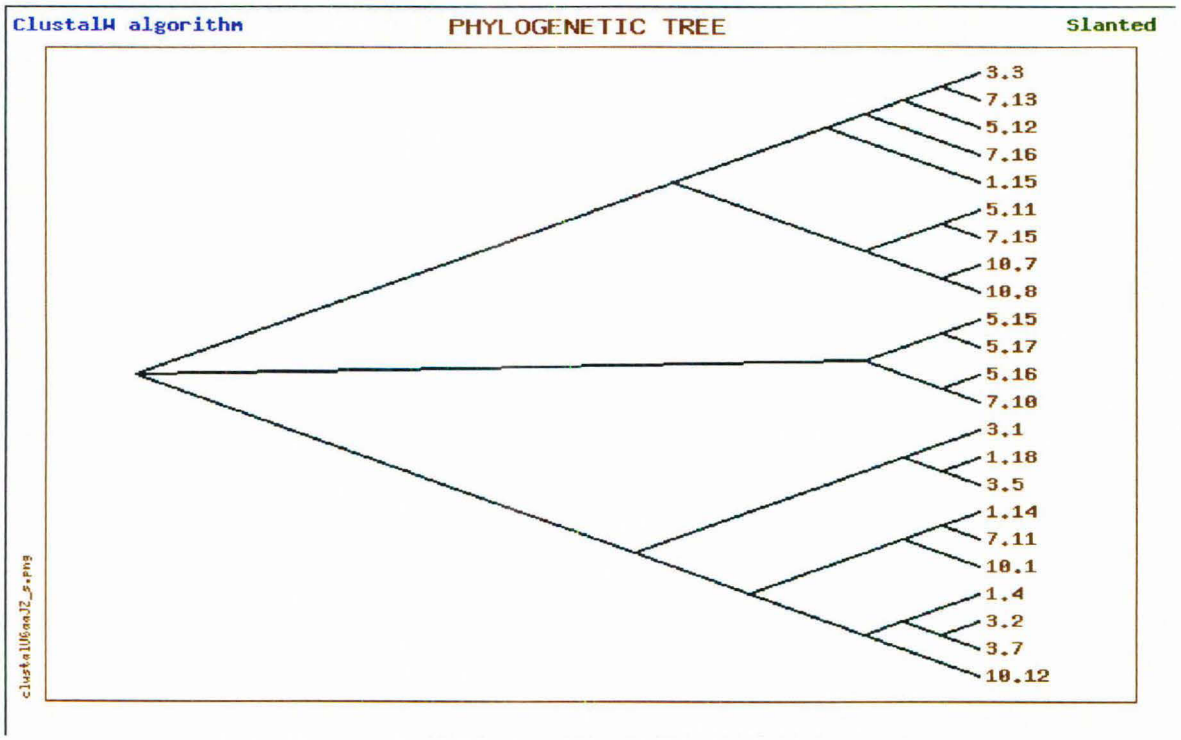
10.7
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGTTACCTTGGTCTG
GATGTGCTAGACATTATCGGAGACAGAGGCTAAGCCCGAATTCGGATCCAAG
CTTTATGATCACTGAAATGTATCGGGAGCTACAGTGTAATGAACATCCCCATA
CCCGGTACCCAGCTTTTGTT

10.8
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGATGTGTGAAAAC
GCGGATATGTTCTCAGCCGTGCACAGGCTAACCCGACTGAATTCGGATCCAAG
CTTCGCTCCCTTAATTGTCATAAAACAATCGCCACCGTAACCCGAAACGTGCA
CCCGGTACCCAGCTTTTGTT

10.12
TAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGAGACGGTAAAT
TAGTTTTGGTGTGCTGCCACGCGACATGTACTAGTAGTGAATTCGGATCCAAG
CTTCAGAAACGACAAGCGAGATCTCAGACATTGATCCGAGCAGCCATTTCAC
ACCCGGTACCCAGCTTTTGTT

III.4.2 Phylogenetic prediction of different classes of glutathione binding DNA aptamers

Phylogenic analysis of the aptamers sequences by CLUSTAL-W algorithm divided them into six major classes (Fig. III.8).



3.3, 7.13, 5.12, 7.16, 1.15	Class-I	
5.11, 7.15, 10.7, 10.8	Class-II	
5.15, 5.17, 5.16, 7.16	Class-III	
3.1, 1.18, 3.5	Class-IV	
1.14, 7.11, 10.1	Class-V	
1.4, 3.2, 3.7, 10.12	Class-VI	

Fig: III.8 ***Phylogenetic classification of glutathione binding DNA aptamers.*** *The phylogenetic tree divided the aptamers into six different classes based on sequence similarity. The different classes were represented as class I-IV from top to bottom. Shown below is the list of aptamers in each class.*

The program divided the sequences into three major groups. The first group was subdivided into two subgroups namely Class-I and Class-II.

The second group had only three sequences with more homology among them hence categorized under one class namely Class-III. The third group was subdivided into three groups namely Class-IV, Class-V and Class-VI.

III.4.3 Multiple Sequence alignment of glutathione binding DNA aptamers

The six class of aptamers emerged from phylogenetic studies were analyzed for Conserved regions by multiple alignment using the web server Multalin.

Class-I

In class-I, there was a 20 mer conserved stretch (TTGTGTCAGCATTTACGCCG) in the first random region. While there were many short conserved stretches in the second random region. (Fig III.9).The 20 mer conserved stretch is part of the conserved structural domain in the secondary structure (Fig III.15 Box A).

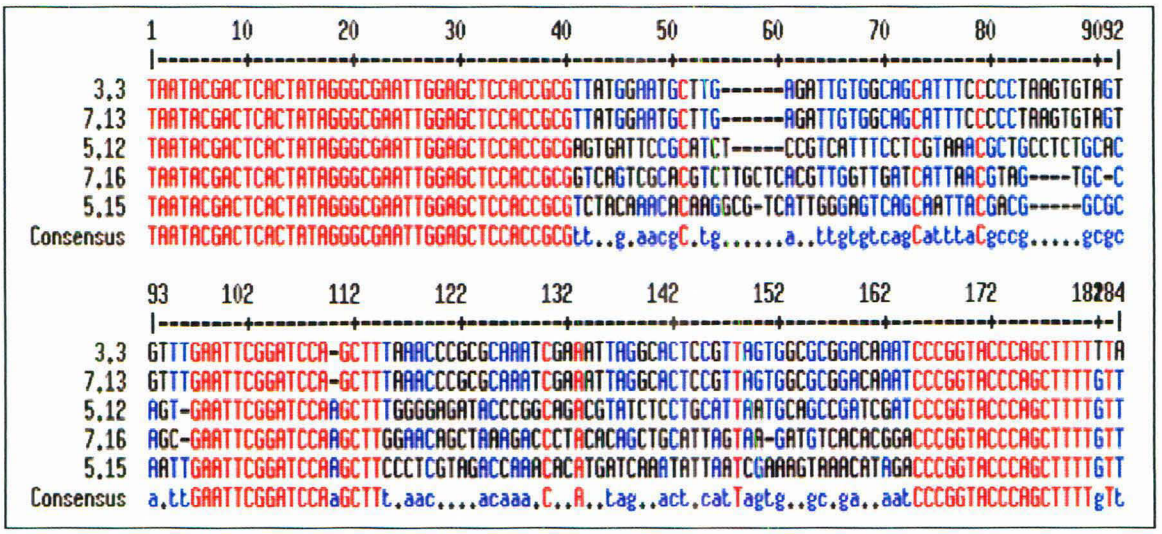


Fig:III.9 ***Multiple sequence alignment of class-I glutathione binding DNA aptamers.*** *Red, blue and black residues represent identical, conserved and non conserved residues respectively. The 20 mer conserved stretch is located between 63 and 83 bp.*

Class-II

In class-II there was a 19 mer conserved stretch (CTACTCGAACAGAGTCTAA) in the first random region and an 11 mer conserved stretch (CAGCGTAACCC) in the second random region (Fig III.10). The 11 mer conserved stretch is part of the conserved structural domain in the secondary structure (Fig: III.16 Box B).

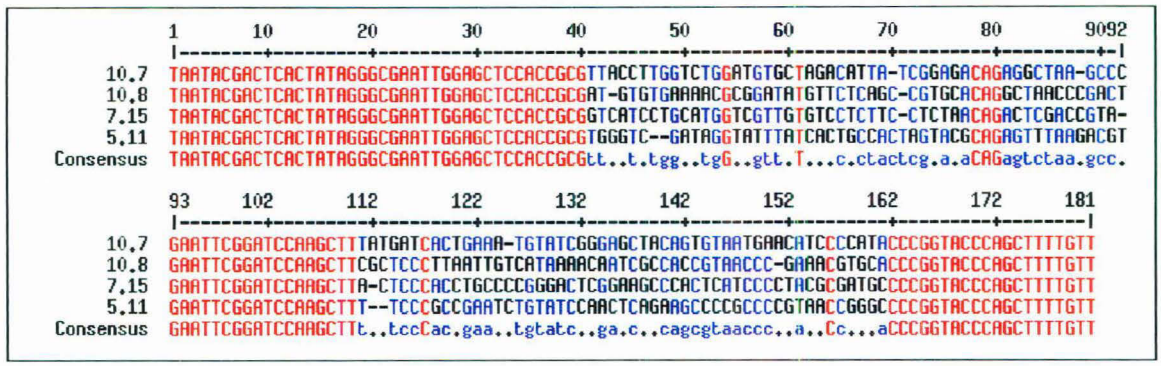


Fig:III.10 ***Multiple sequence alignment of class-II glutathione binding DNA aptamers.*** *Red, blue and black residues represent identical, conserved and non conserved residues respectively. The 19 mer conserved stretch is located between 67 and 87 bp and the 11mer conserved stretch is located between 140 and 150 bp.*

Class-III

In class-III there were many short conserved stretches in the first random region and a 19 mer conserved stretch (CTGAGGCGATAGGCTTACA) and 14 mer conserved stretch (AATCACTTCTGECC) in the second random region (Fig III.11). But both the conserved stretches are not part of the conserved structural domain in the secondary structure (Fig: III.17).

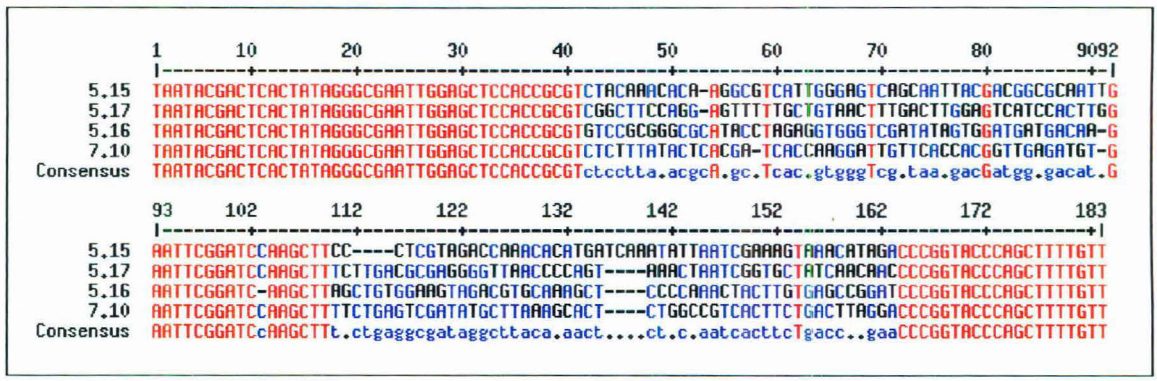


Fig:III.11 ***Multiple sequence alignment of class-III glutathione binding DNA aptamers.*** *Red, blue and black residues represent identical, conserved and non conserved residues respectively. The 19 mer conserved stretch is located between 112 and 131 bp and the 14 mer conserved stretch is located between 144 and 158 bp.*

Class-IV

In class-iv there was a 11 mer conserved stretch (GGGTCGGGGAT) in the first random region besides many short conserved stretches and a 10 mer conserved stretch (AATAAAATAA) in the second random region (Fig III.12). The 10 mer conserved stretch is part of the conserved structural domain in the secondary structure (Fig: III.18).

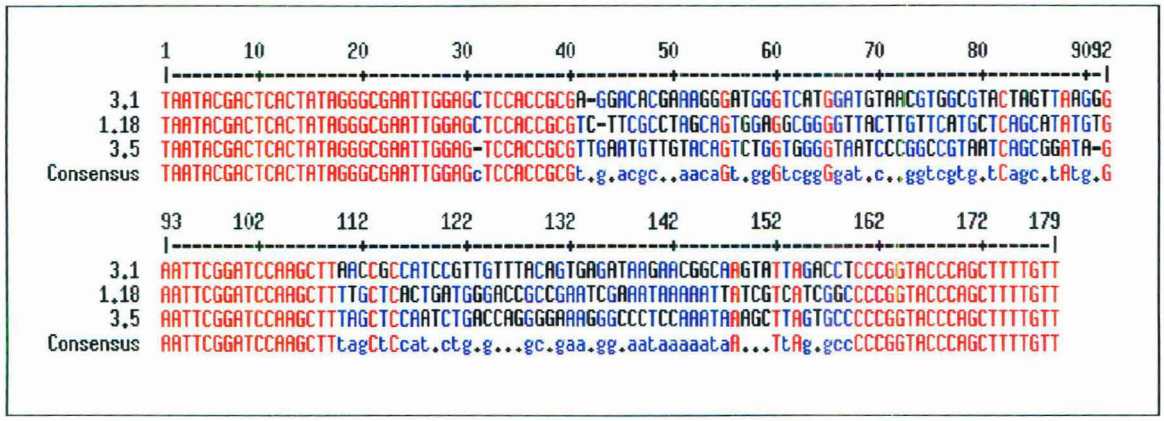


Fig:III.12 ***Multiple sequence alignment of class-IV glutathione binding DNA aptamers.*** *Red, blue and black residues represent identical, conserved and non conserved residues respectively. The 11 mer conserved stretch is located between 58 and 68 bp and the 10 mer conserved stretch is located between 138 and 148 bp.*

Class-V

All the sequences in class-V were similar, even though they were picked from different SELEX cycles (1, 7 and 10) (Fig III.13).

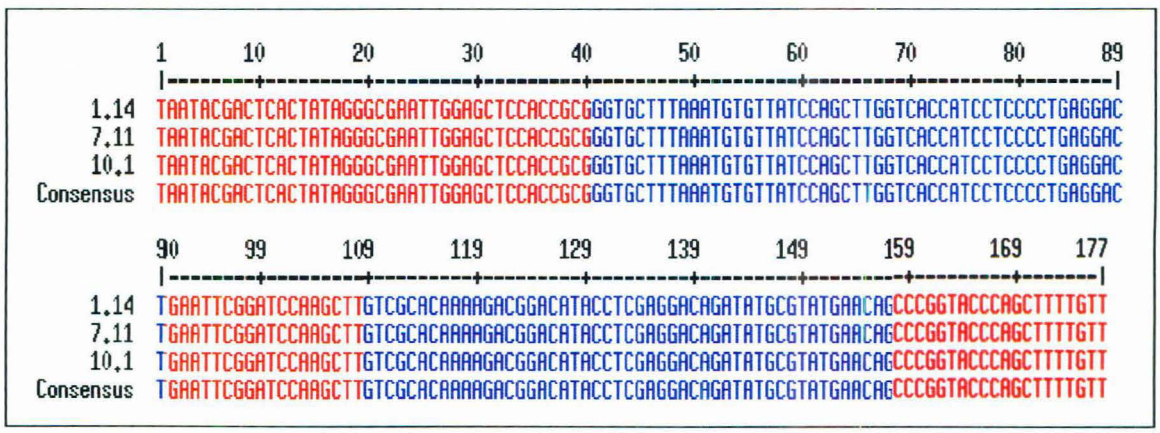


Fig:III.13 ***Multiple sequence alignment of class-V glutathione binding DNA aptamers.*** *The all red and blue residues show the identical and conserved regions respectively. The all blue random regions signify that all are conserved.*

Class-VI

In class-VI there were small conserved stretches in both the random region (Fig III.14).

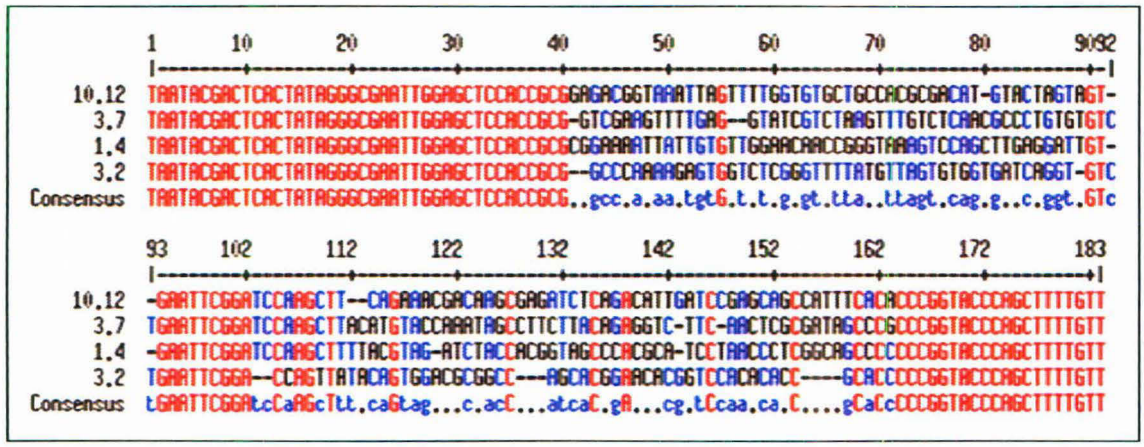


Fig:III.14 ***Multiple sequence alignment of class-VI glutathione binding DNA aptamers.*** *Red, blue and black residues represent identical, conserved and non conserved residues respectively.*

Among all the classes the conserved stretch in the first random region was between nucleotide 60 and 80. In the second random region the conserved stretches were between nucleotide, 102 to125 and 130 to 150. These conserved regions may be having a role in structural stabilization of the aptamers and binding with glutathione. The fifth cycle has all the three member sequences identical this shows the selective advantage to this sequence. The sixth class had no long conserved stretch. Three of the four sequences in this class belong to the first and third cycle. But one sequence (10.12) is from the tenth cycle and that must have some affinity to glutathione to perpetuate through so many rounds of selection. Besides we have sequenced a limited no of clones. Hence the sequence 10.12 might be belonging to another class of aptamers not well represented among the sequences.

III.4.4 Secondary structure prediction of glutathione binding DNA aptamers

The web server MFOLD was used for the prediction of secondary structures of various classes of aptamer. MFOLd predicts secondary structures of nucleic acids based on the Zucker's algorithm of energy minimization.

Class-I

Analysis of the structures showed some shared structural features among the different clones belonging to this class. A three loop three stem structure around 50 bp (Fig III.15 box A) and a two loop – two stem structure around 140 bp (Fig III.15 box B) were the most prominent shared structural domains.

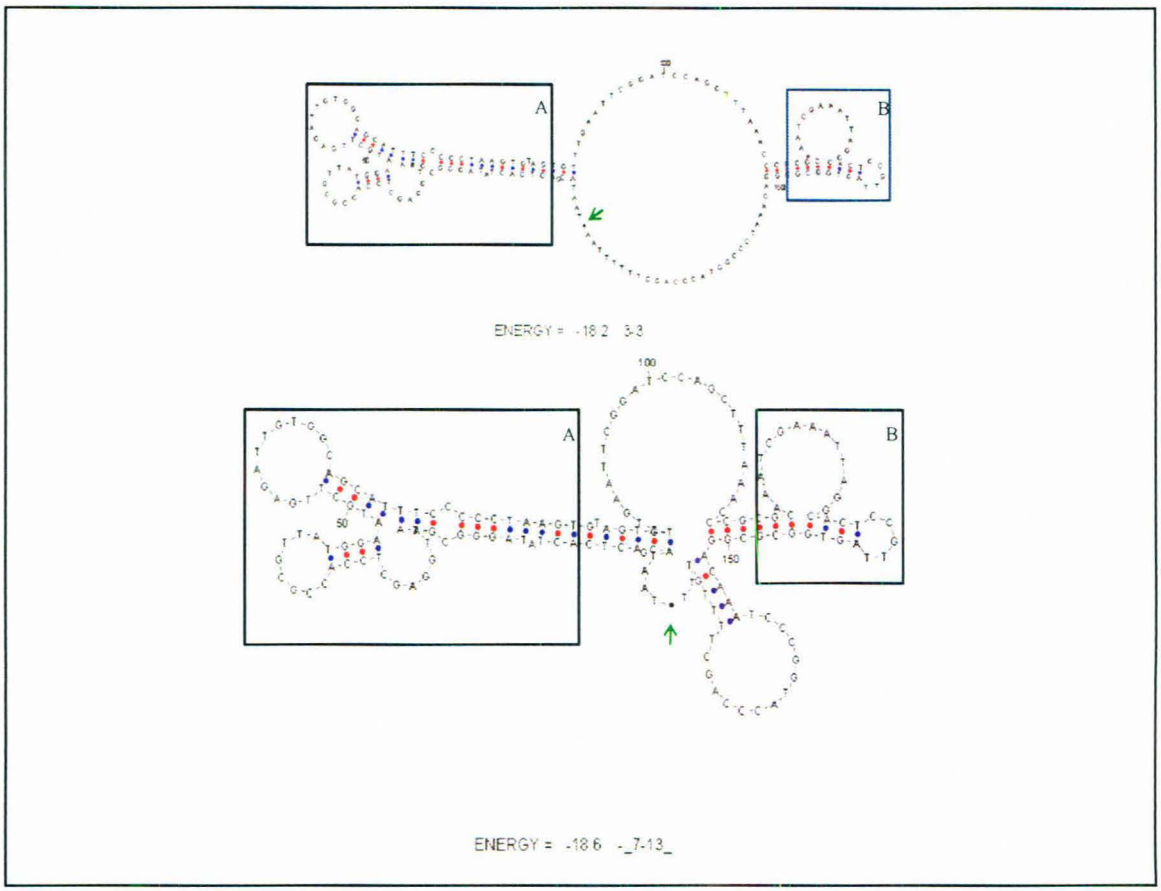


Fig: III.15 ***Representatives secondary structures of class-I.*** *Default parameters given by the program were used. Box A shows the conserved domain around 50 bp and Box B shows the conserved domain around 140 bp. The green arrow shows the two ends. Minimum energies calculated in Kcal/mole.*

Class-II

Representative structures of aptamers belonging to this class are shown below. Structures of this class of aptamers showed not identical but similar shared structural features. A four loop-four stem structure (Fig III.16 box A) and a three loop – three stem structure around 100 bp (Fig III.16 box B) were the most prominent shared structural domains. The four loop-four stem structure (Fig: III.16 Box A) is constituted by different regions of the oligos in different clones and the exact structure varies among different clones but the overall structure is almost similar.

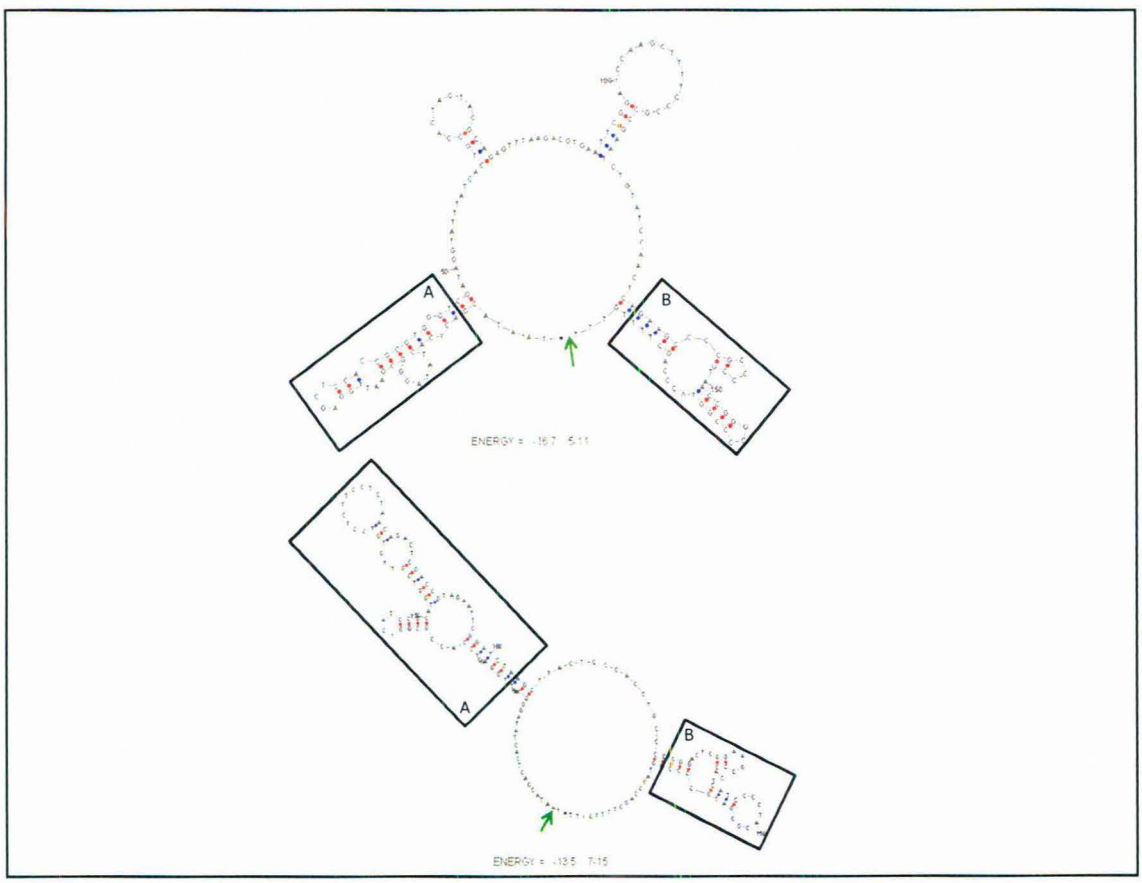


Fig: III.16 ***Representative secondary structures from class-II.*** *Default parameters given by the program were used. . Box A shows the four loop four stem conserved domain and Box B shows the three loop three stem conserved domain around 140 bp. The green arrow shows the two ends. Minimum energies calculated in Kcal/mole.*

Class-III

Aptamers of this class although seem to have similar structure and the overall geometry of them resembles with each other but the exact structures of the domains varies among different clones. A resembling four loop four stem structure (Fig III.17 Box A) in the 50 to 100 bp region, although not identical is shared by the members of this class. Other structural similarities are there but they are not so distinctive (Fig III.17).

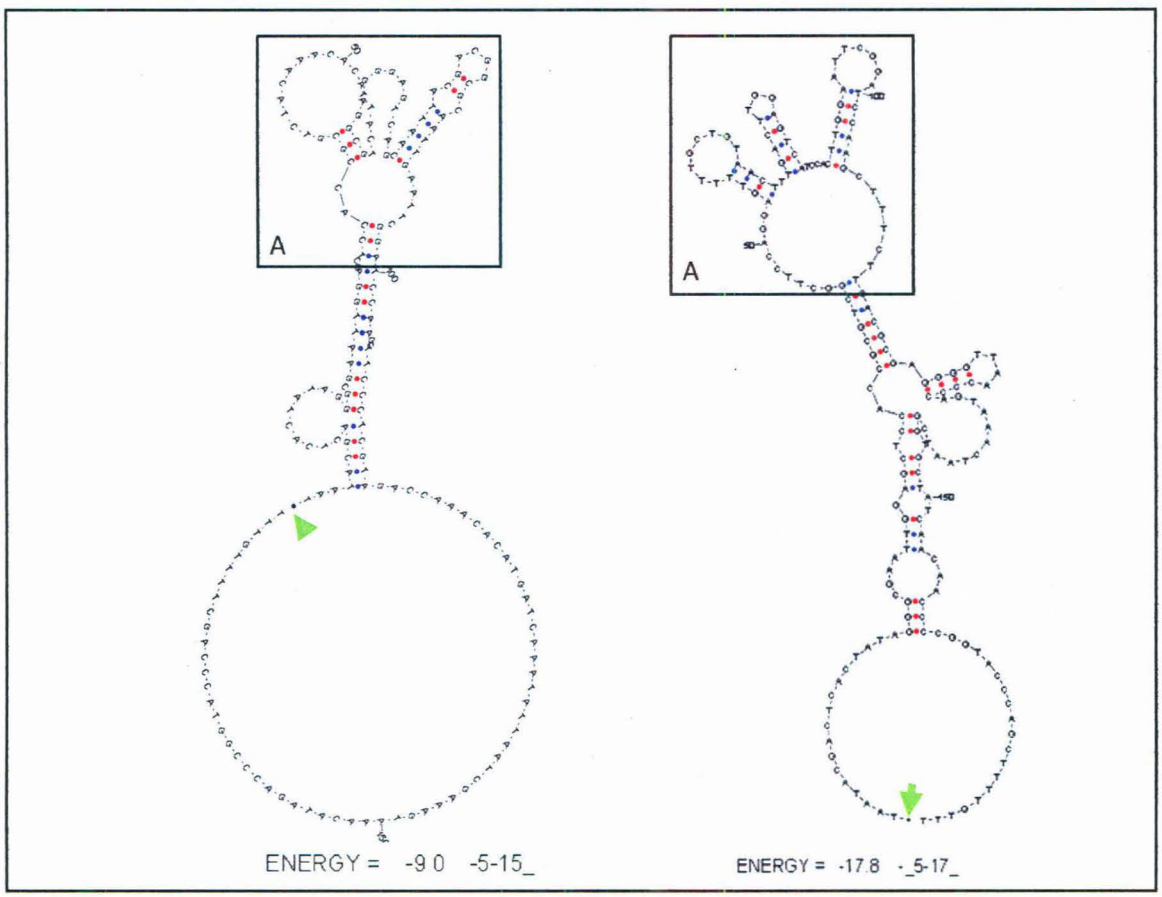


Fig: III.17 ***Representative secondary structures from class-III.*** *Default parameters given by the program were used. . Box A shows the four loop four stem conserved. The green arrow shows the two ends. Minimum energies calculated in Kcal/mole.*

Class-IV

Representative structures of aptamers belonging to this class are shown below. Structures of this class of aptamers showed some similar shared structural features. A two loop-two stem structure with a small purine rich bulge (Fig III.18 box A) and a two loop – two stem structure (Fig III.18 box B) were the most prominent shared structural domains. Both the structures are constituted by different regions in different clones and the exact structure varies among different clones but the overall structures of these domains are almost similar.

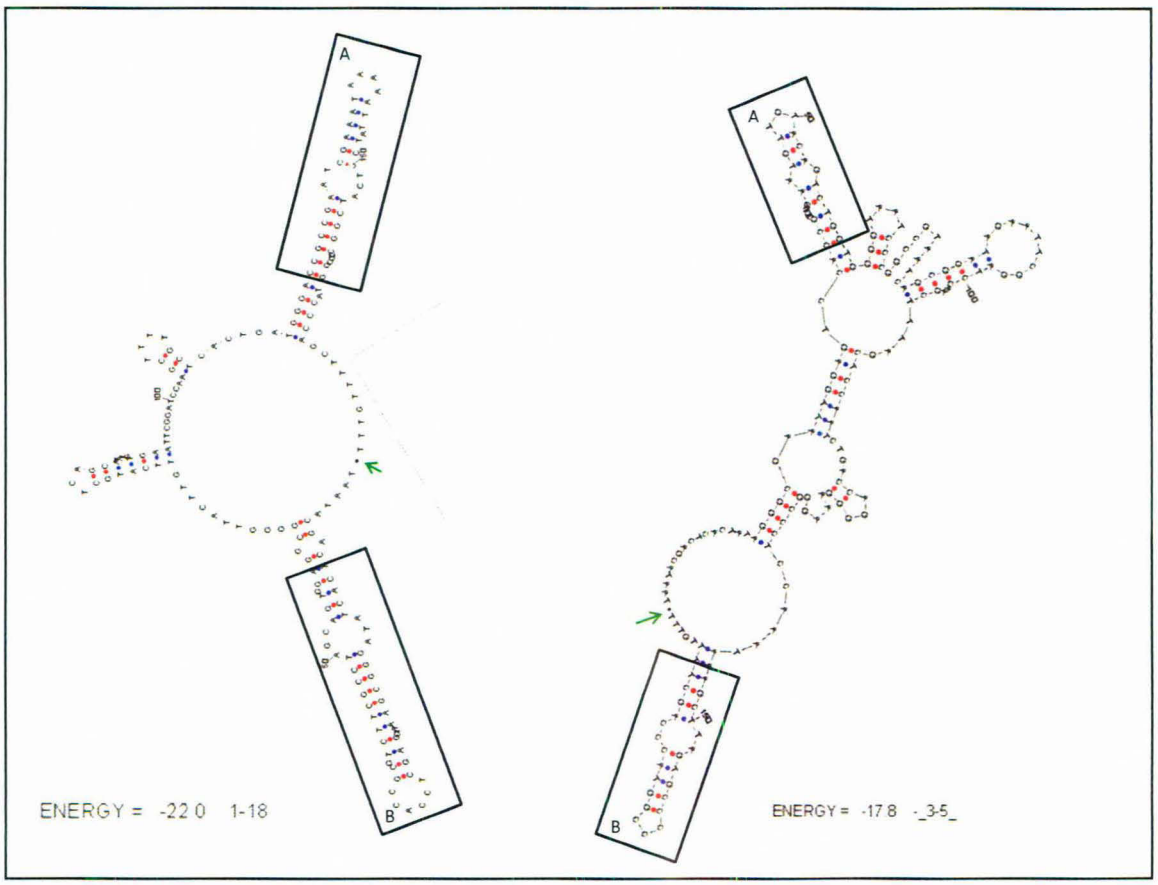


Fig: III.18 ***Representative secondary structures from class-IV.*** *Default parameters given by the program were used. Box A shows the upper two loop two stem conserved domain and Box B shows the lower two loops two stem conserved domain. The green arrow shows the two ends. Minimum energies calculated in Kcal/mole.*

Class-V

Clones belonging to this phylogenitic class of glutathione binding DNA aptamers had identical sequences and hence had identical structures (Fig III.19).

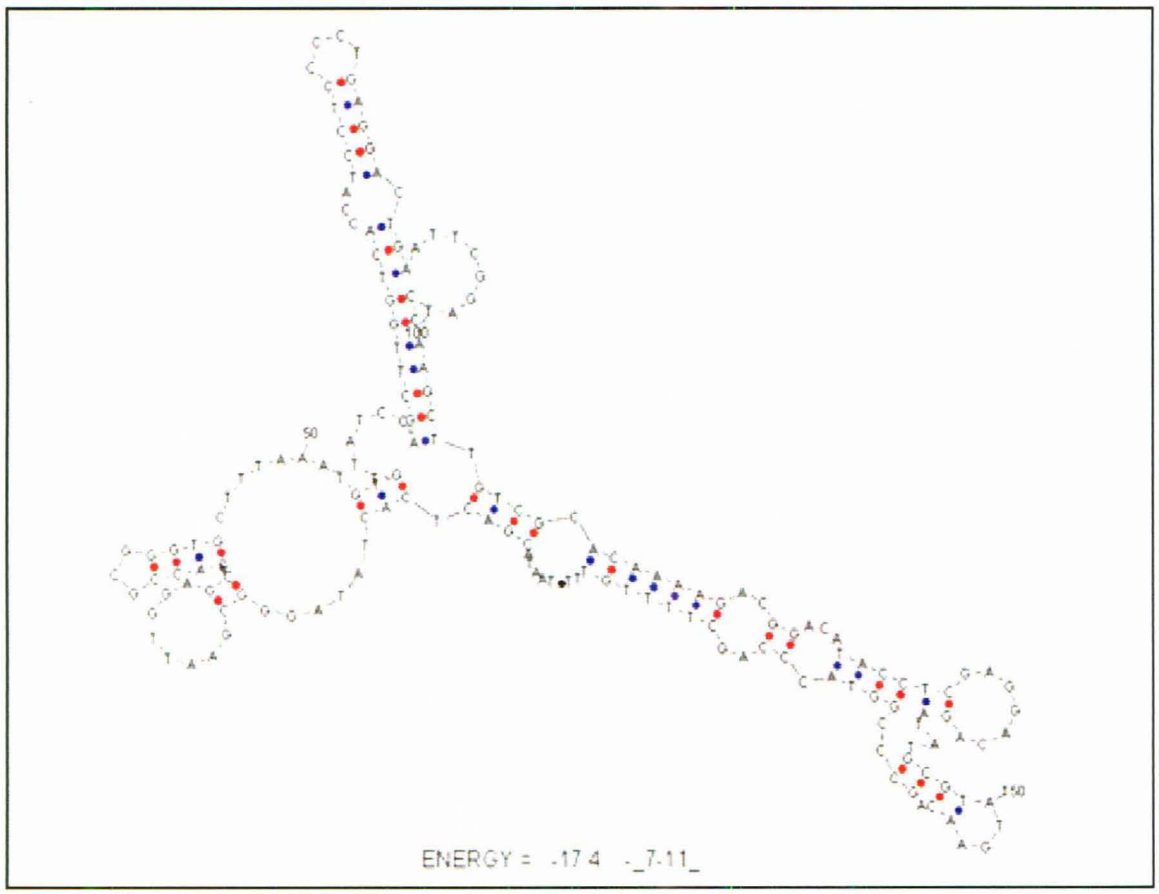


Fig: III.19 ***Representative Secondary structure from class-V.*** *Default parameters given by the program were used. Minimum energy calculated in Kcal/mole.*

Class-VI

Representative structures of aptamers belonging to this class are shown below. Structures of this class of aptamers showed not identical but similar shared structural features. A three loop-three stem structure around 50 bp (Fig III.20 box A) and a three loop – three stem structures around 140 bp (Fig III.20 box B) were the most prominent shared structural domains. Both the structures are constituted by different regions in different clones and the exact structure varies among different clones but the overall structures of these domains are almost similar.

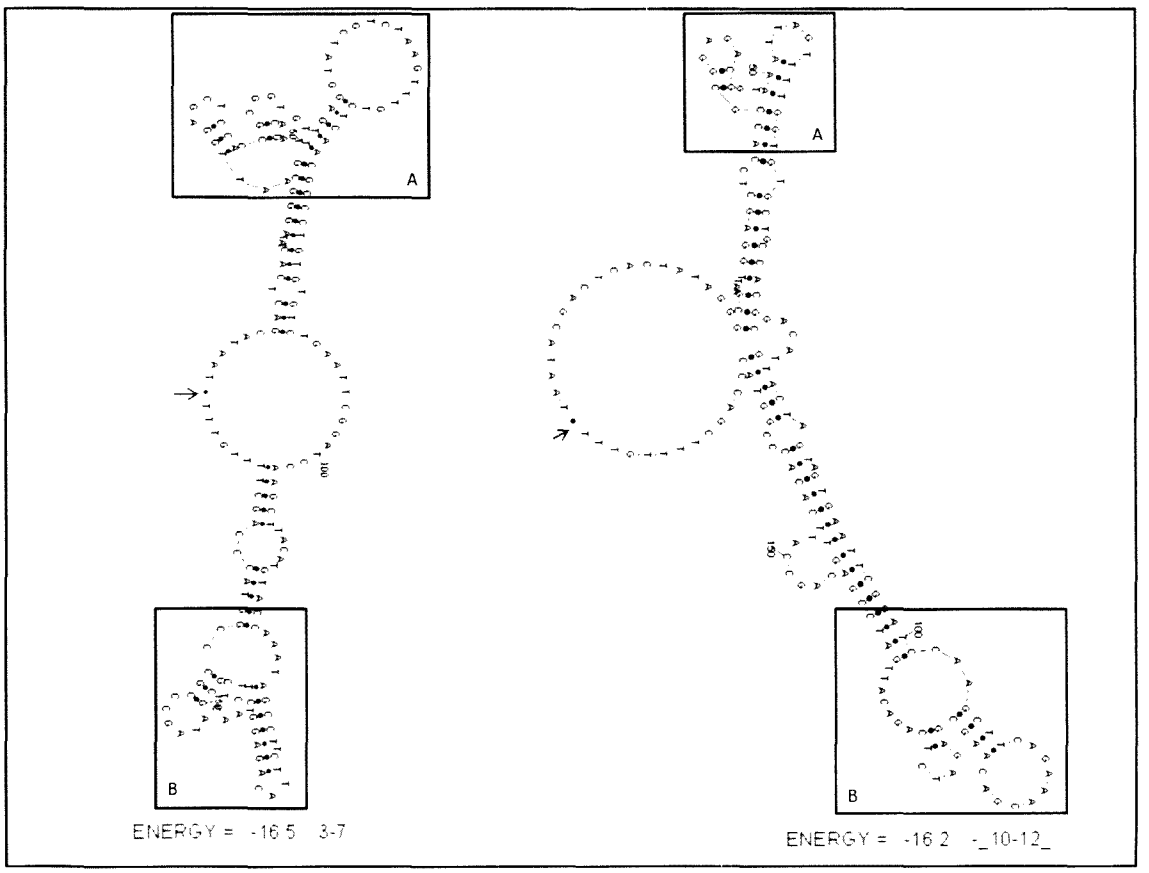


Fig: III.20 ***Representative secondary structures from class-VI.*** *Default parameters given by the program were used. Box A shows the three loop three stem conserved domain around 50 bp and Box B shows the three loop three stem conserved domain around 140 bp. The green arrow shows the two ends. Minimum energies calculated in Kcal/mole.*

III.5 Kinetics

Kinetic studies of three clones (7.10, 10.8 and 10.12) were performing to determine the binding affinity of these selected aptamers to glutathione. α ^{32}P-CTP was used to label the aptamers. The dependence of elution on competitor concentration suggests that the aptamers bind to glutathione. The dissociation constants were calculated using the formula mentioned in material and methods.

Clone 7.10

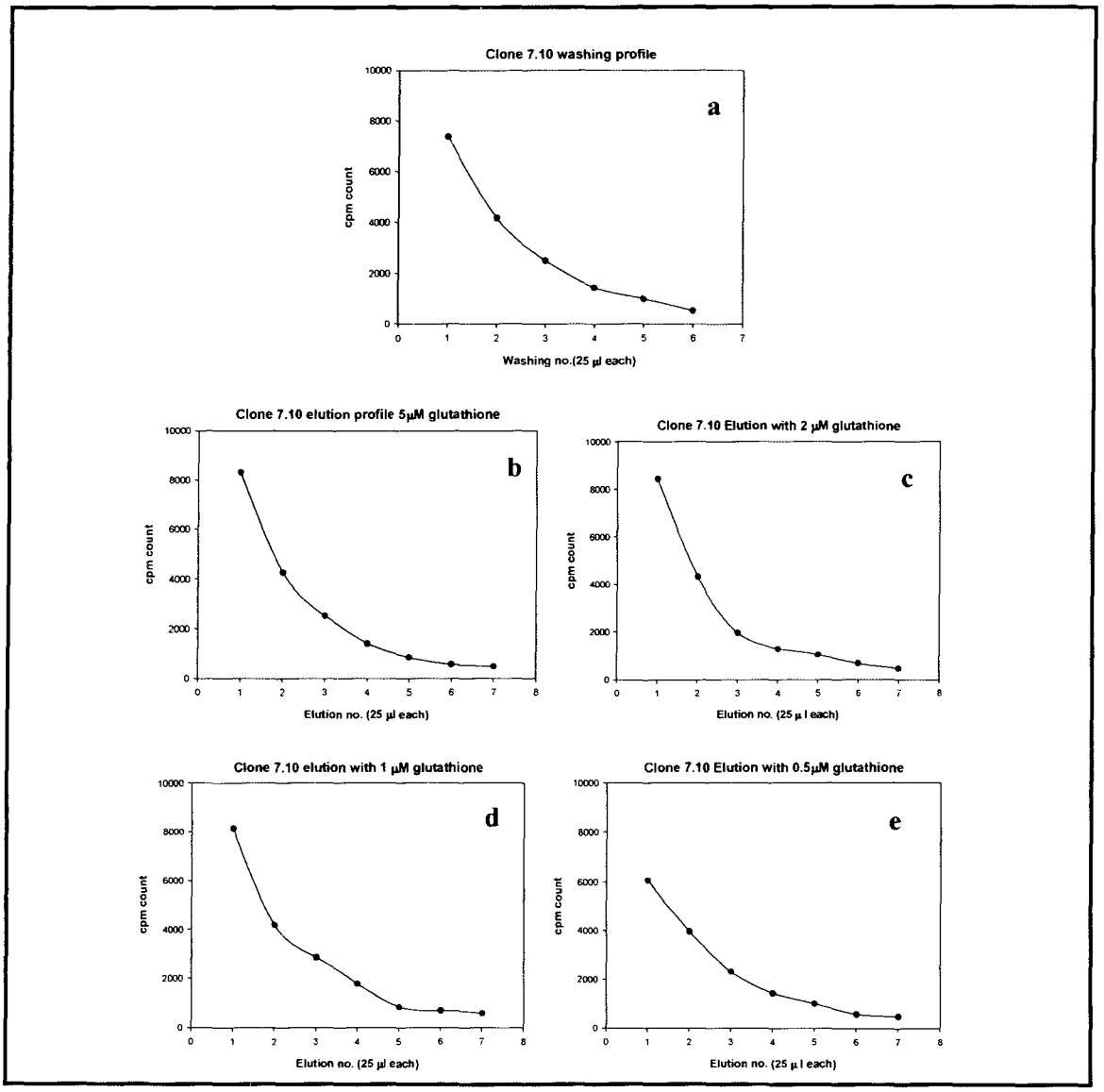


Fig: III.21 ***Clone 7.10. Elution of DNA aptamers clone (7.10)****with 5µM (b), 2µM (c), 1µM (d) and 0.5µM (e), glutathione. The non specifically adhering aptamers were removed by washing (a) before assaying release with varied concentration of competitors (b-e). The Kd for the clone (average of the nearest three) was calculated to be equal to 15µM.*

Clone 10.8

Aptamers were incubated with glutathione beads, washed with six bed volume of washing buffer and finally eluted with elution buffer having different concentration of glutathione which resulted in decreased quantity of bond aptamer as a function of glutathione concentration and time (Fig III.22).

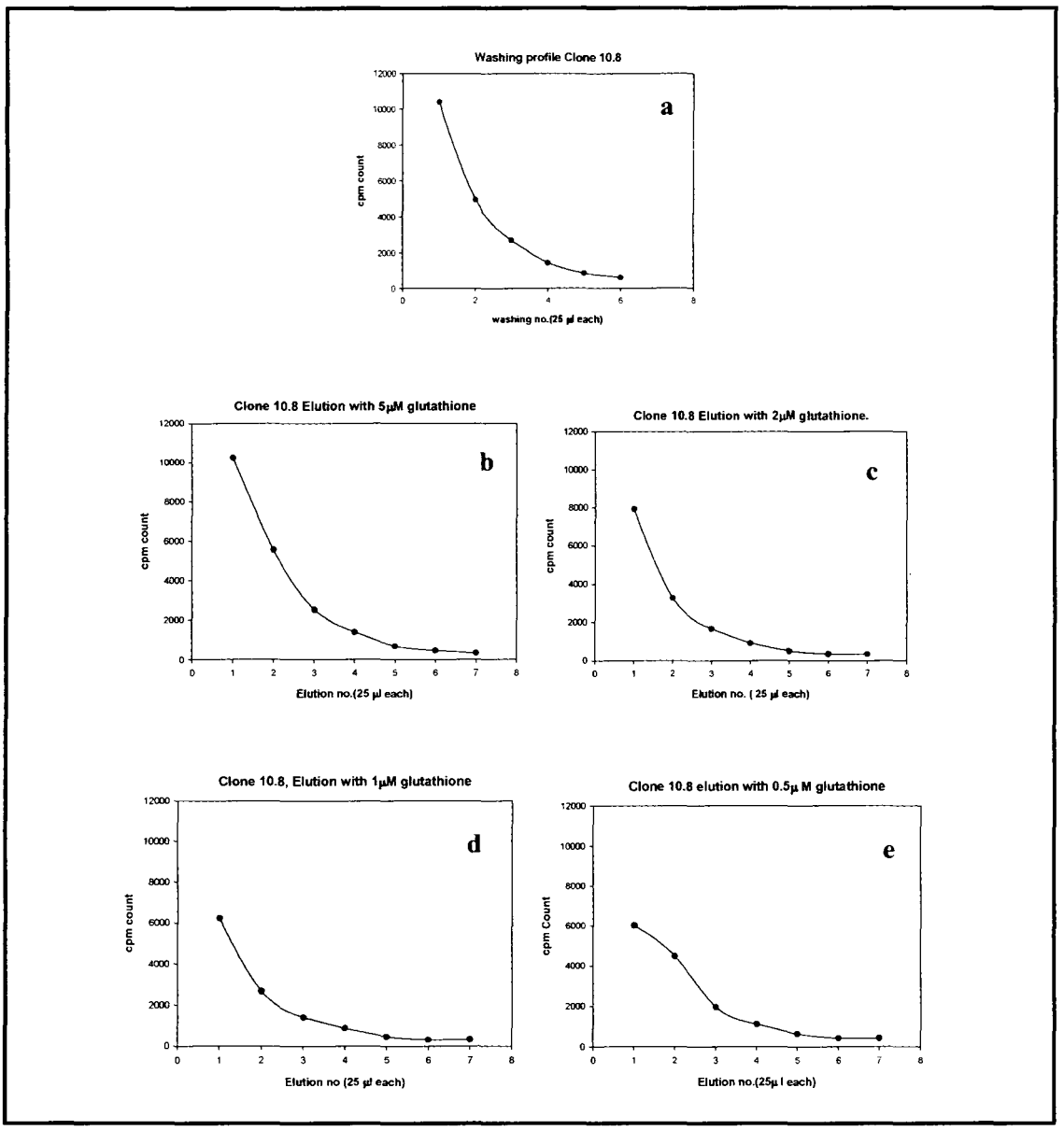


Fig:III.22 ***Clone 10.8 Elution of DNA aptamers clone (10.8)****with 5µM (b), 2µM (c), 1µM (d) and 0.5µM (e), glutathione. The non specifically adhering aptamers were removed by washing (a) before assaying release with varied concentration of competitors (b-e). The Kd for the clone (average of the nearest three) was calculated to be equal to 26µM.*

Clone 10.12

Aptamers were incubated with glutathione beads, washed with six bed volume of washing buffer and finally eluted with elution buffer having different concentration of glutathione which resulted in decreased quantity of bond aptamer as a function of glutathione concentration and time (Fig: III.23).

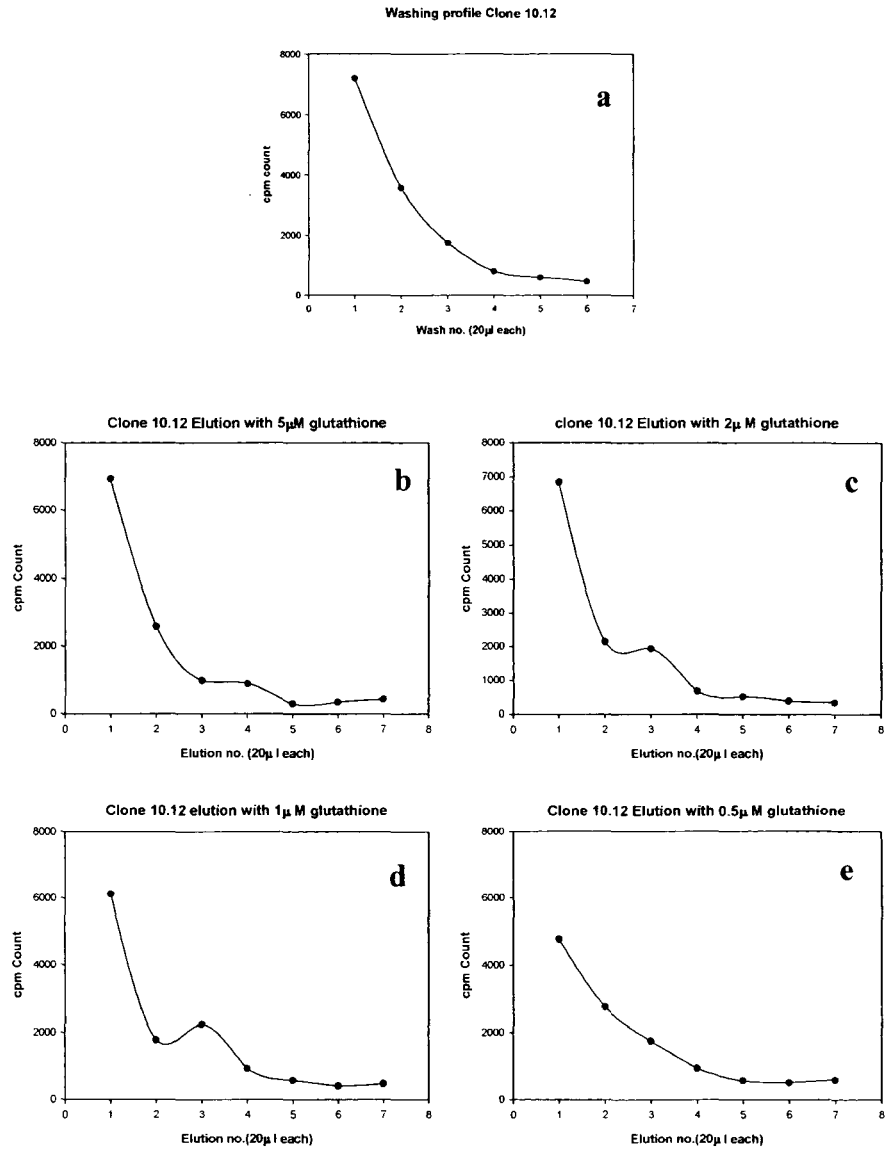


Fig: III.23 ***Clone 10.12. Elution of DNA aptamers clone (10.12)****with 5µM (b), 2µM (c), 1µM (d) and 0.5µM (e), glutathione. The non specifically adhering aptamers were removed by washing (a) before assaying release with varied concentration of competitors (b-e). The Kd for the clone (average of the nearest three) was calculated to be equal to 15µM.*

Discussions

IV. Discussion

Glutathione is a tripeptide thiol made up of the amino acids gamma-glutamic acid, cysteine, and glycine and is also known as gamma-glutamylcysteinylglycine or GSH. This tripeptide is the most abundant intracellular low molecular weight thiol in cells (.5-8 mM) (Kunert et al, 1993), prevalent from prokaryotes to eukaryotes. Glutathione is both a reductant and a neutrophile and hence can react with oxidising species. This property makes glutathione one of the most important water soluble antioxidants. This property is conferred on it by the thiol group and through this thiol group, it participates in redox reactions i.e. disulphide formation and thioesether and thiolester formation. Hence glutathione is suitably the best cellular thiol "redox buffer" to maintain cellular thiol/disulfide redox potential.

The redox state of the cell has an important role in the induction of apoptosis (Green et al, 1998; Marchetti et al, 1997; Coffey et al, 2000; Armstrong et al, 2001). Many agents known to induce apoptosis either induce ROS formation or depletion of antioxidants (Buttke et al, 1994). Investigations have suggested that the latter is most effective apoptosis inducers (Hug et al, 1994).

There are various mechanisms in the cell for the efflux of glutathione conjugates. The formation and efflux of glutathione conjugates of anti-cancer drugs pertain to drug resistance of tumor cells (Hayes 1999).

Many of the presently available anti cancer therapies are limited in their application because of their low efficiency and the most efficient drug is still to come. On another front researchers are engaged in designing strategies to increase the susceptibility of tumor cells to apoptosis which can increase the efficiency of a variety of cancer treatment.

Glutathione sequestration can thus be a good stratagem for increasing the susceptibility of cancer cells to apoptosis as well as to decrease the resistance of the tumor to anti-cancer drugs.

Buthionine Sulphoximine (BSO) is a known inhibitor of gamma-glutamylcysteine synthetase and treatment of cancer cells with BSO has shown to induce apoptosis, commitment to cell cycle arrest, release of cytochrome C and high production of ROS

(Armstrong et al, 2002). This study has also indicated that the efficient scavenging of glutathione can be a good tool in cancer therapy.

Nucleic acid aptamers are one of the most versatile tools of modern research. Aptamers although composed of only four different bases have exceptional ability to assume a vast array of secondary and tertiary structures with different shape. This versatility in structure and the ability of nucleic acids to form many different non covalent bonds enable aptamers to bind to a target with great specificity and affinity. These highly specific aptamers can be selected by the SELEX process. By now aptamers against a vast array of molecules have been selected and many have proven to be very good antagonistic and blocking tool.

From the above research background here we undertook the task of selection of glutathione binding DNA aptamers. They can be good sequestering agents of cellular glutathione and hence an efficient tool for increasing the susceptibility of cancer cells to various anticancer drugs and to decrease the efficiency of cancer cells to become resistant to various drugs.

IV.1. In Vitro Selection of Glutathione Binding DNA Aptamers

Aptamer selection starts with a random nucleotide pool. Most of the aptamer selection pools are designed as a completely random region (mostly 30-60 nucleotides long) flanked by two primer binding consensus regions. The merit of using short nucleotide pools for the selection of aptamers is, they can be aligned easily and the structural and functional motifs can be identified easily. But while selecting aptamers against target not known to bind nucleic acids, pools with longer random nucleotide are preferred since the larger oligonucleotides have greater structural and conformational flexibility besides increasing the number of variants and hence increase the chance of selection of high affinity aptamers. Nevertheless the increase in number of variants with size of oligo is not endless and the ultimate complexity of the population is limited by DNA synthesis chemistry to a total of 10^{13}-10^{16} different sequences. The number of variants is also limited by mass of oligonucleotides that can be handled through the selection. But large oligos withhold the merit of having greater structural versatility which increases the chance of selection of high affinity aptamers.

We started with two oligonucleotides named KpnI and SacI. Each contains from 5' to 3' a primer binding region, a 50 nucleotide random region and a mutually complimentary 18 mer region. The two oligonucleotides were annealed based on complementarity over the defined 18 mer segment and then the annealed product was end filled by klenow filling reaction to get 158 bp double stranded DNA, which was amplified by PCR using the two primers to get the final 178 mer pool. Fair amplification of the pool before starting selection is necessary to give enough representation to each variant.

IV.2 Systematic Evolution of Ligand by Exponential Enrichment (SELEX)

Before starting the SELEX process, the oligonucleotide pool was electrophoresed on 12% denaturing polyacrylamide gel and the band corresponding to 178 mer was cut and eluted to exclude smaller size oligonucleotides and unincorporated nucleotides. This helps in exact quantitation of the starting pool and avoids the selection of truncated oligos. The purified pool was then amplified by asymmetric PCR using a single primer to get single stranded oligos. Asymmetric PCR amplifies the target by arithmetic progression and hence needs to run many cycles to get the desired quantity of DNA. Up to 99 cycles have been reported for DNA aptamers. The problem with so high number of cycle is that the PCR reaction components get degraded and result in production of truncated oligos. So a good practical compromise is to restrict it to 60 cycles while making up the amount of DNA by increasing the volume of reaction. Besides, PAGE purification after every 2-3 cycles helps to avoid their perpetuation through the selection process. Selected aptamers from each cycle were then cloned into pGEMT-easy vector by TA cloning. Positive clones were screened by restriction digestion by Not I and PCR amplification. These clones were then sequenced.

IV.3 Phylogenetic Tree Analysis of Glutathione Binding DNA Aptamers

A phylogenetic analysis of a family of related nucleic acid sequences is a determination of how the different sets have been derived during evolution. Convergent, divergent and parallel evolutionary relationships are expected to emerge. The evolutionary relationship among the sequences is depicted in Fig III.8.

In our case the program (CLUSTAL W) initially classified the sequences into three major groups and these groups were further divided into six classes. The phylogenetic tree

suggested that all the sequences evolved following a common origin. Class-I and class-II are the sub branches of the same initial branch suggesting more homology among them than the rest. Class-III is the single class emerging from the second branch. The third branch gets divided into class-IV, classV and classVI. Most of the sequences come under this branch and three of them (class-V) are identical. Sequences under this branch represent all the cycles. This selective advantage can be interpreted as these sequences may have higher affinity for glutathione.

IV.4 Multiple Sequence Alignment of Aptamers

Alignment is a method for finding similarity between two or more sequences. More homology between two or more sequences implies more structural and functional similarity among them. Alignment can also reveal whether or not there is any evolutionary relationship between the query sequences. Aptamers bind to their target based on the three dimensional structure which is constituted by its nucleotide sequence. All the aptamers binding to a given ligand at a certain facet are likely to have almost similar binding site structure. Thus the region constituting the binding sites are likely to have more similar sequences. Hence multiple sequence alignment of a set of sequences selected for binding a certain ligand can provide information as to the most likely region involved in ligand binding.

The six classes of aptamers emerging from phylogenetic studies were analyzed for conserved regions by multiple alignment using the web server Multalin. The alignment studies showed conserved stretch between 60-80 bp in the first random region and 130-150 bp in the second random region. This suggests the role of these stretches in the stabilization of the aptamer structure and binding to glutathione.

Interestingly the class-V sequences are identical (Fig: III.13); besides they belong to different cycles spanning from first to tenth. They also got well represented among the randomly chosen clones from different cycles (3 out of 23). These points can be interpreted as this sequence have a selective advantages over the other variants and when we are applying conditions to select glutathione binding variants this might be the best binding sequence. Although further kinetic characterization is needed to finally prove the binding affinity. At the moment it is a good sign of binding site conservation.

IV.5 Secondary Structure Prediction of Glutathione Binding DNA Aptamers

Secondary structures of the aptamers were predicted using the web server Mfold. The program provides structures based on energy minimization and gives the energetically most stable structure. The stem regions are important for the stabilization of the structure and the loop regions are more likely to be involved in binding.

Comparison of the secondary structures of different classes showed some structural conservation. In all the classes there were conserved intra-class shared structural motifs. Although these shared structural motifs were not identical and there were variations in the exact shape and size but still the overall geometry was similar (Fig: III.15 Box A, B). At the moment we cannot say which structural variant among those helps in the most specific or stable binding to glutathione. Docking and kinetic studies of the clones can reveal the exact answer. But the conservation of these structural motifs is a good sign of their involvement in stable binding to glutathione.

A variant of the three loop three stem structure (Fig:III.15 Box A) is shared among different clones from different class (Fig:III.15 Box A, Fig: III.19, Fig III.20 Box B). Interestingly this includes class-V (having identical sequences) (Fig: III.19). Hence there is good indication of involvement of this structural motif in stable binding to glutathione.

The minimum energies of the aptamers were found to be around -20kcal/mole. This is as per the average for DNA aptamers although lower than RNA aptamers because RNA has more structural versatility and hence RNA aptamers usually assume more stable structures. Besides the minimum energies are calculated in the absence of glutathione and aptamers selected to bind glutathione can have better stability when bonded with glutathione.

IV.6 Kinetics

The kinetics experiments were carried out by the method described in "the Handbook of RNA Biochemistry". The dissociation constants of various clones were calculated using the formula mentioned in Material and Methods. The graphs were plotted using sigmastat. The cpm count was taken as the x-axis value and the elution fraction number was taken as the Y-axis value. From the graphs we can see a decrease in the cpm count in the early

fractions with the decrease in the concentration of glutathione in the elution buffer. From the data we can see a slight increase in the cpm count of last fractions with the decrease in the concentration of glutathione but the change is just of 100 cpm magnitude <2% of initial counts hence may be part of experimental noise.

We did our first kinetics experiment with clone 10.12 taking 20µl elution buffer for each elution but after seven elution fractions, the elution was not complete (last fraction cpm higher then blank) hence in the next two experiment we took 25µl of elution buffer for each elution.

From the data at different concentration of glutathione (0.5, 1, 2 and 5 µM) we calculated kd of the aptamer at different concentration of glutathione. The final kd was considered as the average of the nearest three of the four kd calculated for each clone.

The assay system used is not an ideal solution or large matrix and hence the calculated values may be taken as an approximation only. It will be necessary to assay the Kd value by other methods such as surface plasma resonance spectroscopy.

At this stage we do not know the exact residues of the aptamers involved in binding GSH. Apparently the different classes of aptamers may interact with different groups of the ligand.

III.7 Prospects

The glutathione binding DNA aptamers offer a more stable alternative to RNA aptamers. It will be important to devise delivery and intracellular amplification methods for such molecules in order to attain concentration required for efficiently sequestering cellular glutathione pool to trigger apoptosis. This could probably be achieved by incorporating the aptamer sequence in a viral DNA replicon. DNA aptamers can also be considered for direct administration in tumor either as synthetic oligonucleotides or as PCR amplicons. It will be of interest to evaluate their influence on ascitic tumor implants to validate their biological activity.

Selection of diverse classes of aptamers suggests for multiple modes of interaction with the ligand. Since all of them have been selected under identical chemical environment, it is likely that they have comparable affinity for the ligand.

Summary and Conclusions

V.1 Summary

Glutathione is a tripeptide thiol made up of the amino acids gamma-glutamic acid, cysteine, and glycine and is also known as gamma-glutamylcysteinylglycine or GSH. It is found in vast majority of prokaryotic and eukaryotic cells and is the biologically most abundant low molecular weight intracellular thiol. Glutathione is both a reductant and a neutrophile and hence can react with oxidising species. This property makes glutathione one of the most important water soluble antioxidant.

Many of the presently available anti cancer therapies are limited in their application because of their low efficiency and the most efficient drug is still to come. On another front researchers are engaged in designing strategies to increase the susceptibility of tumour cells to apoptosis which can increase the efficiency of a variety of cancer treatment. The redox state of the cell has an important role in the induction of apoptosis. Many agents known to induce apoptosis either induce ROS formation or depletion of antioxidants. There are various mechanisms in the cell for the efflux of glutathione conjugates. The formation and efflux of glutathione conjugates of anti-cancerous drugs pertain to drug resistance of tumour cells. Glutathione is thus a good target for increasing the susceptibility of cancer cells to apoptosis as well as to decrease the resistance of the tumour to anti-cancer drugs.

Nucleic acid aptamers are very versatile biomolecules and high affinity aptamers binding to a target molecule with high specificity can be selected.

We selected and characterized glutathione-binding DNA aptamers. We started with a 178 mer random pool and selected glutathione binding DNA aptamer by the SELEX method. The selected aptamers were cloned in pGEMT-easy vector. The positive clones were screened by restriction digestion with Not-I. Five positive clones each from cycles 1, 3, 5, 7 and 10 were sequenced and the sequences were analyzed using different software (CLUSTAL W, Bio-edit, Multalin, and MFOLD).

The aptamers grouped into six classes. There were signs of conserved motifs in each class.

Finally we did kinetic study of three of the clones.

V.2 Conclusions

The results can be summarized as follows.

- Resolution on polyacrylamide gel confirmed the size of the pool before starting SELEX.
- Resolution on 2% agarose gel confirmed the presence of desired size oligos during the SELEX.
- Restriction digestion with Not-I confirmed the presence of inserts of interested size.
- Sequencing results showed that the oligos are of desired size and design without any major aberration.
- Phylogenetic studies showed that six phylogenetic classes of glutathione binding DNA aptamers were got selected.
- Multiple sequence alignment studies showed intra-class and few inter-class conserved regions.
- Secondary structure showed signs of some Intra-class and inter-class conserved structural motifs.
- The conserved regions may be involved in ligand binding.
- As different classes of aptamer have different conserved regions and structural domains, they may be interacting with different epitopes of the ligand.
- Kinetic studies indicate that the aptamers are binding to glutathione.

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