DEVELOPMENT OF CHIMERIC CYTOTOXIN CODING mRNA CONTIGUOUS WITH TEM-BINDING APTAMER

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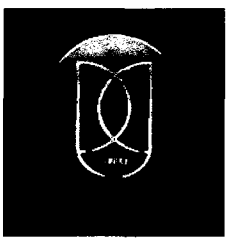

CERTIFICATE

This is to certify that the dissertation entitled "***DEVELOPMENT OF CHIMERIC CYTOTOXIN CODING mRNA CONTIGUOUS WITH TEM-BINDING APTAMER***" has been carried out in Applied Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, submitted by Mrs Abha Kumari. This work is original and has not been submitted so far, in part or in full for the award of any other degree or diploma of any other university.

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Dedicated to my.....

Parents

Commonly used abbreviations and symbols

TEM1	Tumor endothelial marker 1
AP	Alkaline phosphate
APS	Ammonium per sulphate
Bisacrylamide	N, N' –methylene bisacrylamide
Bp	Base pair
BSA	Bovine serum albumin
CIP	Calf intestinal alkaline phosphatase
Ci	Curie
Cpm	Counts per minute
DEPC	Diethyl pyrocarbonate
dNTP	deoxyribonucleoside triphosphate
ddNTP	dideoxyribonucleoside triphosphate
DTT	Dithiothreiotol
EDTA	ethylene diamine tetraacetic acid
fmol	Femto mole
IPTG	Isoprpyl-1-thio- -D-gelactoside
kb	Kilo base
LB	Luria broth
mM	Milomolar
ml	Mili liter

nM	Nanomolar
ng	Nanogram
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pg	Pico gram
pmol	Pico mole
SDS	Sodium dodecyl Sulhate
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
Tris	[Tris(hydroxymethyl) aminomethane]
TEMED	N,N',N'N'-Tetramethyllenediamine
µCi	Micro curie
µg	Microgram
µl	Microlitre
µM	Micromolar
v/v	Volume/Volume ratio
w/v	Weight/Volume ratio
X-gal	5-bromo-4chloro-3-indolyl-ß-galactoside

CONTENT:

Introduction and Review of literature

1. INTRODUCTION:

Cancer is a disease characterized by deregulated cell division. Cancer can affect any part of the body with creation and rapid proliferation of abnormal cells, which grow beyond their development limit and can invade adjoining and distant parts of the body. The cells may spread to other organs, a process referred to as metastasis. Cancer is often associated with genomic instability. Genomic instability is caused by different types of mutation, competition and natural selection. In this disease, a single mutant cell begins by prospering at the expense of their neighbor but in the end, it destroys the whole system. Development of cancer is a micro evolutionary process. This can occur any time in the living organism. Cancer cells are defined by two heritable properties:

(1) Reproduce in defiance of the normal rules

(2) Invade and colonize territories normally reserved for other cells

If the abnormal cell's proliferation is out of control, it will arise as a tumor or neoplasm. As long as the neoplastic cells remain clustered together in a single mass, the tumor is said to be benign and a complete cure can usually be achieved by removing the mass surgically. A tumor is counted as cancerous and cannot be treated surgically if it is malignant, and has the ability to invade surrounding tissue. Invasiveness usually implies an ability to break loose and enter the blood stream or lymphatic vessels and form secondary tumors or metastases at other sites in the body. Cancers are classified according to the tissue and cell type from which they arise. Cancers arising from epithelial cells are termed carcinomas. Those arising from connective tissue or muscle cells are termed sarcomas. Genetically deviant cells are generated as a result of environmental insult or errors in replication. One of historical hypotheses on tumor evolution is that, tumor initiation occurs in single normal cells by induced change which makes it neoplastic and provides it with selective growth advantage over normal cells. From time to time as a result of genetic instability in the expanded tumor population, mutant cells are continuously produced. Through multiple rounds of proliferation, mutation and selection a neoplastic variant evolves

into cancer. Ultimately the fully developed malignancy as it appears clinically has a new genetic constitution e.g. unique aneuploid karyotype associated with aberrant metabolic behavior and specific antigenic properties.

Tumorigenesis thus is a multistep process which involves genetic alteration at different steps of development, resulting in permissive transformation of normal in to highly malignant derivatives. Knudsons two hit hypothesis gave impetus to identification of genes responsible for and genetic changes that lead to cancer. Discovery of tumor suppressor genes and oncogenes has helped to visualize the nature of changes underlying tumorigenesis. Loss of gene function (tumor suppressor gene) and gain of gene function (oncogene) through mutation, deletion and other genetic events are associated with cancer. There are hundreds of cancer types and subtypes of tumors within specific organs, each having its own distinct abnormal regulatory circuits. It is primary question as to what are the regulatory circuits within each type of target cell to be altered to become cancerous cell. Each subset seems to have its own distinct pattern of genetic changes, reflecting on complexities of molecular events to become cancerous. As shown in figure. vast range of alterations resulting in cancerous cells have been cataloged by Hanahan and Robert Weinberg (2000) in to six essential alterations in molecular physiology that collectively dictate malignant growth and those are as follows(Fig 1):

1. Self sufficiency in growth signals.
2. Insensitivity to growth inhibitory signal.
3. Evasion of programmed cell death (apoptosis).
4. Limitless replicative potential.
5. Sustained angiogenesis.
6. Tissue invasion and metastasis.

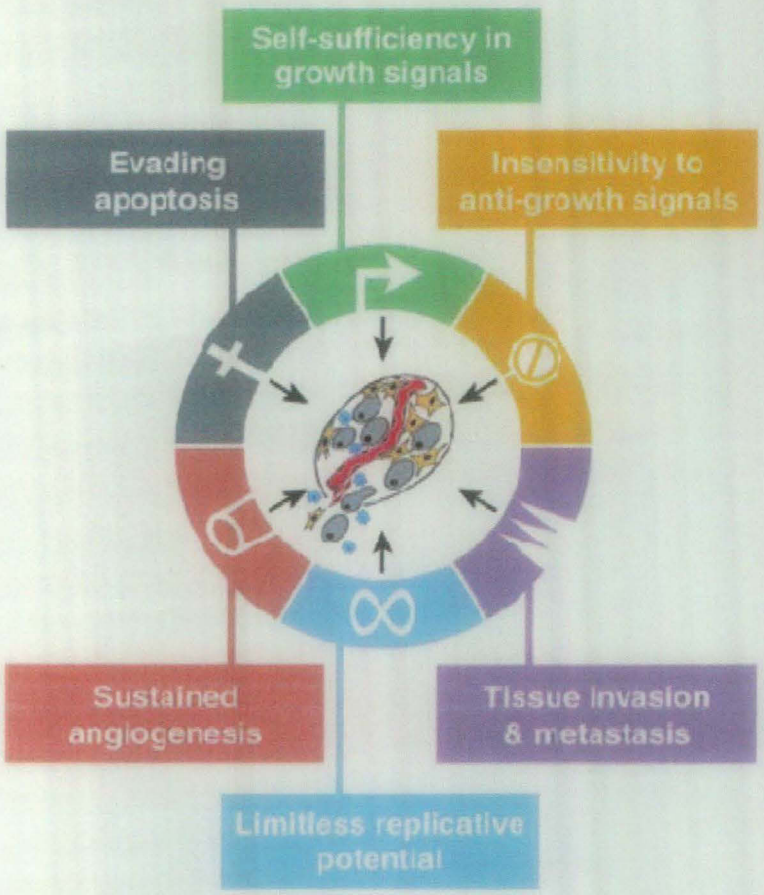


Fig 1: The Hallmarks of Cancer (Taken from Hanahan D and Weinberg RA 2000)

These six capabilities are shared by most of the known type of cancer. Among those, the limitless replicative potential and evasion of apoptosis are of our interest for studying possible therapeutic intervention. Understanding the basic cellular physiology with such an aberration event would be helpful to devise possible therapeutic intervention. Still failure at the front of cancer treatment is attributed to lack of early detection and distinguishable morphological features of cancers from abnormal cells. The six most probable genetic events catalogued by Weinberg provide a window at each step for therapeutic intervention. One such effort is to administer targeted catalytic RNAs. Before exploring the possible interventional modalities, it would be appropriate to understand the developing concepts and molecular players that provide the "limitless replicative potential" to the cells.

2. RNA WORLD:

Origin of life is still a wrestling ground for the science fraternity due to loss of links during the passage of evolution. Many experiment haves been undertaken to reach up to the mark of early life. But it is still an intriguing question, as to how self assembly of all the molecules on early earth occurred and gave rise to a cellular life (Bartel *et al, 1999*).

The term RNA World was given by Walter Gilbert in 1986 when he observed enzymatic activity of various RNAs. It is still hypothetical in context with the origin of life on earth. During early 80s, molecules like DNA and protein had been considered as primary molecules. But after the discovery of catalytic activity of RNA, which is also a genetic material in viruses, it has been hypothesized that RNA can replicate itself and assist various enzymatic reactions. So it could be a primitive molecule originated in primitive soup along with amino acid and nucleotides (Gilbert, 1986).

Ribonuclease P cuts the phosphodiester bond during maturation of tRNA molecule (Takada at el., 1982 and 1983) and *Tetrahymena* in which ribosomal RNA contains self-splicing exons are earlier examples of catalytic RNA molecules. The above citations made a notion in science fraternity that RNA enzymes or ribozymes can synthesize RNA molecule from template RNA and they don't need any protein for catalysis.

RNA world hypothesis envisages that the first stage of evolution proceeds with the RNA molecules that accumulate the other nucleotides and results in to the generation of novel sequences of nucleotides selected evolutionarily for gain in stability and acquisition of new functions Secondarily RNA might have been synthesizing proteins with the help of adapter molecule which further binds with amino acids and then by arranging them according to their RNA template. This would be the first protein ever synthesized on earth which might have a better catalytic efficiency than RNA molecules as ribozymes. It is also seen that the mode of action of proteins are similar to that of RNA molecules so it can be proposed that the information for making protein molecules might have been derived from that in the RNA molecules. These thoughts have been more convincing by the recent progresses in RNA biology like prevalence of various splicing reactions convented with the help of RNA molecules in spite of protein catalysis. The most recent observation which says that the Peptidyl Transferase

reaction on the Psite of the ribosome is performed by RNA rather than ribosomal proteins (Scheiming et al. 2005, Moore and Steitz.2003)

Process of evolution is based on the natural selection and these evolutionary changes can be recapitulated in laboratory leading to artificial catalytic molecules. Even various RNA molecules have been synthesized artificially which have ligase activity in the presence of template. Many investigators have employed in vitro evolution methods to probe the structure and function of existing enzymes or to develop enzymes with novel catalytic properties. So it proves somewhat the level of directed evolutionary processes.

Although by in vitro evolution, various enzymatic nucleic acids have been derived to their natural counterpart, but still these enzymatic molecules have very limited activity and scope with respect to there more evolved counterpart. Even chemical diversity of these molecules is very limited to proteins. Nucleic acids don't have a general acid base with pKa that is near neutral (as in histidine), and there is no specified variety like basic and acidic groups like amino acids. Even the modification sites of amino acids like serine and threonine are more convincing for the various conformational changes during the reaction. So the directed evolutionary approach allows one to explore a wide range of chemical possibilities, not necessarily with the aim of imitating natural enzymes but with the broader goal of understanding what is feasible biochemically (Joyce, 2004).

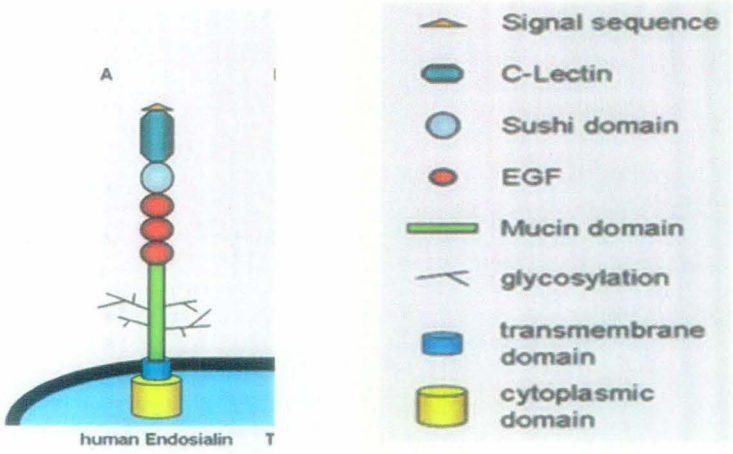
3. REVIEW OF LITERATURE:

3.1. Tumor Endothelial Marker:

Tumor angiogenesis is required for the growth of human tumors to clinically relevant sizes (Iruela- Aripes et al, 1997 and Kerbel et al, 2000). Global analysis of altered gene expression patterns in endothelial cells from human colorectal cancer tissues identified a series of genes termed tumor endothelial markers (TEMs) (St. Croix et al, 2000). These genes were found to be over expressed in the endothelium of both human and animal tumors (Carson-Walter et al, 2001). TEM1 is the prototype of this class, with its mRNA exhibiting the highest differential expression between normal and human tumor endothelium (Rettig et al, 1992). Interestingly, after its discovery as a TEM, TEM1 was found to encode endosialin, the antigen recognized by a monoclonal antibody that had been developed nearly a decade earlier and shown to specifically react with human tumor endothelium and fibroblast-like cells within the tumor stroma (Christian et al, 2001). Endosialin is a 165-kDa single-pass transmembrane glycoprotein that has been classified as a C type lectin-like membrane receptor (Davies et al, 2004). It has multiple extracellular domains consisting of three EGF-like domains, a sushi-like domain, and a C lectin-like domain (Rettig et al, 1992).(Fig.2)

In addition to the importance of TEM gene products as potential therapeutic targets (Marty et al, 2005 & Nanda A et al, 2004), the study of their functional role(s) may provide new insights into angiogenesis. TEM1 is not required for angiogenesis during fetal development, postnatal growth, or wound healing. However, optimal growth, invasion, and metastasis of tumors implanted in abdominal sites of adult mice were found to critically depend on TEM1expression. (Nanda. A et al, 2005)

STRUCTURE OF ENDOSIALIN/TEM1



Rebecca G Bagley: October (2009)

Fig-2: Endosialin is a 757 amino acid protein; the core protein has a molecular weight of 95 kDa. The distinct extracellular domain (aa 1-685) consists of a signal leader peptide (aa 1-20). 5 globular domains followed by a mucin-like region (20-685). The single hydrophobic transmembrane domain spans the (aa 686-706), followed by a short intracellular domain of 50 aa (aa 707-757)

Although the function of endosialin remains to be elucidated, the expression pattern for this protein may be favorable for cancer therapy. Therefore, angiogenesis during embryo development or wound healing was the same as angiogenesis during the growth of malignant cell mass (Folkman J, 2002, 2003&2006). The corollary to this hypothesis was that models of normal embryo development and models working with mature well-differentiated endothelial cells in culture would be sufficient and satisfactory models for tumor endothelial cells. (Herbst et al, 2005) This hypothesis also held that because endothelial cells involved in malignant disease were normal, these cells would be less susceptible to developing drug resistance because they were genetically stable (Riesterer et al, 2006). The current hypothesis is that angiogenesis occurring during malignant disease is abnormal and that therapeutic targets identified by studying endothelial cells isolated from fresh samples of human cancers will be most relevant for developing therapeutic agents to treat human malignant disease (Hida K et al, 2004).

3.2. Ricin:

Purified ricin is a white powder that is soluble in water and stable over a wide pH range.(Cope AC.et.al,1945) It is inactivated by heat, 80°C in aqueous solution for 1 hour,(Parker DT, 1996) and requires higher temperatures or longer periods for inactivation when in powder or crude forms. It is a protein toxin (ie, toxalbumin) derived from the castor bean plant, *Ricinus communis* and has a molecular weight of 60 to 65 kDa.(Balint GA, 1974) Reports on the ricin content of castor beans vary but probably is in the range of 1% to 5%. (Bradberry SM et al. 2003). Ricin is being studied for therapeutic use in cancer chemotherapy, in bone marrow transplantation (Bies C et al, 2004), and in cell-based research. (Godal A et al, 1984) Experimental evidence suggests that malignant cells are more susceptible to ricin toxicity because they express more carbohydrate-containing surface–lectin binding sites than do nonmalignant cells (Lord JM et al, 1994). Antibody-conjugated ricin targets cancer cells (Vitetta ES et al, 1985) and has been investigated as an immunotherapeutic agent (Olsnes S et al, 1972&1976).

Ricin is a glycoprotein lectin composed of 2 chains, A and B, linked by a disulfide bond' (Ishiguro M et al,1976).. The three dimensional structures of Ricin is available in protein data bank. (Fig: 3). A chain has the toxic enzymatic activity & the B chain is a lectin that binds to galactose-containing glycoproteins and glycolipids expressed on the surface of cells, facilitating the entry of ricin into the cytosol (Sandvig et al, 2000). The A chain inhibits protein synthesis by irreversibly inactivating eukaryotic ribosomes through removal of a single adenine residue from the 28S ribosomal RNA loop contained within the 60S subunit. This process prevents chain elongation of polypeptides and leads to cell death (Fig: 4). (Olsnes S et al, 1976) Toxicity results from the inhibition of protein synthesis, but other mechanisms are noted including apoptosis pathways (Griffiths GD et al, 1987), direct cell membrane damage, alteration of membrane structure and function (Hughes JN et al, 1996), and release of cytokine inflammatory mediators (Flexner S et al, 1897 and Kumar O et.al, 2003). A broad group of bacterial and plant toxins have A- and B-chain protein components, such as diphtheria, ricin, botulinum, and anthrax. Ricin belongs to a group of 2-chain toxins possessing ribosomal–inactivating-protein activity (classified as RIP-II) in their A chains, along with such toxins as

shigatoxin, abrin, modeccin, volkensin, and viscumins (Lombard S et al, 2001). Some other plant proteins have no B chain binding component, such as gelonin, trichosantin, and momordin but possess the catalytic RIP activity and are classified as RIP-I (Olsnes S et al, 2004).

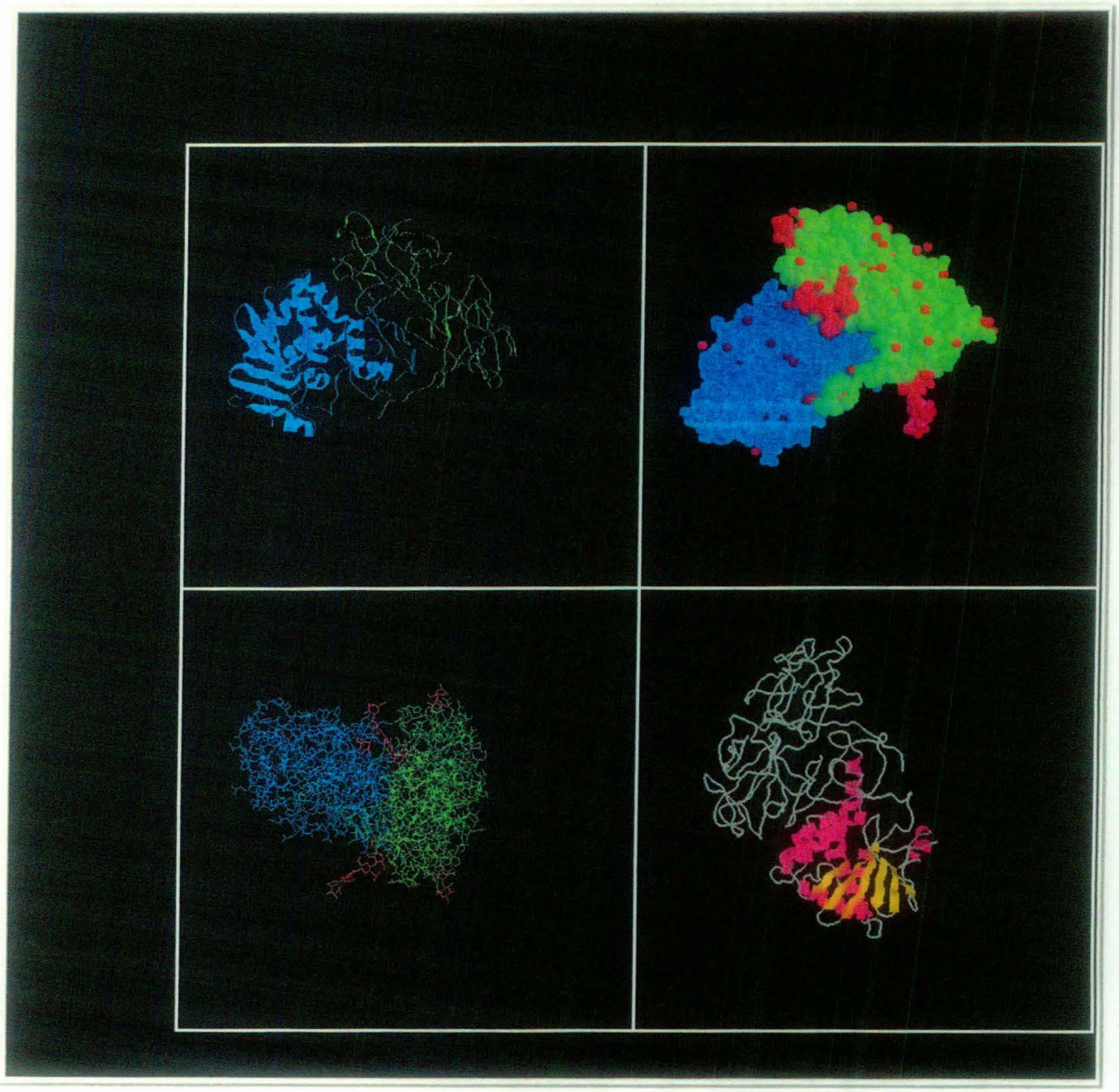
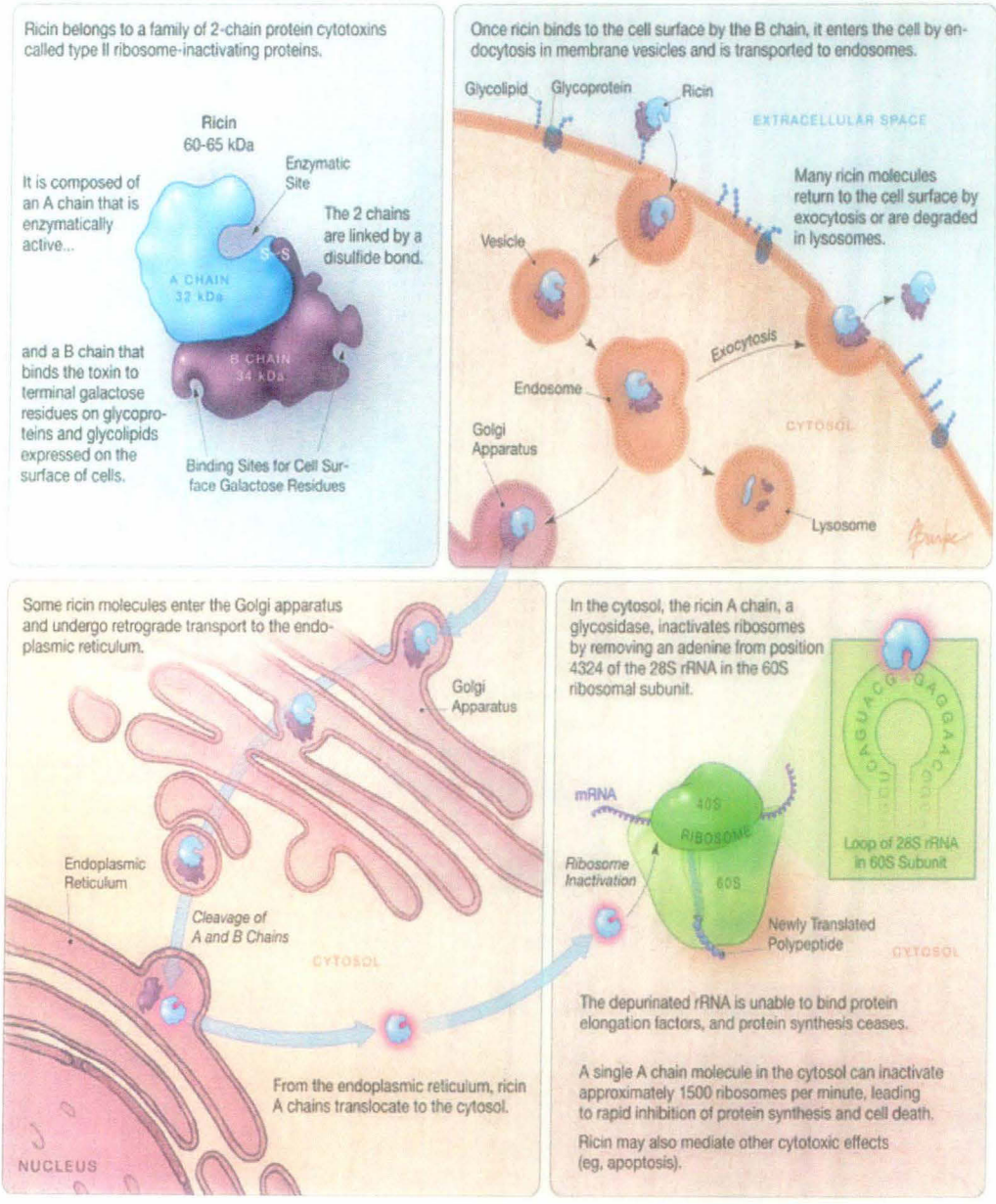


Fig 3: This is a set of four representations of ricin prepared from structural information in the Brookhaven Protein Data Bank (Registry Number 9009-86-3) viewed using the ChIME plug-in for Netscape. The blue strand is the A chain that has the toxic enzymatic activity (an RNA N-glycosidase) of the protein and the green strand is the B chain responsible for binding to cells

and being taken up by them. The ribbon-like winding of the A-chain shows it's highly ordered structure.



Audi,J. et.al. JAMA2005; 294:2342-2351

Fig: 4 Mechanism of Cytotoxicity of Ricin inside the cell (after Audi J.et al. JAMA2005; 294:2342-2351)

3.3. RNA APTAMERS:

Aptamers are an oligonucleotide sequence that forms a precise three dimensional structure and selectively bind with a target molecule. Several aptamers occurring naturally play important roles in monitoring concentration of metabolites and regulating expression of related genes. Aptamer binding ligands of one's choice can also be obtained by in vitro selection. An *in vitro* process called systematic evolution of ligands by exponential enrichment (SELEX) is used to select an aptamer for specific target animal system. The whole process does not depend on *in vivo* system so we can use it to resolve many complicated system.

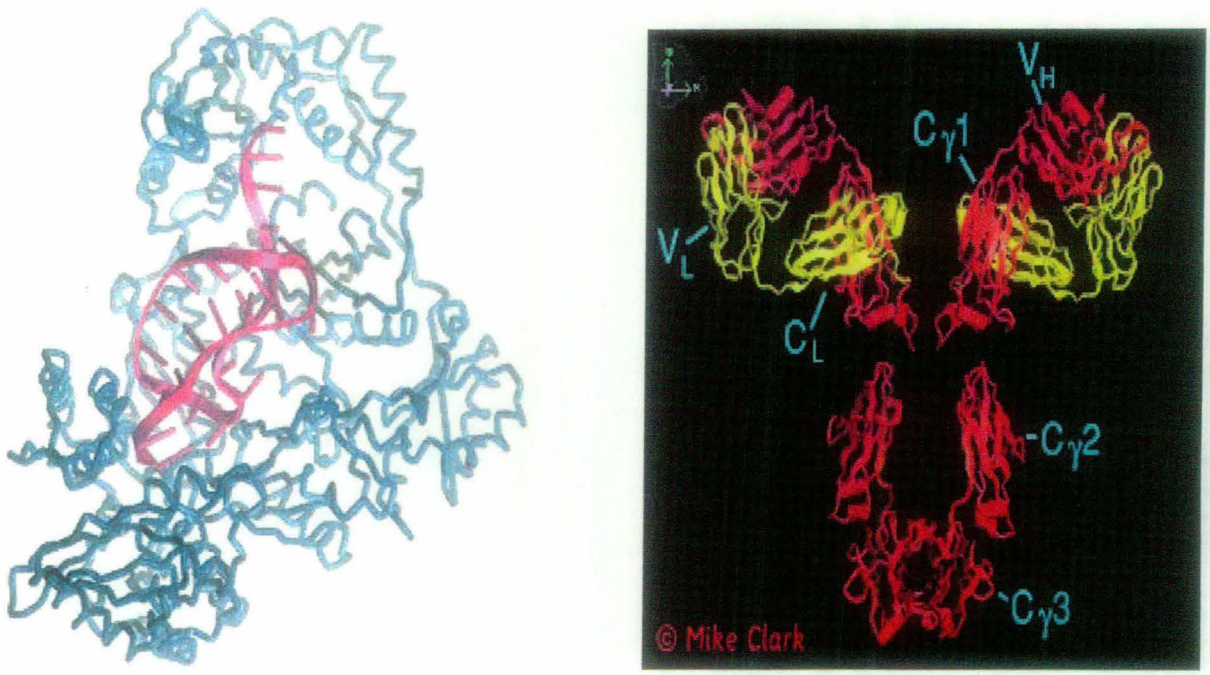


Fig 5: An aptamer binding to its target protein in 3D representation and antibody structure with its heavy and light chains.

Aptamers can be a single stranded, double stranded DNA, RNA, or peptide which forms complex secondary and tertiary structures. Among them, 3D structures of RNA aptamers are most diverse because of the presence of ribose sugar and more flexible structure than DNA. Diversity of RNA aptamers makes them more feasible for screening for a particular target. Even DNA aptamers have almost same characters. Peptide aptamers- are small and simple peptides having single variable loop region that are constrained on both ends to a scaffold protein.(Fig:6) The scaffold nature of peptide aptamers lowers the flexibility of binding to target and can only use their variable region for target protein whereas DNA and RNA aptamers can bind to their target by their whole sequence. These protein aptamers can be generated by high through put screens with yeast two hybrid systems or by retroviral injection in mammalian cells.

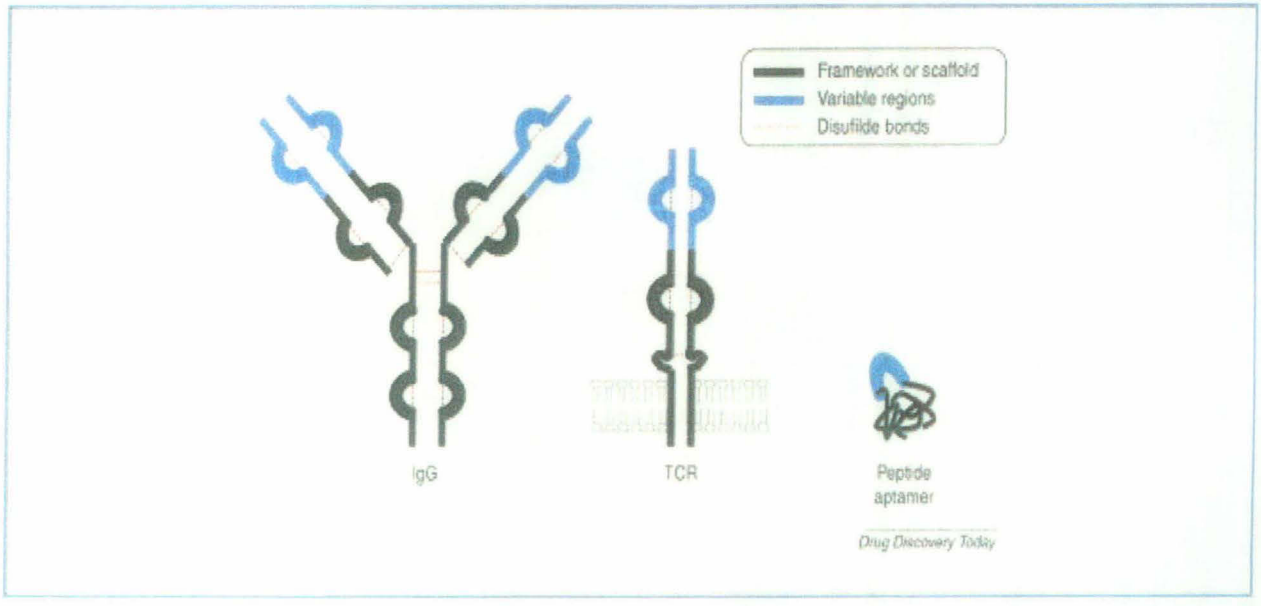


Fig 6: Peptide aptamers are smaller than the others and its structure does not dependent on disulfide bonds so its structure of the variable region is more consistent.

RNA aptamers have an upper hand over the monoclonal antibodies because they can be selected by *in vitro* means. Even antibodies have complications like it can work under the physiological conditions only while aptamers can be selected for various optimized conditions. One more advantage of aptamer is that it has more consistency in their quality like affinity. Time saving is another parameter where aptamers are beneficial than antibodies. (Fig: 5)

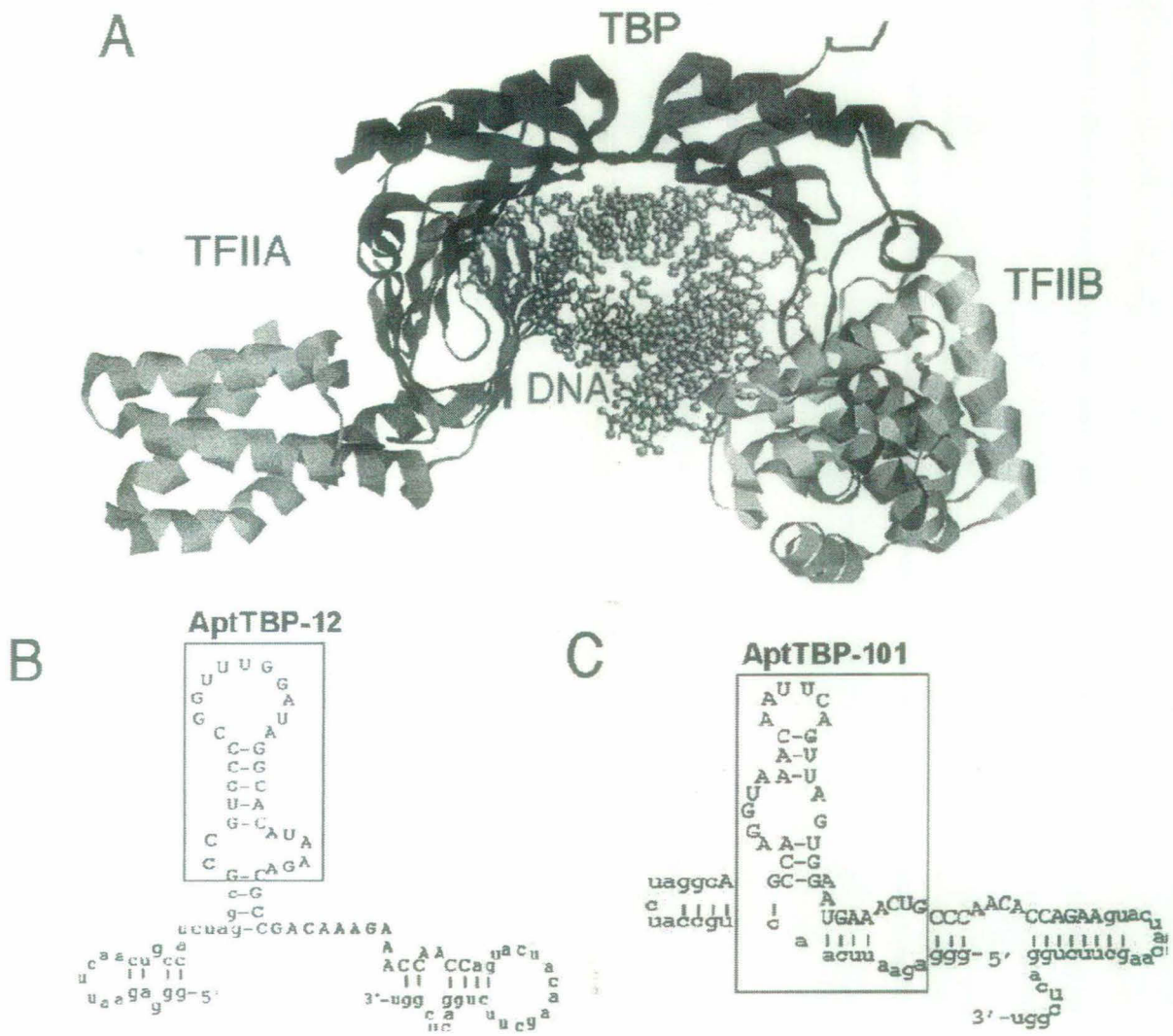
Although RNA aptamers have superiority to DNA and peptide aptamers but there are few disadvantages associated with it. First of them is susceptibility to enzymatic degradation.

However it can be overcome by modifying the 2' position of ribose ring in RNA backbone by modifying the pyrimidines having 2' amino acid and fluoro groups. It makes the RNA more stable and functional during SELEX. Even RNA can be modified by substituting L--ribose form that retains the ability to bind the target and remain stable in cell. For therapeutic uses aptamer can be conjugated with carrier molecule like poly ethylene glycol and liposomal formulation that increases molecular weight and results into an increase in its plasma residence time. Another disadvantage of RNA aptamers is cost to generate them. Small scale synthesis can be performed for research but large scale synthesis is very costly to be performed as the length of RNA increases.

RNA aptamers have very high specificity to their target molecule which makes them a good tool for therapeutic, diagnostic and analytical application. Aptamers are quite promising as biosensor. First aptamer that has been used as therapeutic was Pegaptanib used for age related muscular degeneration. One of the milestones in RNA aptamer history is selection of GSH-binding RNA aptamers by employing *in vitro* selection protocol SELEX. By studying the kinetic properties and observing pro-apoptotic effects on MCF 7 Cell line it has been suggested that glutathione-binding RNA aptamer could be developed into an effective anti-cancer chemotherapeutic agent. (Bala J et al. 2011)

SELEX-generated RNA aptamers are proving highly effective reagents for inhibiting targeted proteins, but conventional methods generate one or several aptamer clones that usually bind to a single target site most preferred by a nucleic acid ligand. One of the main advantages of RNA aptamer is its selection *in vitro* than *in vivo* which provides a high level of control. The RNA aptamers have very high tertiary structure diversity, making a plausibility of finding an RNA aptamers for the target molecule. Such features keep it as advanced source of molecules for analytical, diagnostic and therapeutic applications. An advance generalized scheme for isolating aptamers to multiple sites on a target molecule generated by reducing the ability of the preferred site to select its cognate aptamers. One example of this scheme is generation of "class 1" RNA aptamers, directed to discrete functional surfaces of the yeast TATA-binding protein (TBP), which interfere with the TBP's binding to TATA-DNA. By masking TBP with TATA-DNA or an un amplifiable class 1 aptamer, developed, a new aptamer class, "class 2," that can bind a TBP·DNA complex and is in competition binding with another general transcription factor,

TFIIA. Moreover, both of these aptamers inhibit RNA polymerase II-dependent transcription. (Hua Shi et al, 2007)(Fig- 7)



Hua Shi(2007) PNAS March 6, 10: 3742-3746

Fig 7: an RNA aptamer for discrete functional site of the surface of TATA– binding protein, (b and c) represents the secondary structure of AptTBP-12 AND AptTBP-101.

3.4. The SELEX Process:

SELEX ("Systematic Evolution of Ligands by Exponential Enrichment"), also referred to as *in vitro selection* or *in vitro evolution*, is a combinatorial chemistry technique in molecular biology for producing oligonucleotides of either single-stranded DNA or RNA that specifically bind to a target ligand or ligands. (Tuerk, C et al, 1990) &(Elington et al, 1990). The selected sequences are referred to as aptamers.

The process begins with the synthesis of a very large oligonucleotide library consisting of randomly generated sequences of fixed length flanked by constant 5' and 3' ends that serve as primer annealing sites.(Fig: 8) For a randomly generated region of length n, the number of possible sequences in the library is 4^n.(Four nucleotides (A,T,C,G), with n possibilities). The sequences in the library are exposed to the target ligand - which may be a protein or a small organic compound - and those that do not bind the target are removed by washing and the specific binders can be eluted by affinity chromatography. The bound sequences are eluted and amplified by PCR to prepare for subsequent rounds of selection in which the stringency of the elution conditions is increased to enrich sequences with higher affinity. An advancement on the original method allows an RNA library to omit the constant primer regions, which can be difficult to remove after the selection process because they stabilize secondary structures that are unstable when formed by the random region alone. (Jarosch F et al, 2006)

SELEX was first developed in the early 1990's by the Gold group at the University of Colorado. Since that time, it has been used to select for high-affinity aptamers against hundreds of targets.

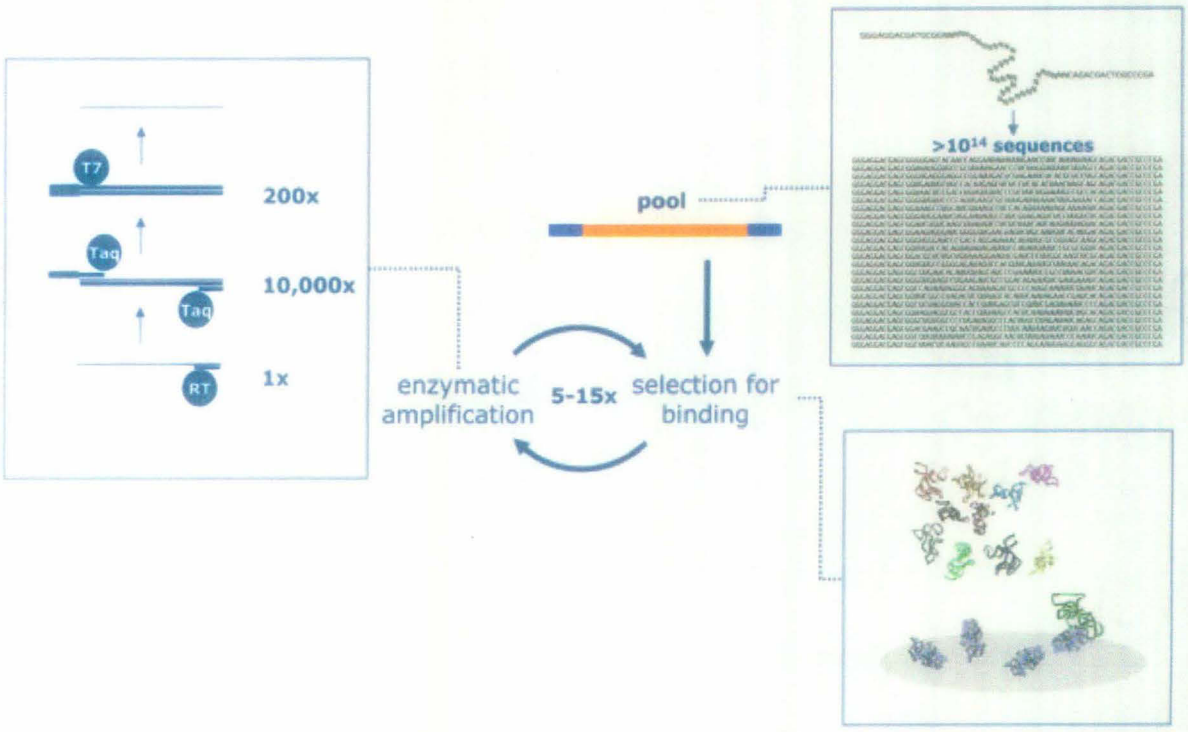


Fig 8: Generation of pool of nucleic acid molecule

Step One. Pool Generation:

At the outset of a SELEX experiment, a pool of nucleic acid molecules is generated using standard automated oligonucleotide synthesis methods. Archemix uses pools comprising a variety of nucleotide compositions for SELEX. Natural RNAs/DNAs have short half-lives in serum due to nuclease degradation. Nuclease activity may be blocked by modifications to the 2'-ribose position (e.g., 2'-O-methyl) on the oligonucleotide backbone. Therefore, in addition to using DNA-based pools, SELEX is also conducted using pools of nucleic acids containing fully 2'-O-methyl nucleotides, or mixtures of 2'-deoxy and 2'-O-methyl nucleotides. At the outset of a selection, each pool contains approximately 10^{14} oligonucleotide sequences, each of which can, in principle, adopt a unique three-dimensional structure. A few of these molecules – the aptamers present a contact surface that is complementary to an "apitope" on the target molecule.

Step Two (Selection):

The selection step is designed to find those molecules with the greatest affinity for the target of interest. The library of nucleotide sequences is exposed to the target protein and allowed to incubate for a period of time. The molecules in the library with weak or no affinity for the target have a tendency to remain free in solution, while those with some capacity to bind will tend to associate with the target. Any one of several methods is used to physically isolate the aptamer-target complexes from the unbound molecules in the mixture, and then the unbound molecules are discarded. The target-bound molecules, among which are the highest affinity aptamers, are purified away from the target and used for the subsequent steps in the SELEX process.(Fig: 9)

Step Three (Amplification):

The captured, purified sequences are copied enzymatically, or "amplified", to generate a new library of molecules that is substantially enriched with oligonucleotides that can bind to the target. The enriched library is used to initiate a new cycle of selection, partitioning and amplification.

Step Four (Aptamer Isolation):

After five to 15 cycles of the complete SELEX process, the library of molecules is reduced from 10^{14} unique sequences to a small number that bind tightly to the target of interest. At this stage, the nucleotide sequences of individual members of the library are determined. The target binding affinity and specificity of selected sequences are then measured and compared.

Oligonucleotides used for SELEX

Oligo I T7+21 (41 mer)
5′ TAATACGACTCACTATAGGGAGACAAGAATAMACGCTCAA 3′
Oligo-II 88 mer
5′ GGGAGACAAGAATAAAACGCTCAANNNNNNNNNNNNNNNNN N NNN
NNNNNNNNNNNNNNNNNNNNTTCGACAGGAGGCTCAC 3′
Oligo-III 24 mer
5′ GCCTGTTGTGAGCCTCCTGTCGAA 3′

SCHEMATIC PRESENTATION OF SELEX

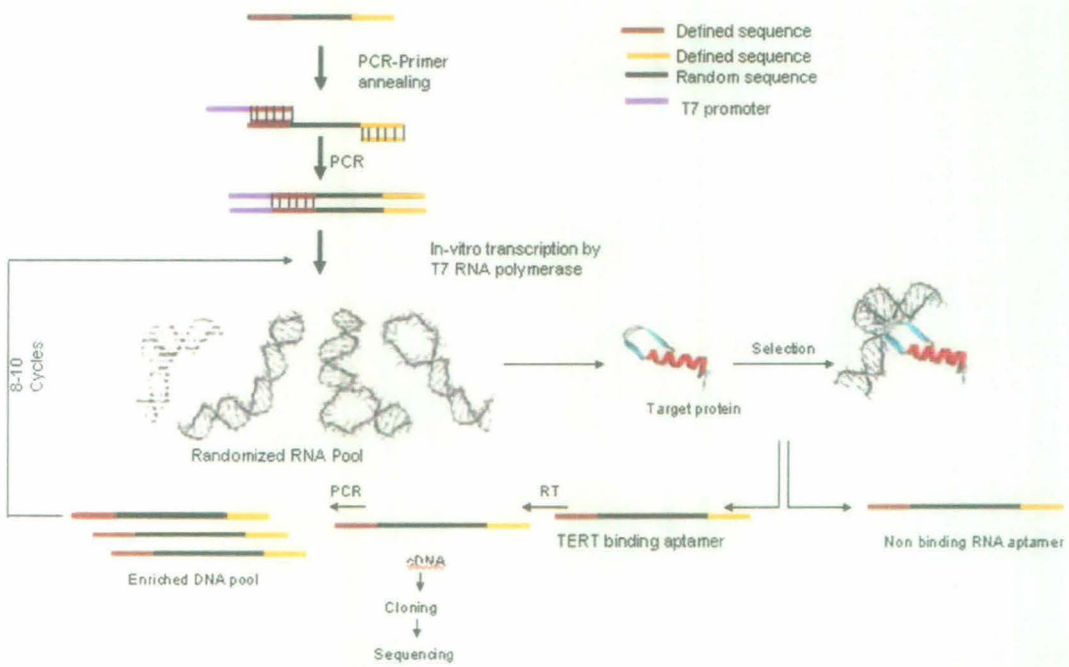


Fig: 9 A general overview of in vitro selection protocol. Enrichment of Nucleic Acids (RNA) start as a random pool, and are enriched through the selection process. (Akhil Varshney, AMBL, SLS, JNU)

The technique has been used to evolve aptamers of extremely high binding affinity to a variety of target ligands, including small molecules such as ATP (Dieckmann et al, 1996) and adenosine(Huizenga et al, 1995) and proteins such as prions (Mercey et al, 2006) and vascular endothelial growth factor (VEGF) (Ulrich et al, 2006). Clinical uses of the technique are suggested by aptamers that bind tumor markers (Ferreira et al, 2006) and clinical trials are underway for a VEGF-binding aptamer trade-named Macugen in treating macular degeneration. (Vavvas D et al, 2006).

Materials and Methods

4. MATERIALS AND METHODS:

4.1. MATERIAL:

4.1.1. Materials used for routine work:

Agarose gels	0.5-2.0% for analysis of DNA, the appropriate amount of agarose was added to a flask witj 1X TAE buffer. This was melted and ethidium bromide (0.5µg/ml) was added and the mixture was poured in to a plate with a slot former.
Agar plates	15g agar per liter medium, autoclaved, cooled down to 45°C and filter sterilized, antibiotics added (Ampicillin: 100µg/ml)
Antibiotics stocks	Ampicillin sodium salt, stock concentration 100mg/ml in water.
DTT	Stock 0.5mM Dithiothreitol. (sigma) in water.
EDTA	0.5M (in double distilled water, pH adjusted to 7.5 with 5M NaOH), autoclave.
Ethidium Bromide	1mg/ml in H_2O (sigma)
H_2O	Bi-distilled water, Mili Q, R.O. and autoclaved.
Media	Luria Broth medium- ingredients (g/l): 10g casein enzymatic hydrolysate 5g yeast extract, 5g NaCl (Hi-media),
PEG	30% polyethyleneglycol 8000 (sigma) in water, autoclaved.
Phenol	Distilled, equilibrated with TE

Restriction Enzymes	Obtained from NEB, Promega, MBI, GIBCO-BRL used at 1-5u/µg DNA in the buffers provided.
RNase A	Pancreatic RNase (sigma), 10mg/ml, dissolved in TNE, incubated for 10min at 85° C, to inactivated DNase, stored frozen.
SDS	100g SDS (Na-laurylsulphate) dissolve in 1000ml of 50mM Tris-HCL pH 8 autoclave in aliquots and stored at room temperature.
Sodium Acetate	3M, pH 5.2, autoclaved
Sodium Azide	0.02% in sterile distilled water
Sodium Chloride	5M NaCl prepared in DEPC water, autoclaved
20X TAE	1.0M Tris-HCL,0.4M Na-Acetate, 0.004 M EDTA, pH adjusted with glacial acetic acid to 7.8, autoclaved.
10X TBE	0.9M Tris base,0.9MBoric acid,0.025M Na_2-EDTA, dissolved in H_2O, pH 8.3, autoclaved.
1X TE	10mM Tris-HCL (pH 7.5),1mM EDTA, M.Q water.
Tris-HCL	1M,pH 7.5 or 8.0, Tris base dissolved in H_2O, adjusted to adjusted to require pH with HCL, autoclaved.

4.1.2. Materials used for RNA work:

Reagent of RNA work:

H2O	DEPC treated and autoclaved milliQ for dissolving all the reagents written below except Tris
0.5M EDTA (pH 8.0)	9.3gEDTA ,(50ml)
1M NaOH	2gNaOH (50 ml)

1M Tris-Cl (pH 8.0)	6g Tris (50ml) pH adjusted with HCl
1M NaCl	2.92g NaCl(50ml)
7.5M Ammonium acetate	28.9g(50ml)
1M KCl	3.72g(50ml)
1M $MgCl_2$	10.16g(50ml)
1.5% SDS	750mg(50ml)
2M sodium acetate	27.2g(100ml)
PBS	**500ml**
NaCl	4g
KCl	100mg
Na_2HPO_4	0.72g
KH_2PO_4	120mg

0.1% DEPC should be added to every chemical except (Tris should be prepared in autoclaved DEPC treated water).

In vitro Riboprobe generation kit	promega
RNAsin(40u/µl)	promega
T7 RNA polymerase(20u/µl)	promega
RNase free DNase(2u/µl)	promega
Sephadex G-50	promega

4.1.3. Materials for generation of nucleic acid library:

Materials:

Oligo I $^{5'}$TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAA$^{3'}$ (+) %GC36.6Tm66

(T7+21)

{ 24 } { 24 }

OlogoII GGGAGACAAGAATAAAACGCTCAA(40N)TTCGACAGGAGGCTCACAACAGGC(+)

(88)

Oligo III $^{5'}$GCCTGTTGTGAGCCTCCTGTCGAA$^{3'}$ (-) %GC56 Tm 60

(24)

Total length of RNA: 88 nucleotides

Total length of DNA: 105 base pair

$^{5'}$TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAA(40N)TTCG 85 base

$^{3'}$ATTATGCTGAGTGATATCCCTCTGTTCTTATTTTGCGAGTT(40N)AAGC

ACAGGAGGCTCACAACAGGC$^{3'}$

TGTCCTCCGAGTGTTGTCCG$^{5'}$

4.1.4. Materials used for PCR reaction:

Material	Concentration	Volume added reaction mix
dNTP mixture	10mM	10.0µl
Taq DNA polymerase (Genei)	(1u/µl)	0.5µl

Primers used for PCR:

Forward primer: T7 + 21 5′ TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAA 3′

Reverse primer: 5′ GCCTGTTGTGAGCCTCCTGTCGAA 3′

4.1.5. Materials used for in vitro transcription:

Material	Concentration(stock)	Final concentration reaction mix
Transcription buffer	5X	1X
Dithiothreitol(DTT)	100mM	10mM
Ribonuclease inhibitor (RNAin)	40u/µl	2u/µl
dNTP mixture (GTP, CTP, ATP, UTP)	2.5mM each	0.5mM
aP^{32} UTP (BRIT)	20µCi/µl	1µCi/µl
T7 RNA Polymerase	20u/µl	1u/µl

Columns: Sephadex G 50 column

4.1.6. Materials used for RT PCR:

Composition	Concentration	Final
Primers for RT		
Forward primer	10picomol/µl	1picomol/µl
Reverse primer	10picomol/µl	1picomol/µl

Bacterial Strains used in this study: DH5a & DH10ß

Both DH5a and DH10ß has several features that make them useful for recombinant DNA methods

1.*Escherechia coli* **DH5a:** fhuA2 ? (argF-lacZ)U169 phoA glnV44 F 80 ? (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*)*

EndA1 mutation inactivates an intracellular endonuclease that degrades plasmid DNA in many miniprep methods, *hsdR17* mutation eliminates the restriction endonuclease of the EcoKI restriction-modification system, so DNA lacking the EcoKI methylation will not be degraded,*(lacZ)*M15 is the alpha acceptor allele needed for blue-white screening with many lacZ based vectors, recA eliminates homologous recombination which makes the strain somewhat sickly, but reduces deletion formation and plasmid multimerization,glnV44 is the systematic name for SupE44, an amber suppressor

2.*Escherechia coli* **DH10ß:** F- endA1 recA1 galE15 galK16 nupG rpsL ? lacX74 F 80lacZ? M15 araD139 ? (ara,leu)7697 mcrA ? (mrr-hsdRMS-mcrBC

The progenitors of DH10ß were all K-12 strains, with the exception of D7091F, in which a region surrounding the (*araA-leu*)*7697* deletion had been derived from *E. coli* B SB3118 by P1 transduction. Among the engineered gene replacements were *recA1* to improve clone stability by inhibiting the homologous recombination system;*endA1*, which inactivates the encoded periplasmic DNA-specific endonuclease, thereby enhancing DNA stability during transformation; and a F 80 derivative containing the *lacZΔM15* mutation for screening by a complementation. DH10 derivative containing the allele *mdoB*::Tn*10* was subjected to fusaric acid treatment to counterselect against the tetracycline resistance gene in the transposon, again in the presence of the RecA-expressing plasmid. DH10ß was one isolate from this selection with a deletion spanning the marker and the flanking region, including the MDRS loci (*mrr*, *mcrA*, *mcrB*, and *mcrC*). Deletion of the MDRS loci was shown to improve the cloning efficiency of mammalian DNA, in which cytosine is commonly methylated.

Plasmid used in this study:

pGEMT Easy

4.1.7. Materials used for vector Construction:

IPTG	Isopropyl-ß-D-thio-galactopyranoside, 100mM in H_2O, Filter Sterilized (GIBCO-BRL)
Ligase	T4 DNA ligase (NEB)
X-gal	5-Bromo-4-Chloro-3-Indoyl-Galactoside (50mg/ml in dimethylformamide) (Sigma)

4.2. METHOD:

4.2.1. Genomic DNA isolation from plant:

Source of sample for genomic DNA isolation: *Ricinus communis* leaves

A small piece of leaf tissue(1cm×1cm) taken and ground with help of mortar and pestle in 1ml of extraction buffer to a homogeneous paste then incubated at 60°c in a water bath for 50min, extracted twice with 650µl chloroform : isoamyl alcohol(24:1) centrifuged at 13000rpm-supernatant was taken (upper aqueous layer) and the DNA was precipitated with 2/3 vol of isopropanol then incubated at room temp for 10minutes, centrifuged at 13000rpm for 5′ at 10°c, washed thrice with 70% alcohol then air dried and dissolved in 30µl milliQ water stored at -20°c. The DNA was checked following agarose gel electrophoresis (0.8%). (Fig: 11).

4.2.2. PCR amplification of gene of ricin A chain:

Gradient PCR was done to ensure at what T_m the amplification of ricin is most appropriate. After confirmation 58°C has been taken as melting temp for amplification of gene of interest. I have used both taq polymerase + pfu to get TA overhang at end.DNA polymerase from *Pyrococcus furiosus*(pfu) was been used in addition to taq because pfu have 3'-5' proofreading activity so that error rate is 1 out of 10^4 bases .as shown in (figure11) I have got sharp band of about 836bp after amplification.

Forward primer- $^{5'}$GAGGATAACAACATATTCCCC$^{3'}$

Reverse primer- $^{5'}$ACCACTGGCCTTATAAGC$^{3'}$

Matetrial	Amount
10XBuffer	10µl
Dntp mix(2.5mm each)	2µl
Taq dna polymerase (1u/µl)	1µl
Pfu dna polymerase (2u/µl)	1µl
$MgCL_2$	2µl
Forward primer (10pmol/µl)	4µl
Reverse primer (10pmol/µl)	4µl
Template DNA (36.13ng/µl)	3µl

Final volume adjusted with water	100.0µl

The PCR reaction was set for 30 cycles under following reaction conditions in thermocycler:

Step 1	94°C	5minutes
Step2	94°C	30seconds
Step3	60°C	30minutes
Step4	72°C	30seconds
Step5	72°C	5minutes

4.2.3. Cloning of Ricin and Aptamer Selection for Tumor Endothelial marker 1:

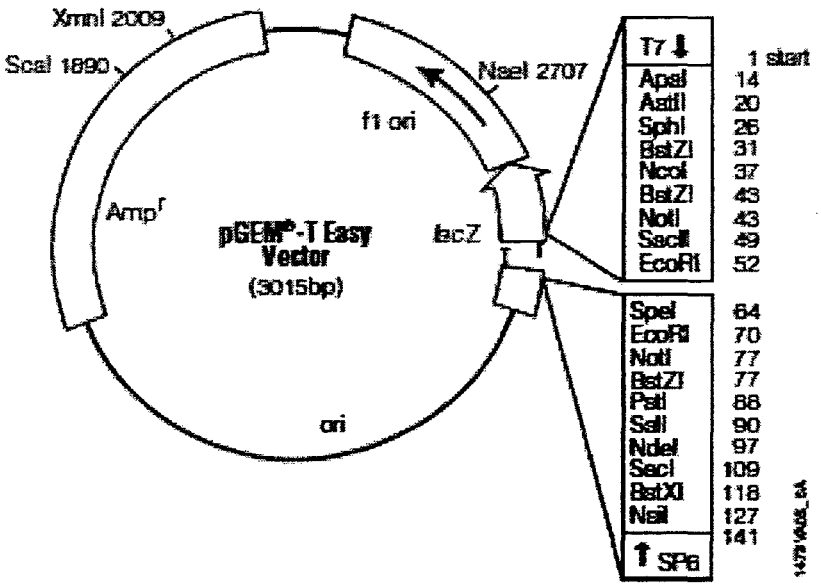


Figure10: pGEMT easy vector map with T overhang

4.2.4. Ligation:

Material	**Amount**
2x Buffer	5µl
pGEMT easy(50ng/µl)	1µl
T4 ligase	1µl
PCR product (160ng/µl)	2µl
Final volume adjusted with water	**10µl**

Incubated at 4°C overnight

4.2.5. Competent cell preparation of bacterial cells:

E.coli strain DH5a &DH10ß was used for cloning the construct. The single preselected colony was grown by shaking overnight in 5ml culture. This culture was inoculated in (1:100,v/v) in fresh LB medium and shaken at 37°C to an OD at 550 nm of 0.6-0.7.This OD corresponds to approximately $6x10^7$cells/ml. The cells were centrifuged at 4500rpm for 10minutes at 4°C, resuspended in half the original culture volume of pre cooled 50mM $CaCl_2$ and incubated for 10-20minutes. The cells were centrifuged as above and suspended in 1/10 of the original volume of precooled 50mM $CaCl_2$ with 20% glycerol. The cells were aliquoted in 200µl volume and stored at -80°C until use.

4.2.6. Transformation of plasmid DNA in E.coli cells:

For transformation typically 10ng of plasmid (pGEMT) DNA was pre treated with 2µl of ice cold TCM solution (100mM Tris and 300mM of $CaCl_2$, 300mM of $MgCl_2$) in ice for 5 mins. 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 mins in ice. After 40 minutes, the transformation mixture was given heat shock at 42°C for 90sec, and immediately kept in ice for 5 minutes. To this 1ml of LB was added (without antibiotic) and incubated under moderate agitation at 37°C for 60 mins, An aliquot of transformed cells 100µl was spread on agar plate containing selective antibiotic and incubated overnight for selection of the transformants.

4.2.7. Blue- white screening:

For blue white selection the transformant cells were spread 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.

4.2.8. Alkaline Lysis Mini-Preparation:

A single colony was transferred into 2ml of LB medium containing the appropriate antibiotic in a15 ml tube. The culture was incubated overnight at 37°C with vigorous shaking (220rpm).The

culture was poured into a microfuge tube, centrifuged at 5000rpm for 5minutes at 4°C in a microfuge. The medium was removed, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 100µl of ice-cold Solution I (50mM glucose, 25mM, Tris-Cl pH-8,10mM EDTA pH-8) by vigorous vortexing. Added 200µl 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 150ul 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 13000rpm for 5 minutes. The supernatant was taken in afresh 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 pellet was dried and finally dissolved in 30µl of water.

4.2.9. Digestion:

Digestion with Not-1was performed at 37°C for1 hour to confirm the positive clones.

Material	Amount
2Xbuffer	5µl
BSA	1µl
Not1(10u/µl)	1µl
DNA	2µg each
Final volume adjusted with water	**50µl**

4.2.10. *Sequencing:*

Some colonies have been selected for sequencing. In pGEMT vector there are two primers at two end of restriction site sp6 primer at one end and T_7 primer other end. Two plates were prepared by streaking to get discrete colonies which were given for sequencing with the SP6 primer.

4.2.11. *Selection of Aptamers:*

General procedures

Routine basic molecular biology procedures involving nucleic acids treatments such as extraction, purification using phenol/chloroform extraction, ammonium acetate, isopropanol precipitation, ligation, and electrophoresis were carried out as per the methods described (Sambrook *et al* 2001). Invitro transcription and ligations were carried out as per recommended by manufactures (New England Biolabs, Promega, GIBCO BRL life Technology, Bangalore Genie and MBI Fermentas).

4.2.12. Generation of duplex template for transcription:

Design of Random oligodeoxynucleotides for generation of variant RNA pool:

The in vitro selection of RNA aptamers is governed by the same principle as natural selection. A population of oligonucleotides with random sequences is screened for fitness, and survivors are pooled and amplified. Mutations in natural process commonly arise as single base deletion or substitution. In case of artificially generated nucleic acids, nearly 10^{15} variants can be screened per generation and sequence can be either partially or completely randomized as desired. A library of 88bp in which 40 mer random sequence with 40bp conserved seq at both ends designed and got from sigma this random seq pool was PCR amplified with olig1(T7+21mer) and oligo 2^{nd} (24mer) and DNA of 105 bp has been generated followed by phenol chloroform purification.

4.2.13. In vitro Transcription

The standard in vitro transcription was carried out with Riboprobe transcription kit (PROMEGA) as prescribed by the manufacturer. Transcription reaction was set at room temperature as DNA can be precipitated with spermidine present in the reaction buffer at low temperature.

4.2.14. *Composition of a typical reaction mixture for in vitro transcription:*

Constituents mix	Volume per 40µl reaction
5 X Transcription optimization buffer	8µl
DTT, 100Mm	4µl
Recombinant RNasin Ribonuclease inhibitor (40u/ µl)	2µl
rATP, rCTP, rUTP and rGTP (2.5Mm each)	8µl
Linearized template DNA	1-2µg
(a-^{32}p) UTP (800Ci/mmol, 20µ Ci/µl)	2µl
T7 RNA Polymerase (20U/µl)	2µl
Final volume adjusted with water	40µl

Prepared by mixing one volume of nuclease free water with one volume each of 10mM rNTPs stocks supplied.

The reaction was incubated at 37°C for 60 minute. The DNA template was digested with 5 µl DNase (1u/µl).

4.2.15. Column Purification (Removal of unincorporated ribonucleotides):

In order to estimate the transcribed RNA it was necessary to separate the unincorporated ribonucleoides. This was done by size exclusion chromatography. Purified transcripts were

obtained by passing the transcription product through a 1 ml column packed with SephadexG-50 (sigma). Sephadex G-50 was previously swelled in sterile DEPC treated MQ water for 4 hrs at room temperature. The suspension was autoclaved and always used fresh. The volume of the transcription reaction (20µl) was adjusted to 100µl with sterile MQ water and loaded on to Sephadex G-50 column.

4.2.16. SELEX:

4.2.16.1. Immobilization of peptide on to beads:

The streptavidin coated magnetic beads were resuspended in to original vial and 40µl was aliquote in to new PCR tube. The PCR tube was placed on magnet and the supernatant was taken out. These beads were washed twice with PBS, with solution 'B' once with solution 'C' followed by.PBS. 4µL of 1ng/µl peptide was added on to beads and incubated for 25 minutes at room temperature. Then supernatant unbound peptide was taken out and stored for further use. The peptide beads were resuspended in 20µl PBS.

4.2.16.2. Addition of RNA pool:

40µl RNA pool was added on to peptide and incubated for 15 minutes to 20 minutes at room temperature. Then unbound RNA was removed and stored for counting, washed 3 times with PBS and each wash product was kept for counting in to 0.5ml eppendrof. Then resuspended the beads in 20µl nuclease free water and eluted at 75° C for 10 minutes. Again resuspend the beads in 20µl water RNA bound to peptide were Eluted at 75° C for 10 minutes.

4.2.16.3. Scintillation Counting:

Precipitation of eluted RNA:

Eluted RNA was precipitated by adding $1/10^{th}$ 7.5M ammonium acetate and 2.5 times 100% ethanol by volume(40µl RNA and 4µl of 7.5M sodium acetate and 100µl 100% ethanol) , stored at -80° C for overnight. The precipitate was collected by centrifugation at 13000 rpm for 20 minute. Washed with 70% alcohol dried the pellet at 37° C for 15 to 20 minutes followed by dissolution in 10µl nuclease free water.

4.2.16.4. RT PCR of eluted RNA:

It has been carried out in 3 steps

Production of cDNA: In 10µl of sample 1µl of 10 picomol/µl oligo 3 was added and in 1µl of RNA +ve control RNA, 4µl of H_2O and 0.5 µl 10 picomol/µl oligo 3 was added and in –ve control 5µl of water and 0.5µl of 10 picomol/µl oligo3 was added and these all set in to 3 PCR tubes at following conditions:

Step 1:

70° C	(5 minute)
4°C	(10 minute)

Step 2: Reverse Transcription

5X RT buffer	8µl
dNTPs (2.5 mM)	2µl
RNasin (40u/µl)	2µl
H_2O	4µl
AMV RT (1u/µl)	2µl
Total	18µl

The above mixture allows addition of RNA in one ninth volume to follow RT reactions under the following conditions.

25° C	5 minute
42° C	60 minute
72° C	15 minute
4° C	hold

Step 3: Final RT PCR

10X reaction buffer	10µl
$MgCl_2$	2µl
dNTPs(2.5mM)	2µl
oligo 2 (10pmol/µl)	2.5µl
oligo 3 (10pmol/µl)	2.5µl
taq DNA polymerase(1u/µl)	2µl
H_2O	69µl
Total	90µl

This reaction was set for 15 cycle on following reaction condition.

Initional denaturation	95° C (5 minute)
Denaturation	95° C (30 second)
Annealing	61° C (30 second)
Extension	72° C (30 second)
Final extension	72° C (5 minute)

TEM 1 binding RNA aptamer were eluted from the streptavidin column varying in their binding affinity and specificity. To select RNA molecules with highest affinity repeated cycles of selection required. For further cycle of the SELEX, RNA molecules were reverse transcribed in to DNA by RT-PCR. The reaction composition follows:

4.2.17. Phenol Chloroform Purification of PCR amplified of first cycle:

The 100µl of PCR amplified product is purified by mixing it with 365µl of H_2O, 200µl of chilled phenol (to denature the protein efficiency) and with 200µl of chloroform (remove traces of phenol from nucleic acid preparation). The contents were mix properly and speen at 13000 rpm for 10 minutes at 4° C. Subsequently add 400µl of chloroform to the aqueous phase in a fresh

eppendrof tube and spin in a microfuge at 13000 rpm for 5 minutes at 4° C. Again to the supernatant add 40µl of 3M Sodium acetate and 1ml of chilled 100% ethanol followed by incubation at - 80° C for 2 hours to overnight for precipitation followed by centrifugation at 13000 rpm for 20 minutes at 4°C. The pellet was washed with 500µl of 70% ethanol. The pellet was dried and resuspended with 10µl of H_2O and DNA quality was checked in 2% agarose gel. Quantification of DNA was done by nanodrop method.

4.2.18. Preparation of competent bacterial cells and transformation:

DH 10 ß was prepared as method described for DH5a

For transformation, competent cells were thawed on ice and to 200 µl cells, DNA was added. After 45min of incubation on ice, the transformant mixture was given a heat shock at 42°C for 90sec, followed by 1 min on ice. The cells were then left at room temperature for 10 min followed by addition of 1 ml LB and incubation at 37°C with moderate shaking at 200 rpm for one hour. Transfection mixture was then plated on LB (amp) plates. If the blue/white selection was desired for screening, LB (amp) plate was prepared by spreading out a mixture containing 50µl of IPTG (100mM), and 20µl of X-gal (50mg/ml in dimethylformamide), letting the plates dry, and by pre-warming them at 37°C for 20 min prior to plating the transfection mixture. The transformed colony appeared after overnight incubation at 37°C.

4.2.19. The LiCl procedure for "Rapid plasmid Minipreps:

Five ml of LB medium were inoculated with a single transformed colony and grown overnight at 37°C/220rpm. The culture was transferred to a microfuge tube and spun at 10,000 rpm for 1 minute, cells were resuspended in 100µl TELT and lysed by adding 10µl freshly prepared lysozyme (10mg/ml) and incubated for 5min in ice followed by incubation in boiling water bath for 45sec. The suspension was chilled on ice foe 5min and cell debris removed by centrifugation (10 min at 12000rpm). The plasmid DNA was precipitated by adding 0.8 volume of isopropanol, for 20min and the pellet washed with 70% ethanol and suspended in 20µl water or TE.

4.2.20. Agarose gel electrophoresis:

According to the size of DNA or RNA to be electrophoresed, agarose was weighed and added to 1 TAE buffer boiled till the solution becomes clear with desired final concentration of 0.5 µg/ml, mixed and the gel poured in casting tray and allowed to settle.

4.2.21. Cloning of RNA aptamers:

All RNA aptamers were cloned immediately downstream of T7 promoter at TA cloning site of pGEMT Easy vector. Vector (pGEMT easy) and insert (105 bp), in ratio 1:3 were ligated by T4 DNA ligase. The ligation product was transformed in DH10ß competent cells as describe above before plating on the LB-agar (ampicillin) plate.

4.2.22. Transformation and blue-white screening:

The frozen competent cells (DH10ß) were kept in ice for thawing. For transformation typically the ligation mixture were added to 200µl of thawed competent cells in a hood and incubated in a ice for 30 minutes. After 30 minutes, the transformation mixture was given heat shocks at 42°C for 90 second and immediately kept in ice for 5 minutes. To this 1ml LB media was added and incubated in a water bath at 37°C for 60 minutes, to allow the expression of antibiotic resistance gene. An aliquot of transformed cells (100-125µl) was spread on agar plates containing selective antibiotics (ampicillin) and incubated at 37°C overnight for selection of the transformants.

For blue white selection of transformants the transformant cells were spread 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.

4.2.23. Alkaline Lysis Mini-Preparation:

A single colony was transferred into 2ml of LB medium containing the appropriate antibiotic in a15 ml tube. The culture was incubated overnight at 37°C with vigorous shaking (220rpm).The culture was poured into a microfuge tube, centrifuged at 5000rpm for 5minutes at 4°C in a microfuge. The medium was removed, leaving the bacterial pellet as dry as possible. The pellet

was resuspended in 100µl of ice-cold SolutionI (50mM glucose, 25mM Tris-Cl pH-8,10mM EDTA pH-8) by vigorous vortexing. Added 200µl of freshly prepared SolutionII (0.2N NaOH, 10% (w/v) SDS).The tube was closed tightly, and contents mixed by inverting the tube rapidly few times. Now 150ul of ice-cold solutionIII (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 13000rpm for 5 minutes. The supernatant was taken in afresh 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 30µl of RNAse (20µg/ml) added water.

4.2.24. Multiple sequence alignment and phylogenetic analysis of TEM 1Binding aptamer:

CLUSTALW is a more recent version of CLUSTAL with the W standing for "weighting" to represent the ability of the program to provide weight to the sequence and program parameters, and CLUSTALX provides a graphics interface. These changes provide realistic alignment that should reflect the evolutionary changes in the aligned sequences and the more appropriate distribution of gap between conserved domains.

CLUSTAL performs a global-multiple sequence alignment by a different method than MSA, although the initial heuristic alignment obtained by MSA is calculated by the similar methods as in CLUSTAL. The step include: (1) Perform pair-wise alignment of all the sequences. (2) use the alignment score to produce a phylogenetic tree and (3) align the sequence sequentially, guided by the phylogenetic relationship indicated by the tree. Thus the most closely related sequences aligned first, and then additional sequences and groups of sequences added, guided by initial alignment to produce a MSA showing in the each column the sequence variation among sequences. The initial alignment used to produce the guide tree may be obtained by a fast *k*-tuple or pattern-pattern finding approach similar to the FASTA that is useful for many sequences or slower, full dynamic programming method may be used. An enhanced dynamic programming alignment algorithm is used to obtain optimal alignment scores. For producing a

phylogenetic tree, an assessment of genetic distances between the sequences is required. Genetic distance is the number of mismatched position in an alignment divided by the total number of matched positions.

CLUSTALW calculates the gap in a novel way designed to place them between the conserved domains. CLUSTALW uses the penalty for opening the gap in a sequence alignment and an additional penalty for extending the gap by one residue. These penalties are user defined (default are available). Gaps found in the initial alignment remain fixed. New gap introduced as more sequences are added also receive this same penalty, even when they occur within an existing gap, but the gap penalties for an alignment are then modified according to the average match value in the substitution matrix i.e., the percent identity between the sequence and the sequence length.

CLUSTALW also has option for adding one or more additional sequences with weights or an alignment to an existing alignment. Once an alignment has been made, a phylogenetic tree may be made by the neighbor-joining method, with corrections for possible multiple changes at each counted position in the alignment.

4.2.25. RNA Secondary structure prediction:

We have used the MFOLD program for secondary structure prediction of RNA aptamers designed by M.Zucker (Zucker 1989). MFOLD prediction is based on the non canonical base pair interaction and predicts several structures having energies close to the minimum free energy. These predictions accurately reflect structure of related RNA molecules derived from comparative sequence analysis. To find these suboptimal structures, the dynamic programming method was modified to evaluate parts of a new scoring matrix in which the sequences are represented in two tandem copies on both the vertical and horizontal axes.

4.2.26 Software and web server used for sequence analysis:

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

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

Gene runner: Primer sequence analysis

ExPaSy: Translation of nucleotide sequence into protein sequence

NCBI: For searching the sequence of DNA, precursor mRNA and protein of ricin A chain

Blastn & Blastp: Generating multiple hits for a sequence. Selection of Aptamer binding with TEM1 by SELEX (Systematic evolution of ligand by exponential enrichment):

Mfold: Secondary structure determination of various classes of TEM1 binding aptamers.

Results

5. RESULTS:

5.1. Genomic DNA isolation, Cloning and sequencing of Ricin A chain:

As Ricin A chain has no intron coding sequence. Ricin A chin genomic DNA was directly amplified from genomic DNA of *Ricinus communis*. First genomic DNA of Ricin was extracted with method already explained. 1µl of this genomic DNA sample was loaded to 0.8% agarose gel (Fig: 11), confirm extraction of genomic DNA. This total genomic sample was used for PCR amplification of Ricin A chain, Primers for this PCR was selected from flanking region sequence of Ricin A chain. Presence of expected size 836bp band was observed in 1% agarose gel (Fig: 12). Further this PCR product was purified by phenol chloroform method and ligated to pGEMT easy vector with help of T4 DNA ligase (PROMEGA). Ligated product were transformed in E.Coli (DH5a) cells and plated in LB agar plate containing ampicilin antibiotics .Before plating of transformed cells x-gal and IPTG was spreaded on plate for blue-white screening. After overnight incubation at 37°C colonies were appeared. 10 White colonies were picked out and inoculated in 5 ml Luria Broth (LB) media with antibiotics ampicilin and prepare the master plate. Plasmid DNA was isolated from 10 cultures and checked on 0.8% agarose gel. Screening PCR was carried out of all 10 recombinant plasmid and PCR product was checked on 1% agarose gel (Fig:13). Expected size of amplified Ricin A chain (836bp) was observed. Further confirmation by restriction digestion with Not1 was carried out. The two bands of expected size (3kb and 836bp) were observed (Fig: 14). Out of 10 clones, clone 7 and clone 4 was selected for sequencing and got the positive result. Multiple alignment of both the clones shows 100% match for both nucleotide and protein sequence (Fig: 15) and (Fig: 16).

5.2. Aptamer selection for tumor endothelial marker1:

Specific peptide sequence (QRCVALEASGEHRWLEGSCTLA) of 22 mer was selected from the conserved region of tumor endothelial marker1 (TEM1) protein for aptamer selection. biotinylated peptide was purchased from GENPRO in lyophilysed form. 88 mer of random library pool with 40 mer random sequence and 24 mer + 24 mer conserved sequence at both end of random sequence was selected and purchased from SIGMA. 100ng/µl of this library will contain approximately 10^{12} variant molecule. This library was PCR amplified with oligo 2nd (T7 + 21) and oligo 3rd (24 mer). PCR product was phenol chloroform purified and used for in vitro

transcription with help of T7 RNA polymerase (PROMEGA). After DNase treatment of the in vitro transcribed product RNA was phenol chloroform purified and used for SELEX after column purification. The scintillation counting result was observed (Fig: 23) for five cycle of SELEX. In 4^{th} and 5^{th} cycle counting of elution 1 was more than counting of wash 3 product indicate increased binding affinity of elute.

Eluted RT PCR product of RNA shows expected size band (105bp) in both 2nd well & 3rd well but no band in –ve control. (Fig: 17). Clones of different cycle were ligated in pGEMT easy vector followed by PCR confirmation. Fig 18 &19 shows successful cloning of different cycle's aptamer. Some of these were sequenced and multiple alignment of Clones of different cycles shows approximately 90% match with 88bp library sequence (Multiple alignment of clones of different classes with each other shows approximately 80-90% match with each other (Fig: 20), Phylogenetic tree in Fig:21 shows different clones of aptamers, evolved in four different classes.

Class I: C4.10, C3-9 and C5-8 Class II: C5-2, C3-6, C2-8 ClassIII: C5-6, C3-8, C2-2

ClassIV: C1-2&C1-5 ClassV: C4-2&C2-3

Secondary structure of different classes of aptamer and their free energy got with help of mfold web server, class vth aptamers C4-2 and C2-3 showing minimum free energy (Fig: 22) w.r.t other classes.

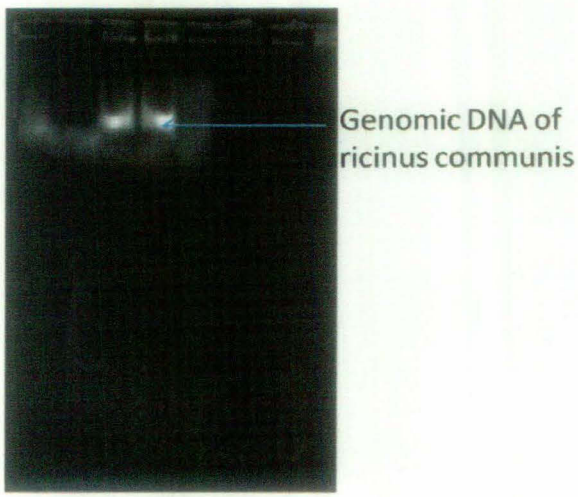


Fig11: Agarose gel electrophoresis of genomic DNA of ricin

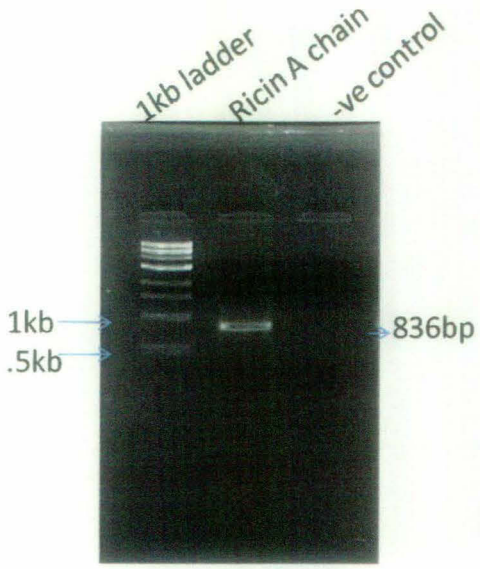


Fig12: PCR amplification of ricinA chain with help of gene specific primer, the band between 1kb and0.5kb in second well show approximately 836 bp gene of ricin A chain.

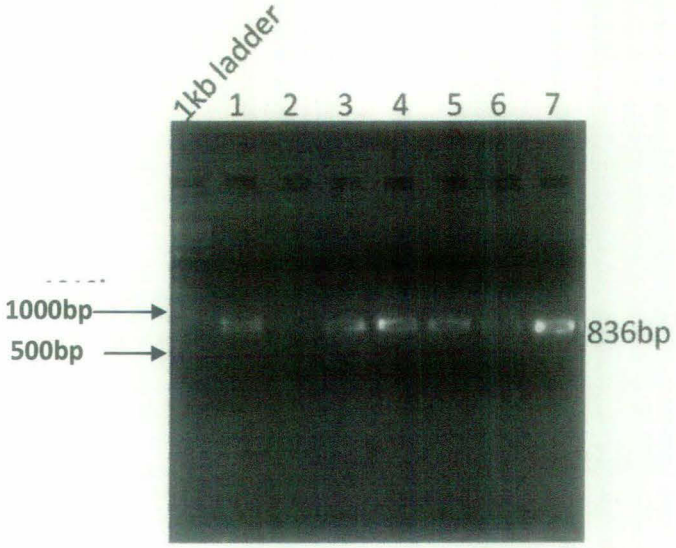


Fig13: PCR amplification result after miniprep isolation of transformed colonies, out of seven six transformed colonies have given positive result.

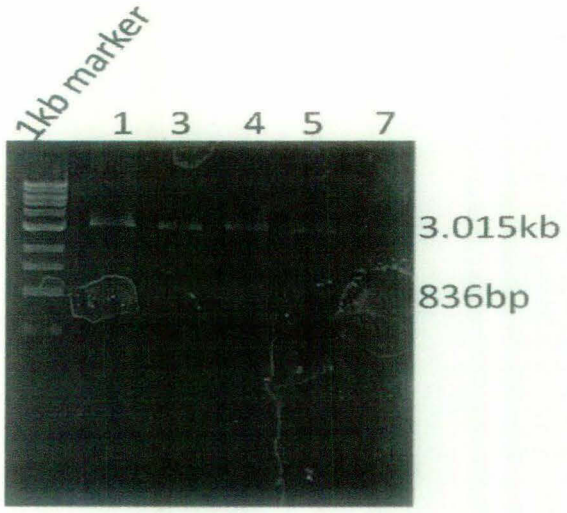


Fig14: Confirmation by restriction digestion with Not1,as both side of gene inside vector two restriction side of not 1 is present two bands appear, one of vector size(approximately 3.015kb) and other of gene of insert (approximately 836bp)

5.3. Result of sequencing of clone 4 and clone7 of Ricin A chain:

CLONE4:

AGGCGGCCGCGAATTCACTAGTGATTACCACTGGCCTTATAAGCAAAGAAAACTGTGACGACGG
TGGAGGTGCGCATCTATACACCATGAGAGCTATGATAGGGATTAATATACTCACATCGTACACA
TTGAATTTGGAACCGTTACGTCTTTGCAGTTGAATTGGACTAGCAAAGGCTCCTTGGTTAGACT
CTTGAATTGCAGTGGAAAGTCTCCCCCAACTATTCTCAAGTGTAATTACGCTAGGATCTGGTGC
AGATCTTCGGTTGTACCTAATTCTCGTGCGCATTTCTCCCTCAATGTACTGGAATCTTGCTGCT
TCTGAAATCATTTGGATGCAAACCATAAAGGAACGAGCCAGAGTTGGAATCTGAGTGCCACAAG
TACTATAATAATAAAGCGCTGAGATAGCGTCCTCTAATGGACCAGTTCCCAACTCAATATTTTC
TCTCAGACCTCCAAGTTGTTCAAGTCTATCATAATTACCACCAAAGGCGAATGTAAATGAATTT
TGAACATCCGTGAAAAGATGAGTGATTGCTTCTGCATCTTCTTGATTGTCAGGATGAAAGAAAT
AGGCGCTATTTCCAGCGCGGTAGCCGACCACATATGCATTGGTGACATCCAGTGCTAATGTAAC
AGAAAGCTCTGCATGATTTGAGAGTTCAACTAAAATAAACCGTTGGCTTATAGGCAAACCAACT
CTGTTTGGCAACACTGGTATTTCATGTCTCACATCACCTCCAGTTGTTAAATGACTGCGCACAG
CTCTGATAAAGTTT

CLONE7:

CAGGCGGCCGCGAATTCACTAGTGATTACCACTGGCCTTATAAGCAAAGAAAACTGTGACGACG
GTGGAGGTGCGCATCTATACACCATGAGAGCTATGATAGGGATTAATATACTCACATCGTACAC
ATTGAATTTGGAACCGTTACGTCTTTGCAGTTGAATTGGACTAGCAAAGGCTCCTTGGTTAGAC
TCTTGAATTGCAGTGGAAAGTCTCCCCCAACTACTCTCAAGTGTAATTACGCTAGGATCTGGTG
CAGATCTTCGGTTGTACCTAATTCTCGTGCGCATTTCTCCCTCAATGTACTGGAATCTTGCTGC
TTCTGAAATCATTTGGATGCAAACCATAAAGGAACGAGCCAGAGTTGGAATCTGAGTGCCACAA
GTACTATAATAATAAAGCGCTGAGATAGCGTCCTCTAATGGACCAGTTCCCAACTCAATATTTT
CTCTCAGACCTCCAAGTTGTTCAAGTCTATCATAATTACCACCAAAGGCGAATGTAAATGAATT
TTGAACATCCGTGAAAAGATGAGTGATTGCTTCTGCATCTTCTTGATTGTCAGGATGAAAGAAA
TAGGCGCTATTTCCAGCGCGGCAGCCGACCACATATGCATTGGTGACATCCAGTGCTAATGTAA
CAGAAAGCTCTGCATGATTTGAGAGTTCAACTAAAATAAACCGTTGGCTTATAGGCAAACCAAC
TCTGTTTGGCAACACTGGTATTTCATGTCTCACATCACCTCCAGTTGTTAAATGACTGCGCACA
GCTCTG

5.4. Results of multiple alignments of clone 4 and clone 7 of Ricin A chain: The nucleotide sequences of ricin A chain of clone 4 and clone7 were checked with help of Sequence massager. Blast ExPaSy & MULTALIN , more than 99% of the bases matched for both DNA and product of translation.

Fig 15: Here from the multiple alignment of clone4, clone7 and precursor mRNA sequence of ricin. 99.9% mach of both clones with precursor mRNA is present so we can take any clone for further work.

Figure 16: This is multiple alignment result of protein sequence of ricin agglutinin precursor with translated protein sequence of clone4 and clone7. Whole sequence getting matched except one amino acid difference represented blue in between red colored sequences.

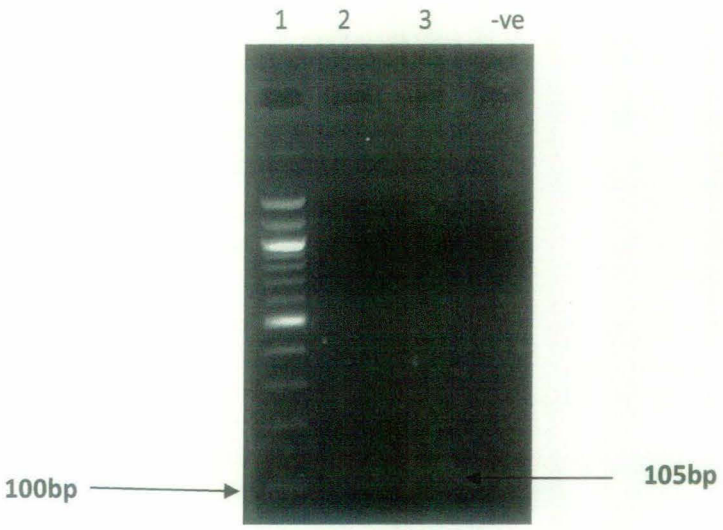


Fig 17: RT PCR product of cycle 1 and +ve control of 105 bp in 2nd and 3rd well while no band in 4th well confirms amplification of cDNA after SELEX.

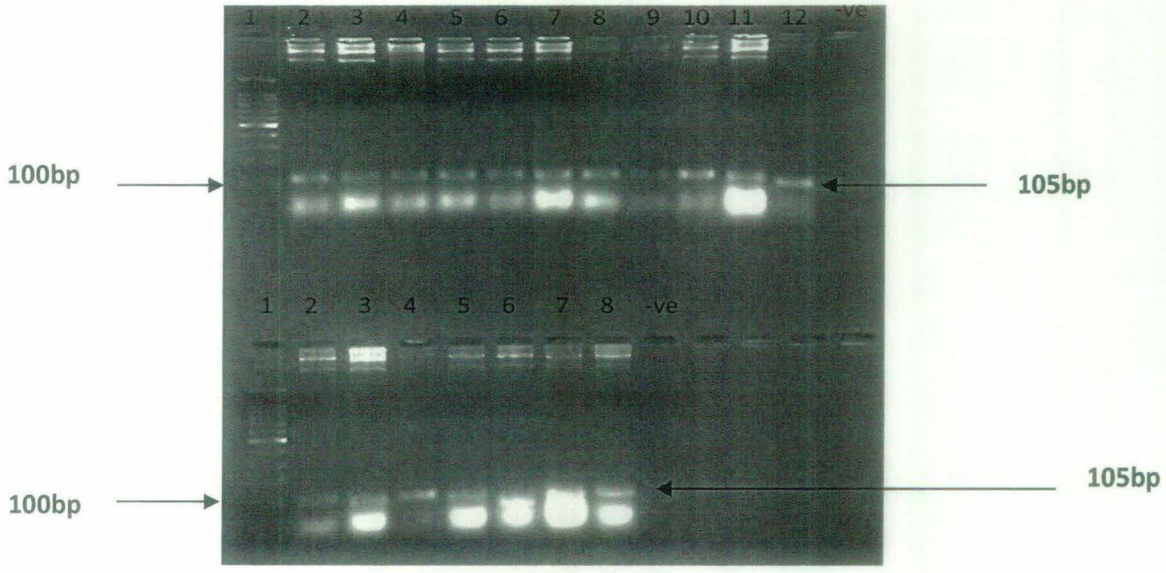


Fig 18: Agarose gel electrophoresis of inserts for cycle1 & cycle 2nd aptamers PCR-amplified from pGEMT easy vector

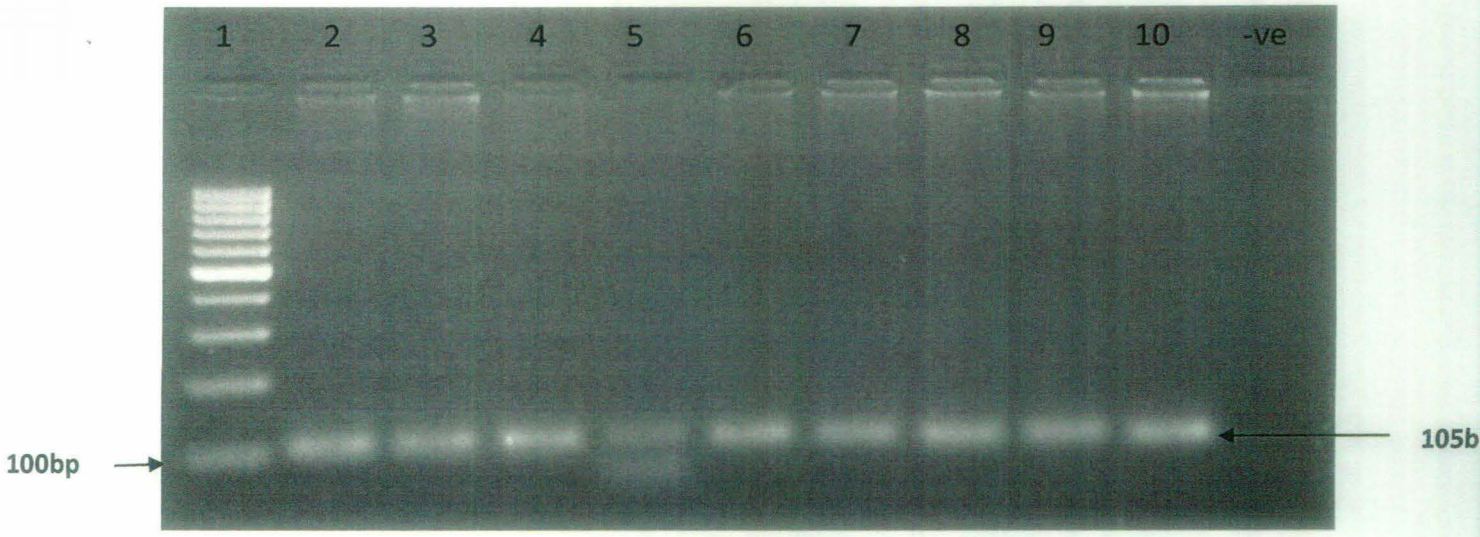


Fig 19: Agarose gel electrophoresis of inserts for 5th cycle aptamers PCR-amplified from pGEMT easy vector

5.5. multiple sequence alignment of different tem1 binding aptamers:

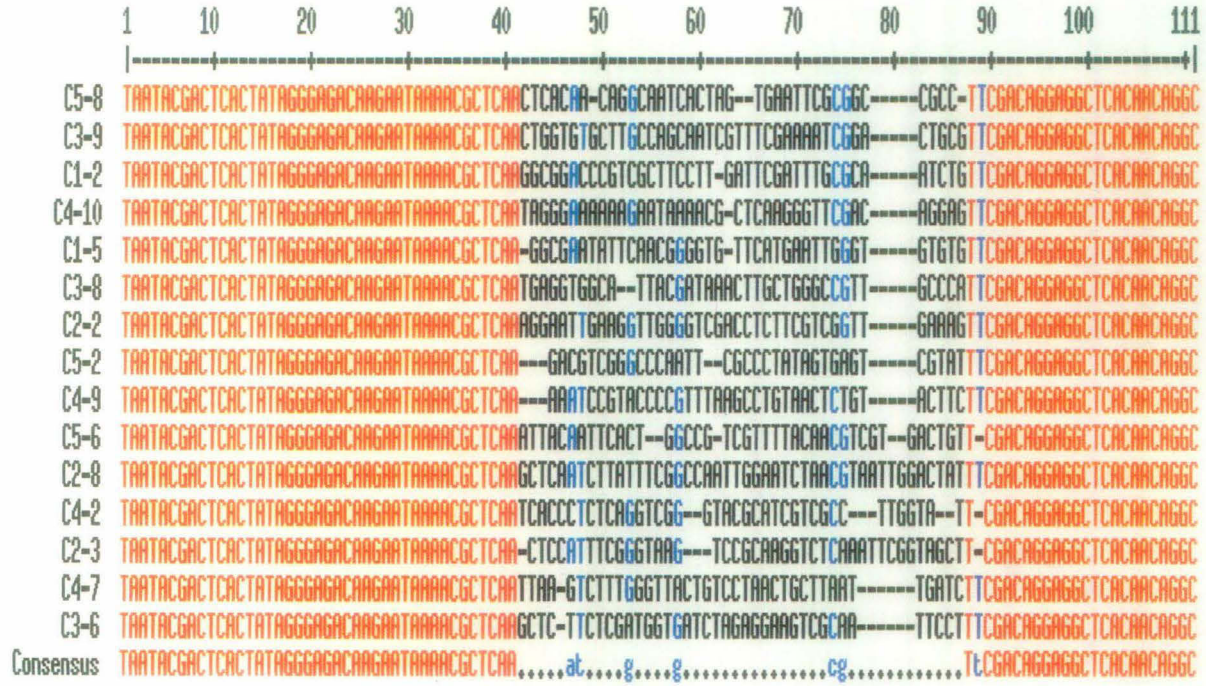


Fig 20: multiple sequence alignment of different TEM1 binding aptamers

5.6. Phylogenetic prediction of different classes of TEM1 binding aptamers: On the basis of phylogenetic tree analysis, aptamers from different cycles are distributed into five classes such that some of them have convergent evolution((C4-2 and C2-3)) while others have divergent evolution (C5-8 and C5-6).

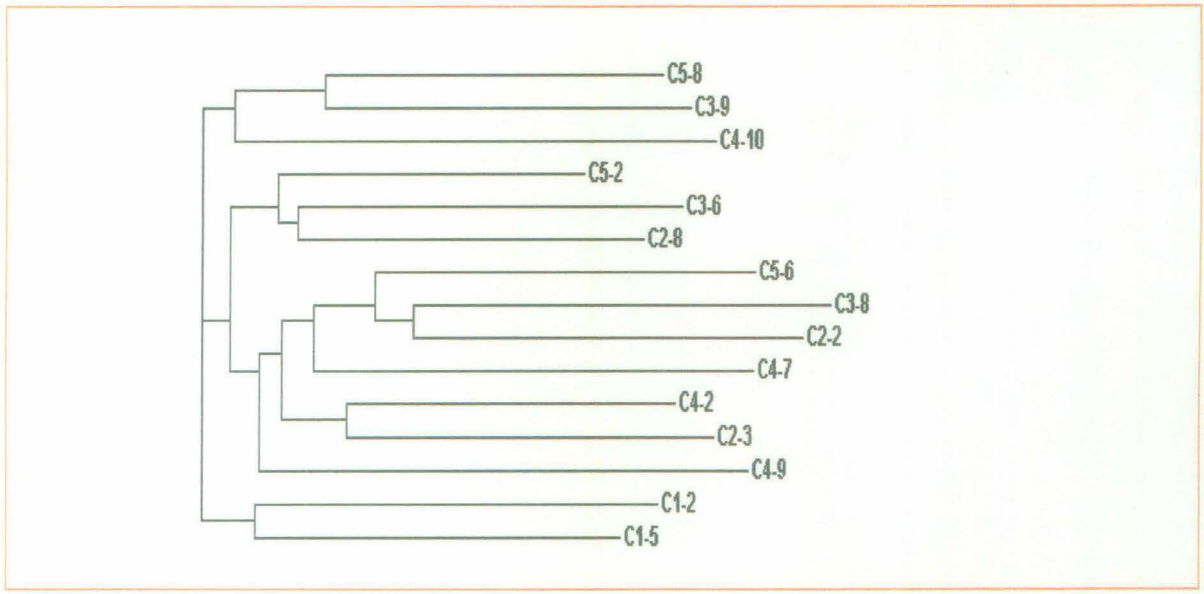


Fig 21: phylogenetic prediction of different classes of TEM1 binding aptamers (on the basis of this phylogenetic relationship clones of different cycles divided into five classes)

CLASS I

C4-10
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAATAGGGAAAAAAGAATAAAACGCTCAAGGG
TTCGACAGGAGTTCGACAGGAGGCTCACAACAGGC
C3-9
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAACTGGTGTGCTTGCCAGCAATCGTTTCGAA
AATCGGACTGCGTTCGACAGGAGGCTCACAACAGGC
C5-8

TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAACTCACAACAGGCAATCACTAGTGAATTCG
CGGCCGCCTTCGACAGGAGGCTCACAACAGGC

CLASS II

C5-2
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAAGACGTCGGGCCCAATTCGCCCTATAGTGA
GTCGTATTTCGACAGGAGGCTCACAACAGGC
C3-6
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAAGCTCTTCTCGATGGTGATCTAGAGGAAGT
CGCAATTCCTTTCGACAGGAGGCTCACAACAGGC
C2-8
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAAGCTCAATCTTATTTCGGCCAATTGGAATC
TAACGTAATTGGACTATTTCGACAGGAGGCTCACAACAGGC

CLASS III

C5-6
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAAATTACAATTCACTGGCCGTCGTTTTACAA
CGTCGTGACTGTTCGACAGGAGGCTCACAACAGGC
C3-8
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAATGAGGTGGCATTACGATAAACTTGCTGGG
CCGTTGCCCATTCGACAGGAGGCTCACAACAGGC
C2-2
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAAAGGAATTGAAGGTTGGGGTCGACCTCTTC
GTCGGTTGAAAGTTCGACAGGAGGCTCACAACAGGC

CLASS IV

C1-2
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAAGGCGGACCCGTCGCTTCCTTGATTCGATT
TGCGCAATCTGCAGGAGGCTCACAACAGGCTTCGA

C1-5
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAAGGCGAATATTCAACGGGGTGTTCATGAAT
TGGGTGTGTGTTCGACAGGAGGCTCACAACAGGC

CLASS V

C4-2
TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAATCACCCTCTCAGGTCGGGTACGCATCGTC
GCCTTGGTATTCGACAGGAGGCTCACAACAGGC
C2-3

TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAACTCCATTTCGGGTAAGTCCGCAAGGTCTC
AAATTCGGTAGCTTCGACAGGAGGCTCACAACAGGC

5.7. Secondary structures and free energy of different aptamers:

Class II:

Fig: C2-8, ΔG= -1.92

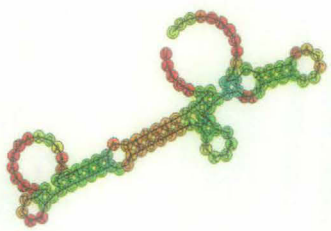


Fig: C3-6, ΔG= -19

Class IV:

Fig: C1-2, ΔG=-17.94

Fig: C1-5, ΔG= -19.70

Class V:

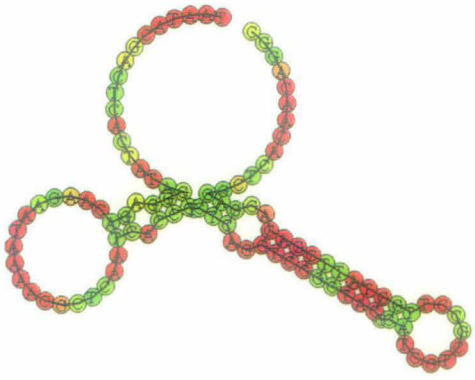


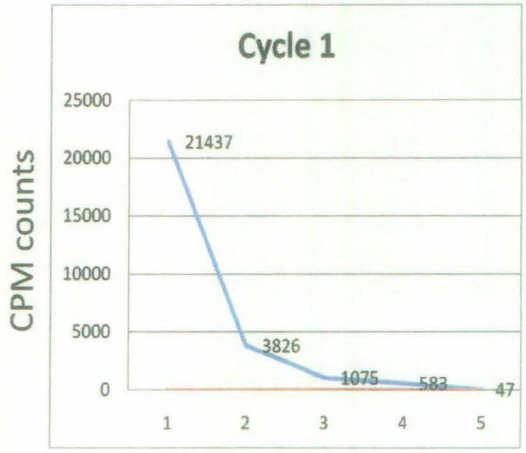
Fig: C4-2, ΔG= -20.80

Fig: C2-3, ΔG= -20.70

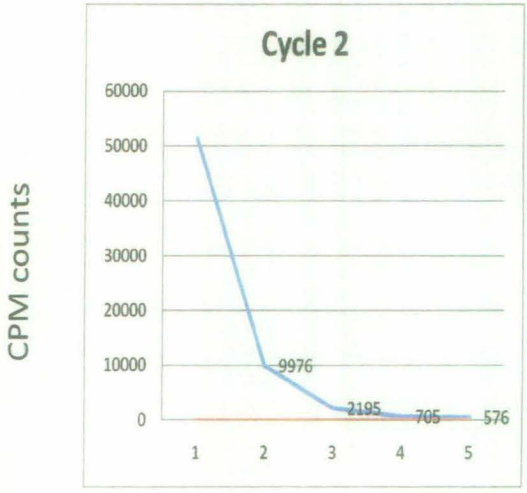
Fig 22: Secondary structures of different clones of aptamer and its free energy. Aptamers of class V have minimum free energy and hence maximum stability while class II aptamers have maximum free energy. The red region in the steam and loop region have maximum probability of pairing and these may be the conserve sequences of the aptamer.

5.8. Scintillation counting:

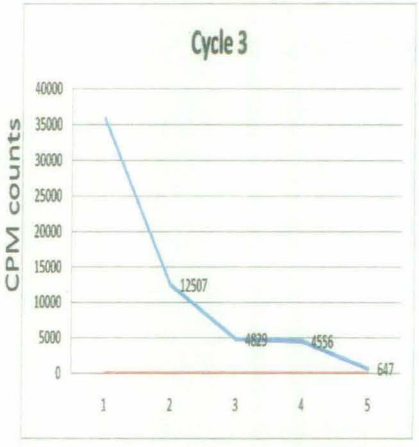
21437	1
3826	2
1075	3
583	4
47	5



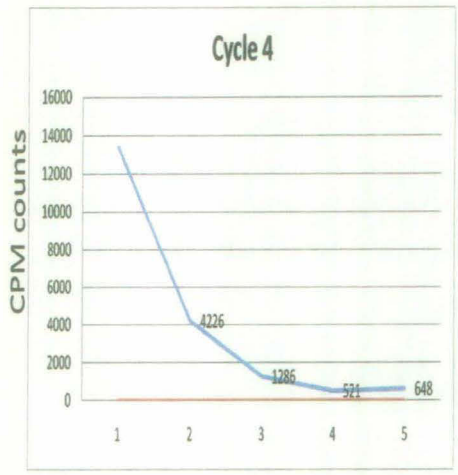
51350	1
9976	2
2195	3
705	4
576	5



35836	1
12507	2
4829	3
4556	4
647	5



13392	1
4226	2
1286	3
521	4
648	5



3246	1
937	2
346	3
629	4
356	5

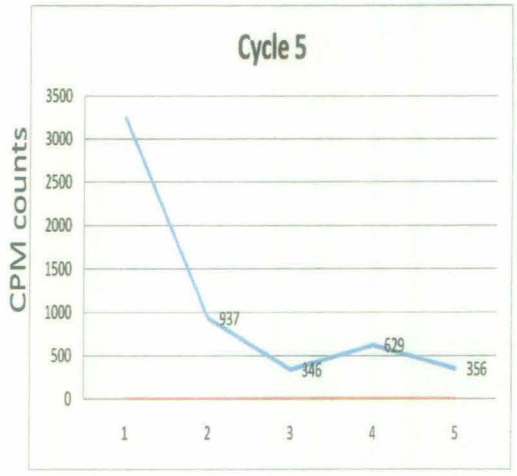


Fig-23: Scintillation counting of five cycle. 1, 2, 3, 4 & 5 used for counting value of wash1, wash2, wash3, eluate1&elutae2. Affinity of TEM1 binding aptamer increased in Cycle 4 and Cycle 5 as counting value of elute increased after wash3.

Discussion

6. DISCUSSION:

6.1. Endothelial Cell Markers:

Endothelial cells line the interior of all blood vessels, composing the endothelium. Targeting the endothelial cells that line tumor infiltrating blood vessels is a new anticancer strategy that has gained widespread support from biologists and clinicians. Here, we highlight approaches currently being used to target tumor endothelium and discuss new avenues for intervention that have been opened through the recent identification of tumor endothelial markers (TEMs). One of these is development of aptamers which is having high affinity for these markers and conjugating toxin coding sequence with them so that they can specifically kill the tumor cells without affecting the normal cells

6.2. Ricin toxin: Potential medicinal uses

Ricin, from castor-oil plant (*Ricinus communis*) is a highly toxic, naturally occurring protein. The LD_{50} of Ricin is around 22 micrograms per kilogram (1.76 mg for an average adult, around 1/228 of a standard aspirin tablet (0.4g gross)) in humans if exposure is from injection or inhalation. Oral exposure to Ricin is far A dose as small as a few grains of salt can kill an adult, less toxic and lethal dose can be up to 20–30 mg/kg.

Some researchers have speculated about using Ricin in the treatment of cancer, as a so-called "magic bullet" to destroy targeted cells (Lord MJ et al, 2003) because Ricin is a protein, it can be genetically linked to an aptamer to target malignant cells recognized by the specific aptamer for specific receptor on these malignant cells. The major problem with Ricin is that its native internalization sequences are distributed throughout the protein. If any of these native internalization sequences are present in a therapeutic, then the drug will be internalized by, and kill untargeted epithelial cells as well as targeted cancer cells.

With this hope that modifying Ricin will sufficiently lessen the likelihood that the Ricin component of these aptamer-Ricin conjugate will cause the wrong cells to internalize it, while still retaining its cell-killing activity when it is internalized by the targeted cells. Here aim of this work is to develop such chimera between aptamers specific to tumor endothelial marker (TEM1)

Our aim is to target any pathway of these hallmarks of cancer, with the help of selected marker. TEMI is claimed to be an exclusively tumor endothelial marker. It has been stated that Endosialin is not present in normal tissue and after doing Blast of both cDNA sequence and protein sequence of tumor endothelial marker1, there is no protein in human cells which has at least 50% match with this protein. So, 22 mer carboxyl termial peptide has been selected for selection of aptamer. This peptide has a =20% match with other peptide sequence in protein data bank. So selected aptamer for this sequence has specific binding affinity with this peptide and it will not bind to any other protein.

RNA aptamer has following characteristics which make it desirable for selection:

- easy to modify,
- relatively small among nucleic acids
- no immunogenicity, and
- moderate half life
- have the capability to fold into specific three dimensional structures.

RNA is much more susceptible for nuclease attack than DNA; it can be modified by modification of 2'OH group in to 2' fluoride or amino group.

In SELEX library of 88 mer has been prepared in which 40 mer random nucleotide has 5' and 3' 2 mer conserved sequence.

40 mer random sequence library has 4^{40} or 10^{24} different sequences and one expects in principle as many possible structures. However, with the limited mass of starting material (i.e. 100ng) ,it will include only about 3.5 picomoles or 2x 10^{12} molecules which may offer aptamers specifically binding the chosen peptide fragment of TEM1

Further by making conjugate of either toxin or toxin coding sequence with these highly specific aptamers to TEM1 will lead to targeting of this tumor marker containing cell. Targeted delivery of toxin in tumor cells will kill them selectively. It will add a new and safe therapy in the world of cancer.

6.3. Multiple sequence alignment of TEM1 binding aptamers:

Alignment of a pair of nucleic acid sequences can reveal whether or not there is an evolutionary relationship between the sequences, similarly the alignment of three or more sequences reveals relationship among them. (Bala J et.al, 2011) Multiple sequence alignment of a set of sequences selected for binding a certain ligands can provide information as to the most likely region in the set involved in the ligand binding. In nucleic acids, such alignment also reveals structural and functional relationship. The alignment provides a prediction as to which sequence character among independently arising clones corresponds. Each column in the alignment predicts the mutation that occurs at one site during the evolution of the sequence family. Within the columns are original character that were present early, as well as other derived character that appears later in evolutionary time. In some cases the position is so important for function that mutational changes become detrimental for selection and are not observed among selected sequences. Deletion and insertion may also be present in the same region of alignment. Thus starting with the alignment, one can hope to dissect the order of appearance of sequences during evolution.

. 6.4. Phylogenetic tree analysis of TEM1 binding RNA aptamers:

A phylogenetic analysis of a family of related nucleic acid sequences is determination of how the different sets of aptamers might have been derived during evolution. Convergent, divergent and parallel evolutionary relationship is expected to emerge. The evolutionary relationship among the sequences is depicted by placing the sequences as outer branches on a tree. The branching relationship on the inner part of the tree then reflects the degree to which the different sequences are interrelated. Two sequences that are very much alike will be located at a neighboring outside branches and will join a common branch beneath them. The object of phylogenetic analysis is to discover all of the branching relationship in the tree and branch length.

Phylogenetic relationship of the aptamers showed some sequences originated from the same root and hence exhibit greater closeness to each other.

6.5. Secondary structure prediction of TEM1 binding aptamer:

Nucleic acid sequences that specify RNA molecules have to be compared differently. Variation in RNA sequences maintains the base pairing patterns that give rise to double stranded region (secondary structure) in the molecule. Thus alignments of two sequences that specify the same RNA molecule will show covariance at interacting base pair positions.

A computational method for predicting the most likely region of base pairing in RNA molecule has been designed, just given the sequences, thus providing the predicted secondary structure. From the many possible choices of complementary sequences that can potentially base pair, the compatible sets that provide most energetically stable structure were chosen. structures with energies almost as low the most stable one may also be produced, and region whose prediction are the most reliable can be putatively identified from such an analysis. Sequence variation found in related sequences may also be used to predict which base pairs are likely to be found in related sequences of each of the molecules. One variation of RNA secondary structure prediction methods which will predict a set of sequences that are able to form a particular structure from the given sequence are also being developed.

The computer program MFOLD was used to predict the secondary structure of the aptamers belonging to the different classes. Secondary structure of all classes showed some stem region which are very important for the stability and conservation of RNA. All classes of RNA aptamers showed at least 1 stem regions.

6.6. FUTURE PROSPECTS:

The production of biomaterials with the capacity to bind tightly and specifically to cell surface receptors of malignant cells can greatly benefit cancer diagnosis and treatment. Whereas antibodies have the ability to specifically recognize some tumor cell makers, their large size and immunogenicity markedly limit their value. The development of nuclease-resistant oligonucleotide agents, such as RNA/DNA aptamers, offers an alternative to antibodies as targeting, diagnostic, and delivery agents. Targeting exclusive marker on tumor cells make ease of drug administration in form of toxin as the probability of internalization of toxin increases many fold inside tumor with respect of normal cells. Using the systematic evolution of ligands by exponential enrichment (SELEX) methodology or other variations, one can select specific sequences that have appropriate binding affinities and specificities against clinically relevant markers from large libraries of oligonucleotide ligands. Aptamers have been found to bind their targets with high specificity and with dissociation constants in the sub-nanomolar or picomolar range. Conjugating toxin with these highly specific aptamer like TEM1 binding aptamer promises a good delivery agent of toxin inside tumor cells. The unparalleled combinatorial chemical diversity, small size, and modification ability of aptamers is expected to meet the criteria for robust, generic drug discovery technology and open new horizons for the development of cancer therapy in future.

Summary and conclusion

Summary:

One approach to target TEM1 containing tumor cells is through RNA aptamers. Aptamers can fold into a variety of three dimensional structures with specific structural, ligand binding and catalytic properties. We isolated and characterize the TEM1 binding aptamers by SELEX. Aptamers duplex was designed based on the random pool sequences. Selection of TEM1 RNA aptamers was done by SELEX methods. C DNA for selected RNA aptamers were PCR amplified and cloned in vector pGEMT. All positive clones were confirmed by PCR amplification. Finally all positive clones were sequenced. On the basis of sequences, we did the evolutionary studies using different software's (CLUSTAL W, Bio Edit and MFOLD) and noticed independent or polyphyletic patterns of selection of TEM1-binding aptamers.

Conclusion:

- Cloning of Ricin A chain.
- Selection of conservative sequence of TEM1.
- Generation of Random sequence library.
- In vitro Transcription.
- Secondary structure prediction.
- Evolutionary studies were done by using multiple sequence alignment and phylogenetic prediction softwares CLUSTALW, and Bio Edit.
- Secondary Structure Predictions of aptamer sequence were done by MFOLD program.

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