IN VITRO SELECTION OF DNA APTAMERS TO GLIOBLASTOMA MULTIFORME

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ABSTRACT

IN VITRO SELECTION OF DNA APTAMERS TO GLIOBLASTOMA MULTIFORME

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Aptamer probes for specific recognition of glioblastoma multiforme were generated using a repetitive and broad cell-SELEX-based procedure without negative selection. The 454 sequencing technology was used to monitor SELEX, and bioinformatics tools were used to identify aptamers from high throughput data. A group of aptamers were generated that can bind to target cells specifically with dissociation constants (K_d) in the nanomolar range. Selected aptamers showed high affinity to different types of glioblastoma cell lines, while showing little or no affinity to other cancer cell lines. The aptamers generated in this study have potential use in different applications, such as probes for diagnosis and devices for targeted drug delivery, as well as tools for molecular marker discovery for glioblastomas.

Keywords: DNA Aptamer, Cell-SELEX, Glioblastoma, Cancer

ÖZ

GLİOBLASTOMA MULTİFORME' YE KARŞI DNA APTAMERLERİNİN İN VİTRO SEÇİLİMİ

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Glioblastoma multiforme'yi özel olarak tanıyan aptamer probları negatif seçilim yapılmadan tekrarlı ve geniş Hücre SELEX'i temelli bir prosedürünün kullanılmasıyla oluşturulmuştur. 454 Sekanslama teknolojisi SELEX yöntemini izlemek için biyoenformatik araçlar ise yüksek kapasiteli verlerden yaralanarak aptamerleri tanımlamak için kullanılmıştır. Özellikle hedef hücreye bağlanabilen ve nanomolar düzeyinde ayrılma sabitine (K_d) sabit bir grup aptamer üretilmiştir. Seçilen aptamerler glioblastoma hücre hattına ait farklı türlere yüksek bağlanma eğilimi gösterirken, diğer kanser hücre hatlarına az ya da hiç bir bağlanma göstermemektedir. Bu çalışmada üretilen aptamerler glioblastoma hücreleri için moleküler işaretleyici keşfi için araç olmanın yanı sıra teşhis için kullanılan prob ve hedeflenmiş ilaç dağıtımı gibi farklı uygulama alanlarında kullanılma potensiyeline sahiptirler.

Anahtar Kelimeler: DNA Aptamer, Hücre-SELEX, Glioblastoma, Kanser

To ceo,

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

ABBREVIATIONS

DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMT	Dimethoxytrityl
DOX	Doxorubicin
dsDNA	Double Stranded Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma multiforme
HBSS	Hank's balanced salt solution
HPLC	High Pressure Liquid Chromatography
K_d	Dissociation constant
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PET	Positro Emission Tomography
RT-PCR	Reverse transcription polymerase chain reaction

SDS	Sodium dodecyl sulfate
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SPECT	Single-photon Emission Computed Tomography
ssDNA	Single Stranded Deoxyribonucleic Acid

CHAPTER 1

INTRODUCTION

1.1. Aptamers

Aptamers are DNA or RNA molecules, which are capable of binding different classes of targets with high affinity and specificity,(Larry Gold et al. 2002),(A D Ellington and Conrad 1995). They bind to the target with high selectivity and specificity because of their specific three-dimensional structure (R Stoltenburg et al. 2005). Binding capacity is the result of oligonucleotides' three-dimensional conformation not nucleotide base-base complementarity (Sampson 2003). This three-dimensional shape is a result of stems, loops, bulges, hairpins, pseudoknots, triplexes, or quadruplexes formed by single-stranded nucleic acids. Aptamers can well-fittingly bind to a variety of targets from single molecules to whole organisms. This well-fitting binding is a result of; structure compatibility, stacking of aromatic rings, electrostatic and van der Waals interactions, and hydrogen bonds, or from a combination of these effects (Figure 1.1) (Hermann 2000).

Aptamers have been defined in Encyclopedia of Analytical Chemistry as; "…artificial nucleic acid ligands that can be generated against amino acids, drugs, proteins and other molecules. They are isolated from complex libraries of synthetic nucleic acid by an iterative process of adsorption, recovery and re-amplification."

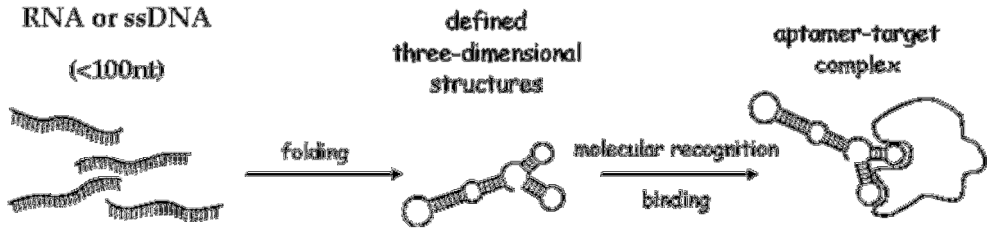


Figure 1.1 Molecular recognition of targets by aptamers with defined three dimensional structures (Regina Stoltenburg et al. 2007)**.**

1.2. Generation of Aptamers

There are two main approaches to design ligands that can bind with high affinity and high specificity; rational design and combinatorial chemistry. Rational design relies on chemical wisdom, intuition and to some degree luck to identify molecules capable of interacting with the target (Michael T Bowser 2005). On the other side, in combinatorial chemistry, enormous numbers of molecules are produced to form libraries and these molecules are tested to find potential candidates to be ligand. Aptamers are produced and selected with the use of combinatorial chemistry. In 1990 a different combinatorial technique named in vitro selection (A D Ellington and J W Szostak 1990), in vitro evolution (Joyce 1989) or SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Tuerk and L Gold 1990) is introduced for production of aptamers.

1.2.1. SELEX

Since its first description in 1990, the SELEX technology is widely applied to many targets to evolve nucleic acid ligands, called aptamers.

1.2.1.1. General Principles

First aptamer discovery via SELEX is achieved by two different groups contemporaneously in 1990. Tuerk and Gold used a combinatorial nucleic acid library to select RNA oligonucleotides (aptamers) that bind very tightly and selectively to non-nucleic acid target. They firstly studied the interaction between bacteriophage T4 DNA polymerase (gp43) and the ribosome binding site of the mRNA. They selected gp43 binding sequences from an RNA pool randomized at specific positions and named this selection procedure as SELEX (Tuerk and L Gold 1990). As well Ellington and Szostak independently used a similar strategy to find RNA molecules from a random sequence RNA library that can fold in to specific 3-D structure so have specific binding sites for small ligands such as organic dyes e.g. Cibacron Blue (A D Ellington and J W Szostak 1990). After two years the first DNA aptamer is selected from chemically synthesized random pool (A D Ellington and J W Szostak 1992). Since this time SELEX technology became an important and widely used tool in molecular biological, pharmaceutical, and medical research.

Basic steps of a SELEX are presented in figure 1.2. SELEX starts with a chemically synthesized random DNA library consisting of 10^{13} to 10^{15} different sequence motifs (W James 2000). If the aim is to get DNA aptamers, this library can be used directly without any treatment, whereas in order to get RNA aptamers one should convert DNA library into RNA library. After incubation of this library with the target the binding complexes are partitioned from unbound and weakly bound oligonucleotides. Target bound oligonucleotides are eluted and amplified by PCR for DNA aptamers and RT-PCR for RNA aptamers. For DNA aptamers newly formed dsDNA is converted into ssDNA. This new enriched pool is used for the next cycle of SELEX round. By consecutive and successive cycles of selection and amplification the initial random pool is reduced to relatively few sequences that have affinity to target. Generally SELEX is modified and some extra steps are

included to be able to get more usable or specific aptamers, the most common extra steps are negative selection or subtraction. By these additional steps aptamers that can specifically bind to a molecule and do not bind to other one can be selected in a very distinctive manner. Also target concentration, stringency of selection can be modified through the course of the SELEX to be able to get better aptamers.

After getting enough enrichment for binding of selected pools to target SELEX process is finished. Generally the last PCR products are cloned in to vectors and transformed in to bacteria to get specific aptamer clones. These individual clones are sequenced and sequences are analyzed to find representative aptamer sequences. But also with the progression of new sequencing technologies cloning steps can be omitted and pools can be directly sequenced by using specific primers for pyrosequencing (AT Bayrac et al. 2011). Finally selected aptamers can be subjected to some post-SELEX modifications for different reasons e.g. immobilization, enhancing the stability, reporting ability.

In general it can be seen that SELEX can be summarized into five common, universal steps; binding, partition, elution, amplification, and conditioning. There is no standardized SELEX method for any target. The SELEX design is highly depend on target molecule, starting library, selection, amplification, and conditioning. The following subchapters will give more detailed information about targets, libraries and the particular process steps.

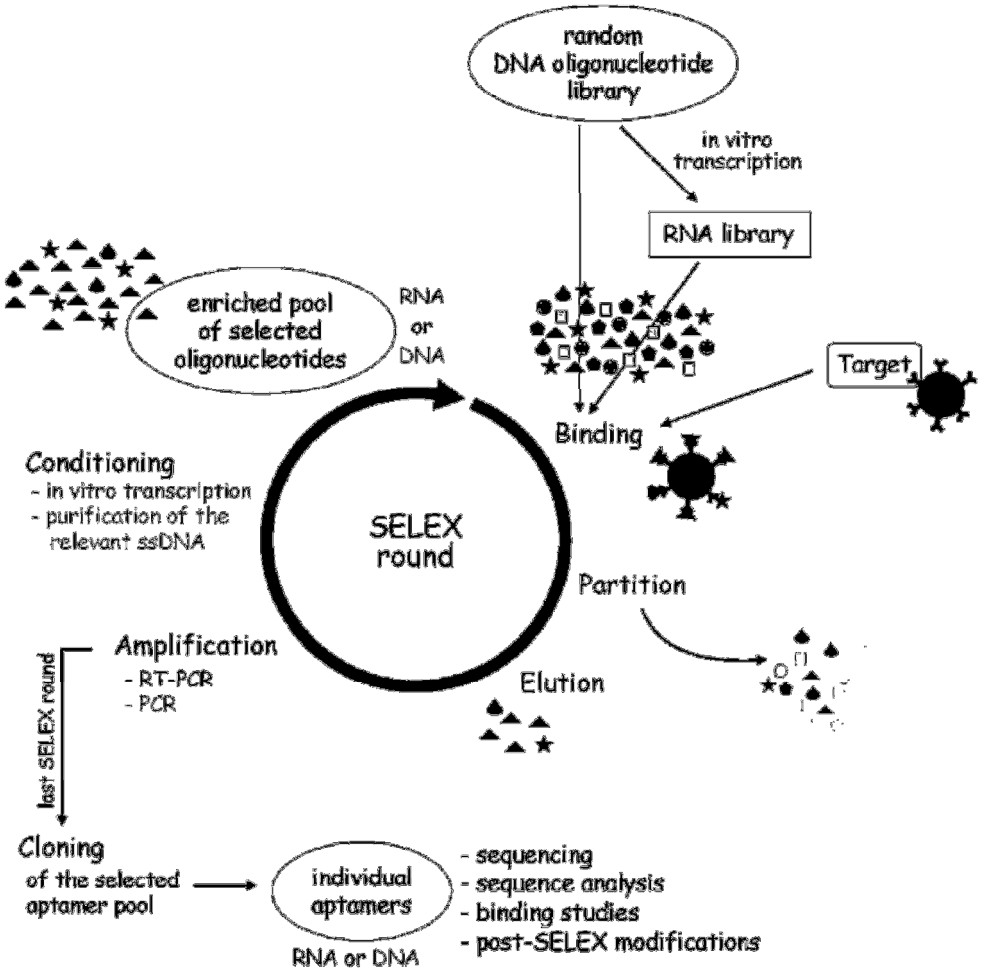


Figure 1.2 Schematic representation of SELEX.(Regina Stoltenburg et al. 2007)

1.2.1.2. Target Molecules

Since 1990, the SELEX has been applied to many different classes of targets. Inorganic molecules, small organic molecules, peptides, proteins, carbohydrates, antibiotics, and complex targets as whole cells, bacteria, and viruses used as a target in aptamers selection. Main examples of targets used in SELEX since 1990 can be found in table 1.1.

Table 1.1 Examples of targets used for aptamer selection.

Target for aptamer selection	Type	KD	References
Inorganic components			
Zn^{2+}	RNA	1.2 mmol/L	(Ciesiolka et al. 1995)
Ni^{2+}	RNA	0.8-29πmol/L	(Hofmann et al. 1997)
UO_2^{2+}	DNA	84.6pmol/L	(J Kim et al. 2011)
Small organic molecules			
Ethanolamine	DNA	6-19nmol/L	(D Mann et al. 2005)
Theophylline	RNA	100nmol/L	(Jenison et al. 1994)
Malachite Green	RNA	1μmol/L	(Grate and C Wilson 2001)
Organic Dyes	RNA	100-600μmol/L	(A D Ellington and J W Szostak 1990)
	DNA	33-46μmol/L	(A D Ellington and J W Szostak 1992)
Sulforhodamine B	DNA	190nmol/L	(C Wilson and J W Szostak 1998)
Hematoporphyrin	DNA	1.6μmol/L	(Okazawa et al. 2000)
Ricin toxin	DNA	58-105nmol/L	(J Tang et al. 2006)
Cholic acid	DNA	5-67.5μmol/L	(Kato et al. 2000)
4,4′-Methylenedianiline	RNA	0.45-15μmol/L	(Brockstedt et al. 2004)

Table 1.1 (continued)

Dopamine	RNA	2.8μmol/L	(Mannironi et al. 1997)
Cocaine	DNA	n.s.[a]	(Stojanovic et al. 2000)
Bisphenol A	DNA	8.3-208nmol/L	(M Jo et al. 2011)
Acetamiprid	DNA	4.98μmol/L	(He et al. 2011)
2-anilinophenyl acetic acid	DNA	42.7-166.3nmol/L	(Joeng et al. 2009)
Nucleotides and derivatives			
Adenine	RNA	10μmol/L	(Meli et al. 2002)
ATP	RNA	0.7-50μmol/L	(Sassanfar and J W Szostak 1993)
Adenosine/ATP	DNA	6μmol/L	(Huizenga and J W Szostak 1995)
ATP	RNA	4.8-11μmol/L	(Sazani et al. 2004)
Xanthine	RNA	3.3μmol/L	(Kiga et al. 1998)
cAMP	RNA	10μmol/L	(Koizumi and R R Breaker 2000)
8-OHd6	DNA	0.1μmol/L	(Miyachi et al. 2009)
Cofactors			
Coenzyme A	RNA	n.s.[a]	(Saran et al. 2003)
Cyanocobalamin	RNA	88nmol/L	(Lorsch & J W Szostak 1994)

Table 1.1 (continued)

Riboflavin	RNA	1-5µmol/L	(Lauhon and J W Szostak 1995)
NF-KB	RNA	11-149nmol/L	(Wurster and Maher 2008)
FMN	RNA	0.5µmol/L	(Petra Burgstaller and Michael Famulok 1994)
FAD	RNA	137-273µmol/L	(Petra Burgstaller and Michael Famulok 1994)
NAD	RNA	n.s.[a]	(Petra Burgstaller and Michael Famulok 1994)
	RNA	2.5µmol/L	(Lauhon and J W Szostak 1995)
S-adenosyl methionine	RNA	n.s.[a]	(D H Burke and L Gold 1997)
S-adenosyl homocysteine	RNA	0.1µmol/L	(Gebhardt et al. 2000)
Biotin	RNA	5µmol	(C Wilson and J W Szostak 1995)
Nucleic acids			
TAR RNA element of HIV-1	DNA	50nmol/L	(Boiziau et al. 1999)
	RNA	20-50nmol/L	(Ducongé & Toulmé 1999)
	DNA	50nmol/L	(Sekkai et al. 2002)

Table 1.1 (continued)

Yeast phenylalanine RNA	RNA	12-26nmol/L	(Scarabino et al. 1999)
E.coli 5S RNA	RNA	6-12μmol/L	(J-hyeong Ko et al. 1999)
	RNA	3μmol/L	(J Ko et al. 2001)
HIV-1 LTR DNA duplex	DNA	0.1-2μmol/L	(Srisawat and Engelke 2010)
Amino acids			
L-Arginine	RNA	330nmol/L	(Geiger et al. 1996)
	DNA	~2.5mmol/L	(K Harada and Frankel 1995)
L-Citrulline	RNA	62-68μmol/L	(M Famulok 1994)
L-Valine	RNA	12mmol/L	(I Majerfeld and M Yarus 1994)
L-Isoleucine	RNA	1-7mmol/L	(Lozupone et al. 2003)
	RNA	0.2-0.5mmol/L	(I Majerfeld and M Yarus 1998)
D-Tryptophan	RNA	18μmol/L	(M Famulok & J W. Szostak 1992)
L-Tyrosinamide	DNA	45μmol/L	(Vianini et al. 2001)
L-Histidine	RNA	8-54μmol/L	(I Majerfeld et al. 2005)
Carbohydrates			
Cellobiose	DNA	600nmol/L	(Q Yang et al. 1998)

Table 1.1 (continued)

Sialy Lewis X	RNA	0.08-10nmol/L	(S Jeong et al. 2001)
Chitin	DNA	n.s.[a]	(Fukusaki et al. 2000)
Sialyllactose	DNA	4.9μmol/L	(Masud et al. 2004)
Sephadex	DNA	n.s.[a]	(Srisawat et al. 2001)
(1-3)-β-D-glucon	DNA	303nmol/L	(Low, Hill, and Peccia 2009a)
Cellulose	RNA	n.s.[a]	(B. J. Boese et al. 2008)
	DNA	n.s.[a]	(B J Boese & R R Breaker 2007)
Antibiotics			
Kanamycin A	RNA	≤300nmol/L	(Lato et al. 1995)
Kanamycin B	RNA	180nmol/L	(Kwon et al. 2001)
Streptomycin	RNA	n.s.[a]	(Wallace and Schroeder 1998)
Neomycin	RNA	~100nmol/L	(Wallis et al. 1995)
Tobramycin	RNA	2-3nmol/L	(Y Wang and Rando 1995)
Lividomycin	RNA	≤300nmol/L	(Lato and A D Ellington 1996)
Moneomycin A	RNA	300-400nmol/L	(Schürer et al. 2001)
Tetracycline	RNA	1μmol/L	(Berens et al. 2001)

Table 1.1 (continued)

Chloramphenicol	RNA	25-65μmol/L	(D H Burke et al. 1997)
Tetracycline	DNA	63-483nmol/L	(Niazi, SJ Lee, and Gu 2008)
Oxytetracycline	DNA	9.6-56.8nmol/L	(Niazi, SJ Lee, YS Kim, et al. 2008)
Peptides and Proteins			
T4 DNA polymerase	RNA	5-30nmol/L	(Tuerk and L Gold 1990)
α-thrombin	DNA	200nmol/L	(Bock et al. 1992)
	RNA	<1-4nmol/L	(R White et al. 2001)
Bovine thrombin	RNA	164-240nmol/L	(Xuemei Liu et al. 2003)
Neurotensin receptor NTS-1	RNA	0.37nmol/L	(Daniels et al. 2002)
Immunoglobulin E	DNA	23-39nmol/L	(Mendonsa & Bowser 2004)
	RNA	30-35nmol/L	(Wiegand et al. 1996)
	DNA	10nmol/L	(Wiegand et al. 1996)
Interferon-γ	RNA	2.7nmol/L	(Kubik et al. 1997)
MCP-1(mouse)	RNA	180-370pmol/L	(Rhodes et al. 2001)
PDGF	DNA	0.1nmol/L	(L S Green et al. 1995)
VEGF	RNA	0.1-2nmol/L	(Jellinek et al. 1994)
	RNA	0.05-0.1nmol/L	(Ruckman et al. 1998)

Table 1.1 (continued)

HIV-1 integrase	RNA	10-800nmol/L	(Allen et al. 1995)
HIV-1 RT	RNA	~5nmol/L	(Tuerk et al. 1992)
	DNA	~1nmol/L	(DJ Schneider et al. 1995)
	DNA	180-500pmol/L	(Mosing et al. 2005)
HIV-1 nucleocapsid protein	RNA	0.84-1.4nmol/L	(SJ Kim et al. 2002)
TTF1	DNA	3.3-67nmol/L	(Murphy et al. 2003)
HGF	DNA	19-25nmol/L	(Saito and Tomida 2005)
Streptavidin	RNA	7nmol/L	(Tahiri-Alaoui et al. 2002)
	RNA	70-200nmol/L	(Srisawat et al. 2001)
	DNA	57-85nmol/L	(R Stoltenburg et al. 2005)
L-Selectin	DNA	1,8-5.5nmol/L	(B J Hicke et al. 1996)
Taq DNA polymerase	DNA	0.04-9nmol/L	(Dang and Jayasena 1996)
Prion protein (PrP^{C})	RNA	0.1-1.7nmol/L	(Daniela Proske et al. 2002)
PrP^{Sc} fibrils	RNA	23.4nmol/L	(Rhie et al. 2003)
$rPrP^{c}$	RNA	n.s.[a]	(Weiss et al. 1997)
	DNA	n.s.[a]	(K Takemura et al. 2006)
C5 protein	RNA	2-5nmol/L	(Biesecker et al. 1999)
Hepatis C virus RdRp	DNA	1.3-23.5nmol/L	(LA Jones et al. 2006)

Table 1.1 (continued)

Heaptis C virus NS3	RNA	n.s.[a]	(PK Kumar et al. 1997)
Hepatis Cvirus NS3 helicase	DNA	140nmol/L	(Zhan et al. 2005)
ppERK2/ERK2	RNA	4.7-50nmol/L	(Seiwert et al. 2000)
Protein kinase C delta	DNA	122nmol/L	(Mallikaratchy et al. 2006)
RNase H1	DNA	10-80nmol/L	(Pileur et al. 2003)
Colicin E3	RNA	2-14nmol/L	(Hirao et al. 2004)
Oncostatin M	RNA	7nmol/L	(Rhodes et al. 2000)
Tumor marker MUC1	DNA	0.1-34nmol/L	(Ferreira et al. 2006)
GnRH	RNA	190nmol/L	(Leva et al. 2002)
	DNA	45nmol/L	(Leva et al. 2002)
Vasopressin	DNA	1.2μmol/L	(KP Williams et al. 1997)
Amyloid peptide βA4(1-40)	RNA	29-48nmol/L	(Ylera et al. 2002)
Microcystin	DNA	1mmol/L	(C Nakamura et al. 2006)
DNA-dealkylating enzymes	DNA	20-240nmol/L	(Krylova et al. 2011)
Interleukin 17A	RNA	72.2nmol/L	(Adachi et al. 2011)
HCV Minus IRE5 Domain	RNA	32nmol/L	(Konno et al. 2011)
EpCAM	RNA	55nmol/L	(Shigdar et al. 2011)
Mycobacterium tuberculosis polyphosphate kinase 2	DNA	870nmol/L	(Shum et al. 2011)
Prostate specific antigen	DNA	40-100nmol/L	(Savory et al. 2010)

Table 1.1 (continued)

Plasminogen activator	RNA	n.s.[a]	(Dupont et al. 2010)
Hyalurinic acid binding domain	DNA	180-295nmol/L	(Somasunderam et al. 2010)
Leismenia infantum H3	DNA	n.s.[a]	(Ramos et al. 2010)
Mutant KRAS protein	RNA	4.04nmol/L	(Sujin Jeong et al. 2010)
Egg white lysozyme	DNA	2.8-52.9nmol/L	(DT Tran et al. 2010)
Hepatitis B virus surface antigen	RNA	n.s.[a]	(Jia Liu et al. 2010)
Plasmodium falciparum erythrocyte membrane protein	RNA	33nmol/L	(Barfod et al. 2009)
Asp f 1	DNA	0.2-400nmol/L	(Low, Hill, and Peccia 2009b)
Amyloid β-peptide	RNA	10.9-21.6μmol/L	(Takahashi et al. 2009)
Yeast sup35 protein	DNA	0.1-1μmol/L	(Surina et al. 2009)
Phospholamban	DNA	20nmol/L	(Y Tanaka et al. 2009)
Insulin	DNA	n.s.[a]	(Yoshida et al. 2009)
MUC-1	DNA	n.s.[a]	(Ferreira et al. 2008)
Quino protein glucose dehydrogenase	DNA	n.s.[a]	(Kazunori Ikebukuro et al. 2007)
Dendritic cell SIGN protein	DNA	21.73nmol/L	(Hui et al. 2007)
GluR2 glutamate receptor channel	RNA	0.419nmol/L	(Z Huang et al. 2007)

Table 1.2 (continued)

Complex Structures			
Anthrax spores	DNA	n.s.[a]	(Bruno and Kiel 1999)
Ribosomes/ribosomal protein S1	RNA	4-5nmol/L	(Ringquist et al. 1995)
Rous sarcoma virus (RSV)	RNA	n.s.[a]	(Pan et al. 1995)
Differentiated PC12 cells	DNA	n.s.[a]	(C Wang et al. 2003)
YPEN-1 endothelial cells	DNA	n.s.[a]	(M Blank, T Weinschenk, et al. 2001)
A-172 gliblastoma cells	RNA	61-158nmol/L	(AT Bayrac et al. 2011)
U251 glioblastoma cells	DNA	150nmol/L	(Daniels et al. 2003)
	RNA	5nmol/L	(B J Hicke et al. 2001)
	DNA	33-700nmol/L	(Castronovo et al. 2008)
Live African trypanosomes	RNA	60nmol/L	(Homann and Göringer 1999)
Jurkat T cell leukemia	RNA	n.s.[a]	(Lee Young and S-W Lee 2006)
Receptors of Trypanosoma cruzi	RNA	40-400nmol/L	(Ulrich and Wrenger 2009)
CCRF-CEM leukemia cells	DNA	0.8-229nmol/L	(Shangguan et al. 2006)
M types of Streptococcus pyrogenes	DNA	4-86nmol/L	(Hamula et al. 2011)
Small Cell Lung Cancer	DNA	n.s.[a]	(Kunii et al. 2011)

Table 1.3 (continued)

Vibrio alginolyticus	DNA	n.s.[a]	(Zheng et al. 2010)
Compylobacter jejuni	DNA	292.8nmol/L	(Dwivedi et al. 2010)
Lactobacillus acidophilus	DNA	13nmol/L	(Hamula et al. 2008)
Liver cancer cell (MEAR)	DNA	4.51-157nmol/L	(Shangguan et al. 2008)
Colorectal Cancer Cells	DNA	0.7-302nmol/L	(Sefah, Meng, et al. 2010)
Ovarian Cancer Cells	DNA	0.25-132nmol/L	(Van Simaeys et al. 2010)
Vaccinia virus infected cells	DNA	1.4-122.6nmol/L	(Parekh et al. 2010)

[a] *n.s., not specified*

Aptamers can be developed for targets that naturally have interaction with nucleic acids, nucleotides, cofactors, and nucleic acid binding proteins like polymerases. Beside these molecules there are many aptamer targets that naturally do not have any interaction with nucleic acids.

1.2.1.3. Random DNA Library

One of the most important factors of SELEX is the starting library. All SELEX experiments start with chemically synthesized random DNA oligonucleotide library. This library is synthesized by a standard DNA synthesizer by just placing a central random region of 20 to 80 nucleotides flanked by 18 to 21 nucleotides of

appointed sequence which function as primer binding site in the PCR. Since the library is synthesized as a single stranded DNA, this library can be used directly in DNA SELEX process. Some researchers carry out a large scale amplification of this DNA library before initiating the aptamer selection in order to eliminate damaged DNA synthesis products, which can't be amplified by PCR (Marshall and A D Ellington 2000).

For selection of RNA aptamers synthesized random DNA library has to be transcribed into a RNA library before starting the first round of an RNA SELEX process. Differing form DNA library, in RNA libraries T7 promoter sequence must be added to sense primer. Firstly ssDNA is used as a template and converted into a dsDNA by PCR. Than the dsDNA library is transcribed into RNA library by T7 RNA polymerase. Now RNA library can be used as a starting library in RNA SELEX. For each round of SELEX selected RNA pools must be reverse transcribed and amplified by RT-PCR using the same primers and the new RNA pool for the next round is then generated again by in vitro transcription as mentioned above.

Although primer binding sites seems to take role in only PCR steps, they are present in all sequences and effect 3-D shape of all. For this purpose design of primer binding sites are also very important. Primers must have reasonable annealing temperatures, must have ≤50% GC. Also primer selfdimers, and heterodimers take into account and must be avoided. The design of primers and library is very significant, as the efficiency of PCR amplification is the key to a successful selection so avoid a library with strong interaction between the constant regions (Sefah, Shangguan, et al. 2010).

Size of the randomized region is an also important step in design of library. Oligonucleotide libraries 20 to 80 nucleotides are generally used in SELEX procedures. The size of random region increases the number of DNA types in the

pool so increase the possibility of finding good binders. Some researchers have found that functionality of some aptamer variants are only 20 to 30 mer. For example, sgc8 aptamer that has 88 nucleotide and has an K_d of 0.8 nM has successfully optimized to 41 nucleotide with a K_d of 0.78 nM (Shangguan et al. 2007). This suggests that short randomized regions are enough for a successful SELEX. However, libraries with longer randomized region have more structural complexity and better for complex targets and targets that are not naturally bind to nucleic acids.

1.2.1.4. Selection

Selection step of the SELEX is incubating the target molecules with random library, partitioning of unbound sequences, and elution of bound sequences to be used in next steps. Since random library has many types of sequences it has sequences with different affinity and specificity. A true selection strategy has to ensure the maintenance of high specificity and affinity sequences to the next round while getting out of no and low affinity sequences.

As mentioned the most crucial part of selection is partitioning. The power of SELEX is directly correlated with the design of partitioning and the rounds of SELEX to get efficient aptamers diminish with the better designs. Generally most of the SELEX procedures include immobilization of target to have better partitioning. In this case after incubation of random library with target, immobilized target can easily be washed and nonbinding sequences can be eliminated easily. First researchers generally used affinity chromatography with immobilization of target on column material like agarose or sepharose (Jiajian Liu and Stormo 2005) (Tombelli et al. 2005). Also micro magnetic beads are commonly used because of their high surface to size ratio and easy handling with small amount of target molecule (R Stoltenburg et al. 2005) (Kikuchi et al. 2003) (Lupold et al. 2002) (Murphy et al. 2003). Also there

are separation methods without target immobilization. Mostly used one is ultrafiltration by nitrocellulose filters (Bianchini et al. 2001) (D Schneider et al. 1993). Although it is very easy to use filtration it has many drawbacks such as unspecific interaction between oligonucleotides and membrane. Unwanted interaction of these sequences can easily result in non-specific enrichment of some sequences throughout the SELEX. Beside filtration many groups developed different techniques with separation during the SELEX, e.g. capillary electrophoresis (S D Mendonsa and M T Bowser 2004) (Mosing et al. 2005) (J Tang et al. 2006), flow cytometry (Davis et al. 1996) (X Yang et al. 2003), electrophoretic mobility shift assay (Tsai and Reed 1998), surface plasmon resonance (Misono and PKR Kumar 2005), or centrifugation (Rhie et al. 2003) (Homann and Göringer 1999).

For progress of the SELEX throughout the selection binding and/or nonbinding sequences have to be quantified. Mostly the radioactive nucleotides are used for quantifying the bound sequences since it is very sensitive and able to detect very small amounts of nucleic acids (Beinoraviciūte-Kellner et al. 2005)(A D Ellington and J W Szostak 1990)(Hua Shi et al. 2002). Since it is not environmentally friendly, needs an isotope laboratory, and have health risks for employee nowadays fluorescence labeling is used for quantification (R Stoltenburg et al. 2005) (Davis et al. 1996) (Rhie et al. 2003).

After removing nonbinding sequences binding sequences are generally eluted by simple denaturing techniques such as heat treatment or special chemicals like urea, SDS or EDTA. Also some researchers used affinity elution and competitive binders if available.

1.2.1.5. Amplification

In each SELEX round, the selected sequence number is directly correlated with the number of targets and the population of sequence in the pool. To be able to enrich the binding sequence through course of the SELEX, PCR is used. Since the starting library is very complex each sequence is rare in the pool but using PCR to amplify the eluted binders exponentially increases the amount of these rare binding sequences. Beside the enrichment PCR also helps for other purposes, for example by using modified nucleic acids or attachment to primers one can easily add additional groups to sequences for detection, immobilization, etc. The most commonly used modifications are biotin and fluorescence attached primers. Biotin is commonly used to immobilize dsDNA sequences on to streptavidin beads to be able to elute ssDNA for next rounds. Fluorescence attachments are also used for measuring the progress of SELEX by quantifying the binders.

1.2.1.6. Cloning and Characterization

The last part of the SELEX is cloning and characterization of the selected sequences. Depending on the efficiency of the SELEX and the specificities of the target, generally after 6 to 20 selection rounds, enough enrichment can be attained and selection is stopped. Generally the pool of the last round is cloned to get aptamer to be able find best affinity sequences. Generally 50 or more aptamer clones are analyzed by sequencing. Sequence alignment programs are used for finding most common and similar sequences. Generally the most common sequences are the best affinity sequences but one must consider the contaminant or parasite sequences that survive throughout the SELEX and check for the affinity and specificity. Secondary structure analysis also gives important information about structures or domains for binding. Generally mfold (Zuker 2003) is used for determination of secondary structures. Nowadays as in our study cloning sequence can be removed from the SELEX. Pyrosequencing technologies like 454, Illumina GAIIx, and SOLID

are very suitable for SELEX. Using these technologies any pool can easily be sequenced without cloning and in high numbers. For using these sequencing technologies the pool that will be sequenced can easily be amplified using primers with 454 flanks and they are ready for sequencing. In 454 with one chip one can read up to 10000 sequences and it is only around 1000$. This many sequences also guarantee to find all selected sequences and give statistical data for selection. Even with using enough chips and sequencing different pools evolution of sequences in the course of SELEX can be revealed.

After determining aptamer candidates by sequencing next step is to find the best aptamers with the best affinity and specificity. For this first important test is binding. The affinity constant (K_d) is a good parameter for aptamers to state their binding efficiency. A small K_d value means a high affinity to target. There are picomolar to micromolar K_d aptamers in the literature. Other than binding, specificity is an also important factor. To find that researchers generally look for the binding affinities of aptamers to similar molecules to the target.

1.2.2. Cell-SELEX

So far SELEX is explained in detail with different applications. As mentioned, SELEX can be carried out for small molecules to whole cells. Cell-SELEX is a modified SELEX in which target is whole living cells. Although it can be used for all types of cells, cell-SELEX is generally used with the selection of aptamers for cancer cells.

Several kinds of aptamers have been selected against cancer related proteins, such as PDGF, VEGF, HER3, NFkB, and PMSA. By using cell-SELEX an aptamer can be selected without prior knowledge. Since cell-SELEX will select specific aptamers that can bind to that cell, it will indirectly select the aptamers for specific proteins on the surface of the cell. Cell-SELEX can be used for many purposes; the most

common aim to use cell-SELEX is biomarker discovery. Since researcher doesn't need any prior knowledge about target in cell-SELEX it is one of the best strategies for development of biomarkers. Aptamers developed by cell-SELEX can easily differentiate cancer cells from normal ones and also they can differentiate between different cell lines. So that developed aptamers can be very specific biomarkers for that type of cancer. Since their specificity is very high, aptamers developed by cell-SELEX can easily be used in cancer cell detection also they can be used in cancer cell sorting and enrichment. Another important application of aptamers developed by cell-SELEX is targeted drug delivery. Again high specificity gives advantage of delivering the drug specifically to target.

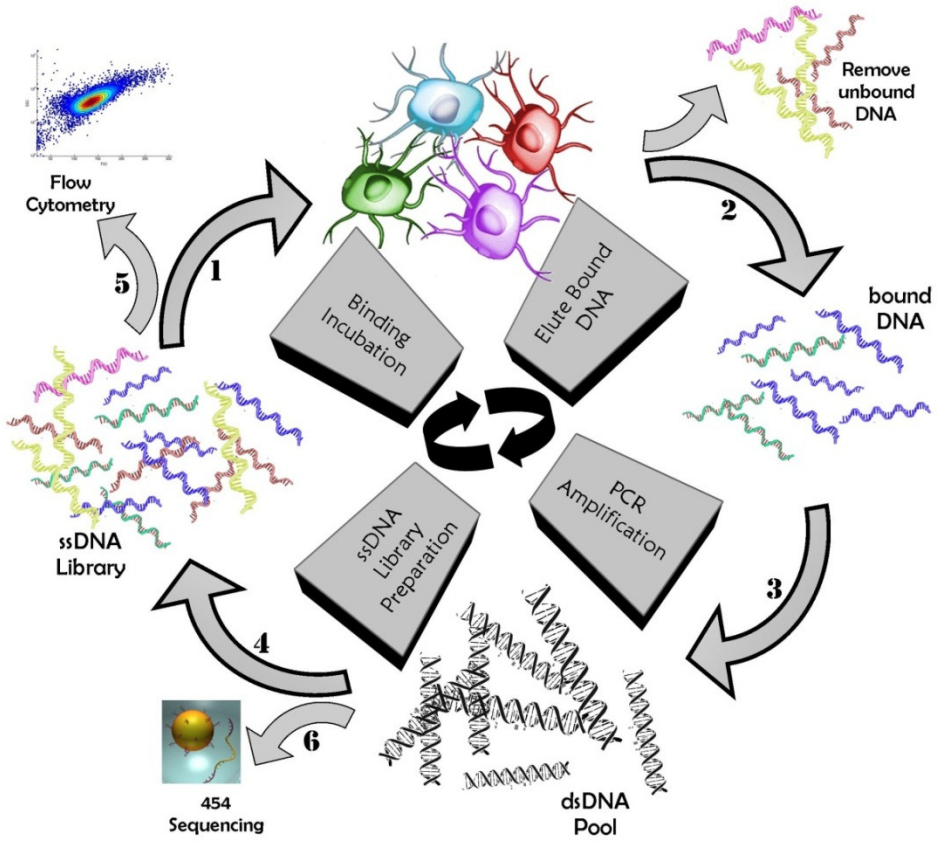


Figure 1.3 Schematic representation of cell-SELEX.

1.3. Application of Aptamers

1.3.1. Aptamers as Detection Reagents

If aptamers are considered as binding molecules, they are inevitably rival antibodies, which are still the ubiquitous reagents in the field of diagnostics (Daniela Proske et al. 2005). Monoclonal antibodies (mAbs) are widespread in use since their discovery and they made a tremendous contribution to a wide range of applications. Aptamers also started to demonstrate their potential where they substituted for mAbs.

Both antibodies and aptamers can bind the target with high affinity. However, aptamers have advantages over antibodies that make them very promising.

The most important advantage of aptamers over antibodies is their production; aptamers are isolated by in vitro methods, independent from animals. Antibodies are produced in biological systems and are results of immune response to the target analyte. This restricts the target variety; target molecules that have similar structures to endogenous proteins cannot be produced by animal antibody production techniques, also toxic targets have the difficulty of production because of the damage given to the animal. Another parameter that restricts the usage of antibodies is their production in physiological conditions thus limiting the functionalization conditions (Luzi et al. 2003). Aptamers supersede the antibodies by these parameters because aptamers can be produced for each target whether it is toxic or endogenous. Also selection conditions (pH, buffer, salts, etc.) of aptamers can be changed according to the application. This gives the advantage of production of aptamers that can work in any environmental conditions.

Aptamers are produced by chemical synthesis and purified to a very high degree and this eliminates the batch-to-batch variation which is a common problem in antibodies. Aptamers can also be modified easily in accordance with the requirement; fluorescent chemicals, stabilizers, spacers, biotin, amino modifiers, thiol modifiers, etc.

Another advantage of aptamers can be seen in their higher temperature stability over antibodies. Most of the antibodies undergo irreversible denaturation. By contrast, aptamers are very stable and can recover their native active conformation after thermal denaturation.

Aptamers are now used for detection in many areas. Somalogic Inc. (Boulder, USA) reported the development of an aptamer chip that nearly asses for 50 different analytes in patient samples. Aptamers can reach to very low detection limits and can be performed in solution by simple one-tube reactions without the need for washes and separations. Fredrikson (Fredriksson et al. 2002) demonstrated the detection of zeptomolar (10^{-21}) concentrations of protein, which corresponds to a 1000 fold increase in sensitivity compared to a conventional ELISA (Baldrich et al. 2004). Also nanoparticle-aptamer bioconjugates increased the sensitivity of detection. With the usage of quantum dots multi-analyte electrochemical biosensors are produced with subpicomolar (attomole) detection limits (JA Hansen et al. 2006).

Since the advent of aptamers, many examples show that aptamers can substitute for antibodies in diagnostic applications. For instance they have proven to be appropriate tools in ELISA like assays (Baldrich et al. 2004) as well as in flow cytometry (Michael Blank, Toni Weinschenk, et al. 2001) Also aptamers have shown to be used in microscopy studies as histological markers in place of mAbs (Michael Blank, Toni Weinschenk, et al. 2001), (Chu et al. 2006), (Farokhzad et al. 2004).

1.3.2. Use of Aptamers in Affinity Chromatography

Aptamers are synthesized chemically and can be easily modified at their 3′ or 5′ end with biotin, primary amino or other functional groups. This property of aptamers makes them suitable for immobilization on carrier material for affinity chromatography.

First example of aptamers in affinity chromatography is a fluorescently labeled DNA aptamer produced for human immunoglobin E (Ig E) used for competitive assay (Luzi et al. 2003), (German et al. 1998). Another example is the purification of recombinant L-selectin receptor globulin (LS-Rg) from cell medium using human L-selectin specific DNA aptamer. This purification system is resulted in 1500 fold

enrichment of LS-Rg with 83% recovery (Romig et al. 1999). Also thyroid transcription factor (TTF1) is purified from soluble fraction of bacterial lysate by streptavidin magnetic beads coated with DNA aptamer.

1.3.3. Aptamers for Target Validation

Verifying that a DNA, RNA, or protein is involved in a disease process and is a suitable target for therapeutic compound development is called target validation. There are many competing technologies addressing the process of target validation such as gene knockout, antisense oligonucleotides, ribozymes, and RNA interference. All of these technologies are genetic inactivation methods at genomic or transcriptional level though they give limited information on functional relevance of a protein in disease development. Up to now protein level target validation was only available through antibodies and phage display libraries but their long development times, poor specificity were big problem. But development of aptamers opens a new window for this area; intracellular targets are more accessible with low molecular weight aptamers which can be labeled also by fluorescents or nanoparticles such as quantum dots. There are many examples of aptamers for intra and extracellular targets that are highly specific such as aptamers against $\alpha v\beta 3$-integrin (Mi et al. 2005), PDGF (Floege et al. 1999), tenascin-C (B J Hicke et al. 2001), human epidermal growth factor receptor-3 (C-HB Chen et al. 2003), and IFN-γ-inducible CXCL10 (Marro et al. 2005). There are even aptamers that can even discriminate between phosphorylated and nonphosphorylated conformations of targets such as aptamer produced for Erk-2 MAP kinase (Seiwert et al. 2000). This means that posttranslational conformations of targets or inhibitory mechanisms can be studied by aptamers in high order protein complexes or networks without altering the proteomic status of model (Daniela Proske et al. 2005), (Gavin et al. 2002). Also there are examples of functional analysis of

intracellular target validation including HIV proteins (Konopka et al. 2000), (NICKENS et al. 2003), splicing factors (H Shi et al. 1999) etc.

1.3.4. Aptamers as Therapeutics

Numerous aptamers have been selected against therapeutic targets, such as IgE, IFN-g, alpha thrombin, PTPase and they showed great prosperity in tissue culture experiments and animal models (JF Lee et al. 2006). Most successful aptamer used for therapeutics to date is anti-vascular endothelial growth factor (anti-VEGF) aptamer (Cunningham et al. 2005), (Ng and Adamis 2005), (Siddiqui and Keating 2005). VEGF promotes the development of abnormal new blood vessels in the eyes, causing vision loss. The aptamer is used by directly applying to the vitreous cavity and by binding to VEGF it inhibits binding of VEGF to its receptor. Clinical trials showed that more than 80 % of the patients showed stable or improved vision three months after treatment (Siddiqui & Keating, 2005). The market name of this aptamer is Macugen® (by Pfizer and Eyetech) and it is fully approved by the FDA. Another candidate aptamer to be used is the anticoagulant aptamers. Heparin has long lasting and its pharmacokinetics are difficult to control but Gilead (Bock et al. 1992) promotes transient anticoagulant activity during coronary artery bypass graft surgery and has passed the Phase I clinical trials. Also a RNA aptamer developed for coagulation factor IXa has a great deal on replacing heparin. This aptamer is containing 2'fluoropyrimidines and conjugated to a cholesterol moiety which extends its half-life in blood. Additionally 17 base pair 2'-O-methyl RNA oligonucleotides antidote is developed. Antidote binds to aptamer, disrupts its structure and renders it inactive allowing more control over and better timing of the reversal of anticoagulant activity (CP Rusconi et al. 2002), (Michael Famulok 2004), (CP Rusconi et al. 2004).

1.4. *Glioblastoma Multiforme*

1.4.1. *General Information*

1.4.1.1. *Incidence*

A glioma is a type of cancer that occurs in brain or spine. It is called glioma because it arises from glial cells. Glioma is categorized in to 4 grades by World Health Organization (WHO) based on prognostic and survival correlates. Glioblastoma multiforme (GBM) is the most malignant, WHO grade IV glioma. Lower WHO grades of gliomas are called secondary GBM since they can progress into GBM. GBM is the most malignant brain tumor in humans. Although it comprises just 1.35% of all primary malignant cancers in the US(Yohan Lee et al. 2008), by accounting >51% of all gliomas, GBM is the most common primary central nervous system tumor in the US and European countries(Adamson et al. 2009).

1.4.1.2. *Survival*

The median survival of patients after diagnosis is 17-30% at 1 year and only 3-5% at 2 years with no difference between man and women(Adamson et al. 2009), (Ohgaki et al. 2004), (A a Brandes et al. 2008). Survival rates are obviously higher for younger patients. Five year survival rates decreases from 13% to less than 1% from the youngest group (15-45 years) to the oldest group (≥75 years)(A a Brandes et al. 2008).

1.4.1.3. *Aetiology*

Aetiology is the study of cause of diseases. Known risk factors for glioblastoma are therapeutic ionizing radiation, working in synthetic rubber manufacturing or petroleum, and exposure to vinyl chloride or pesticides (Wrensch et al. 2001). Prior therapeutic irradiation especially for leukemia as children has higher risk of

glioblastoma. Also passive smoking is associated with increased risk of 1.2 (Wrensch et al. 2001). Filter cigarettes, residential electromagnetic fields, formaldehyde and cell phone use are not proven factors. A recently published meta-analysis is showing consistent pattern of an increased risk for the use of cell phones for ≥10 years (Hardell et al. 2007).

1.4.2. Pathology and Biology

1.4.2.1. Definition

GBM has histopathological indications like poorly differentiated neoplastic astrocytes, cellular and nuclear atypia, extra mitotic activity, reduced apoptosis, vascular thrombosis, microvascular hyperproliferation and necrosis. Vascular hyperproliferation and necrosis is essential diagnostic features that differs GBM from lower grades gliomas (Adamson et al. 2009)(A a Brandes et al. 2008).

1.4.2.2. Genetics

Although GBM has common pathological, histological and clinical indications it is clearly genetically heterogeneous tumor. Current knowledge for molecular characteristics of GBM demonstrates that there are unlikely to be single genetic or cellular events that can be effectively targeted for all patients. There are many common mutations or deletions throughout the patients. Yet in a recent study that is sequencing the genome of U87MG glioblastoma cancer cell line it is shown that there are more single-nucleotide variations, insertions/deletions and translocations than expected. In the study Clark *et al.* found 2,348,470 single nucleotide variations, 191,347 small indels, 1,315 structural variations, and 35 interchromosomal translocation events(Clark et al. 2010).

The gene for phosphatase and tension homolog (PTEN) located on chromosome ten (10q23) is commonly mutated or deleted in glioblastoma. Recent studies indicate

that 80% of the human glioblastoma has a deletion or mutation in PTEN (R Zhang et al. 2008), and a homozygous mutation in PTEN has been robustly identified in the genomic sequence of U87MG (Clark et al. 2010). Absence of PTEN in glioblastoma promotes angiogenesis and prevents apoptosis, even after treatment with powerful chemotherapeutic drugs. Due to its high occurrence rate, high VEGF expression and poor response to chemotherapeutic drugs, the PTEN-negative glioblastoma cell line A172 was chosen as a model target for aptamer selection in this study.

1.4.3. Diagnosis

Most common clinical presentations of GBM are progressive neurological deficit, motor weakness, headache, and seizure. Since first symptoms are common for many basic health problems diagnosis is done generally several months after the appearance of initial symptoms. Generally MRI (Magnetic Resonance Imaging) or CT (Computed Tomography) is carried out for precise diagnosis after epileptic seizures or advanced neurological disorders. Beside MRI and CT; positron emission tomography (PET), single-photon emission computed tomography (SPECT), and magnetic resonance angiography (MRA) is used for diagnosis.

1.4.4. Treatment

The current standard of care for GBM is maximal safe surgical resection, followed by a combination of radiation and chemotherapy with temozolomide (Clarke et al. 2010).

1.4.4.1. Surgery

Surgery is the first and main therapeutic treatment for GBM. The ultimate and optimal aim of surgery is removing the complete tumor. However complete removal is virtually impossible and reoccurrence is inextricable. In this

circumstances surgery is not curative but it helps in bulk reduction, decreasing the cranial hypertension causing improve in the quality of life, and possibly prolong survival. Type of surgery is also important for survival. In a study it is discovered that 18-month survival is 15% for patients who underwent biopsy alone, 25% with partial resection, and 34% for total resection (CH Chang et al. 1983). In another study median survival is 6.6 months for biopsy alone, 10.4 months for partial resection and 11.3 months for total resection (Simpson et al. 1993). In their sequent study the same group showed that post-operative residual tumor size is highly correlated to median survival with >4cm^2 with 11 months, 1-4 cm^2 15 months, and 0-1 cm^2 18 months.

For more convenient surgery new methods are also used such as 5-aminolevulinic acid. 5-aminolevulunic acid is a florescent label that assists surgeons for complete resection. Complete resection was achieved in 65% of patients in the treatment compared with the 35% in the conventional study (W Stummer et al. 2006), (Uwe Pichlmeier et al. 2008). This clearly shows that aptamers with fluorescent labels are suitable for complete resection of tumors.

1.4.4.2. Radiation Therapy

Radiation therapy (RT) is a chief support of treatment after surgery. The standard RT for GBM is focal, fractionated external beam RT (EBRT). RT after surgery is the best supportive are and roughly doubles survival (Kristiansen et al. 1981), (Walker et al. 1978). Intensity modulated RT is widely used and fairly advantageous to 3-D EBRT (Narayana et al. 2006).

1.4.4.3. Chemotherapy

Since the late 1970s, many clinical trials have examined the chemotherapy in improving survival of GBM patients. Since long term survivors (36 months) is only 2.2% of the population and heterogeneity of the disease is very high, strategy of the

experiment and the higher power statistical design is very important. In order to get reliable and larger patient populations single randomized studies may be combined in meta-analysis with enhanced statistical power. Fine *et al*.(Fine et al. 1993) in meta-analysis about GBM with more than 3000 patients from 16 randomized clinical trials showed that combined radio and chemotherapy will cause an increase in survival 10.1% at 1 year and 8.6% at 2 years. Also in a recent study by The Glioma Meta-analysis Trialist Group (GMT) it was shown that chemotherapy would yield a 15% relative decrease in the risk of that with an increase in 1-year survival of 6% and an increase in medium survival time of 2 moths(Stewart 2002). Nowadays addition of temozolomide (TMZ) to radiotherapy became the standard treatment for GBM. It is shown in a phase-III study that TMZ with radiotherapy increases median survival from 12.1 months to 14.6 months, also 2 year survival is increased to 26% from 10 %(Stupp et al. 2005).

1.5. Aim of the Study

The aim of this study is the selection of aptamers as a probe for specific recognition of glioblastoma multiforme cell line A-172 with cell-based SELEX methods.

The selected aptamers will be used in the preliminary studies as;

i) diagnostic agent for detection of cancer cells using microscopy,

ii) carrier for drug delivery.

CHAPTER 2

MATERIAL AND METHODS

2.1. Cell Culture

2.1.1. Cell Lines and Growth Conditions

Cell lines A-172 (ATCC.CRL-1620™, brain, glioblastoma), CaOV-3 (ATCC.HTB-75™, ovary, adenocarcinoma), GMBJ1 (kindly provided by Dr. P.J. Tofilon (Moffitt Cancer Center, Florida), primary brain glial cell line, glioblastoma) were grown in Dulbecco's modified Eagle medium (DMEM, ATCC.30-2002). The MCF-7 (ATCC.HTB-22™, mammary gland, adenocarcinoma) and U87MG (ATCC.HTB-14™, brain, glioblastoma) cells were grown in Eagle's minimum essential medium (EMEM, ATCC.30-2003). Cell lines CCRF-CEM (ATCC.CCL-119™, T lymphoblast, acute lymphoblastic leukemia), Ramos (ATCC.CRL-1596™, B lymphocyte, Burkitt's lymphoma) and H23 (ATCC.CRL-5800™, lung, non-small cell lung cancer) were grown in Roswell Park Memorial Institute 1640 medium (RPMI-1640, ATCC.30.1001). The HT-29 (ATCC.HTB-38™, colon, colorectal adenocarcinoma) cells were grown in McCoy's 5A medium (ATCC.30-2007), and HBE135-E6E7 (ATCC.CRL-2741™, lung epithelial, HPV-16 E6/E7 transformed) cells were grown in keratinocyte free medium (Keratinocyte-SFM, GIBCO.17005-042). All cell lines were supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO.26140079) and 100 units/mL penicillin-streptomycin (Cellgro.30-002CI).

2.1.2. *Cell Harvesting (Passaging)*

Cells were grown in 25cm^2 or 75cm^2, filter cap culture flasks (Greiner Bio-One, Germany). When the confluency of attached cells reached to 80% of the flask surface cells were trypsinated with 2 mL of 0.05% trypsin/0.53 mM EDTA in Hank's balanced salt solution (HBSS).

2.2. *Design of Library and Primers for SELEX*

The DNA library used for aptamer selection consisted of a central, continuous stretch of 42 randomized sequences flanked by PCR primer sequences (5'-TGACGAGCCCAAGTTACCT-42N-AGAATCTCCGCTGCCTACA-3'). FITC labeled 5' primer (5'-FITC-TGACGAGCCCAAGTTACCT-3') and biotinylated 3' primer (5'-Biotin-TGTAGGCAGCGGAGATTCT-3') was used in the PCR. All sequences were synthesized by standard phosphoramidite chemistry using an ABI 3400 DNA synthesizer (Applied Biosystem Inc., Foster City, CA, USA) and purified by reversed-phase ion pairing HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA)

2.3. *SELEX*

In this study, human glioblastoma cell line A-172 was used as the taroiget cell line. The initial pool containing 7nmol of DNA library was dissolved in 500μL of binding buffer (4.5 g/L glucose, 5mM $MgCl_2$, 0.1mg/mL yeast tRNA and 1mg/mL BSA in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$ (Sigma.D1283)). The library was denaturated by heating at 95°C for 5 min and cooled on ice for 10 min before incubation. Then the ssDNA pool was incubated with A-172 cells on a 5.0 cm diameter cell culture dish (Corning) for 60 min at 4°C. After incubation, cells were washed twice with 3mL washing buffer (4.5 g/L glucose, 5mM $MgCl_2$ in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$) for 30 second at 120 rpm. Cells were harvested using a

cell scraper (Corning) and transferred into 500μL water. The bound DNA sequences were eluted by heating at 95°C for 15 min. The eluted sequences were amplified by PCR with FITC and biotin labeled primers (denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec). For each round of SELEX, the number of PCR cycles was optimized between 10 and 20. The PCR products were separated on 3% agarose gel, and the highest numbered cycle without any nonspecific band was chosen as cycle number for amplification of the selected sequences. After amplification, selected ssDNA was separated from the biotinylated antisense ssDNA by alkaline denaturation using 200mM NaOH and affinity purification with streptavidin-coated sepharose beads (GE Healthcare Bio-Sciences Corp.). The separated ssDNA solution was passed through a desalting Nap-5 column (GE Healthcare Bio-Sciences Corp.) to remove the NaOH. The entire selection process was repeated according to the course of enrichment. To acquire more specific and high affinity aptamers the incubation time was decreased from 60 min to 30 min as the number of selection rounds increased, and the washing time was also increased gradually from 30 sec to 60 sec. Additionally, 10% FBS was added to the incubation mixture after the 4^{th} round of selection (Table 2.1).

Table 2.1 Parameters used in SELEX.

SELEX	Pool Used	Incubation Time	Washing	FBS
1	100 pmol	60 min	2x3mL (5 sec)	-
2	100 pmol	55 min	2x3mL (5 sec)	-
3	100 pmol	50 min	2x3mL (5 sec)	-
4	100 pmol	45 min	2x3mL (5 sec)	10 % FBS
5	100 pmol	40 min	2x3mL (30 sec)	10 % FBS
6	100 pmol	35 min	2x3mL (35 sec)	10 % FBS
7	100 pmol	30 min	2x3mL (40 sec)	10 % FBS
8	100 pmol	30 min	2x3mL (45 sec)	10 % FBS
9	100 pmol	30 min	2x3mL (50 sec)	10 % FBS
10	100 pmol	30 min	2x3mL (55 sec)	10 % FBS
10-18	100 pmol	30 min	2x3mL (60 sec)	10 % FBS

2.4. 454-Sequencing

After 18 rounds of selection, 4 selected ssDNA pools were amplified using 19-mer 454 fusion and multiplex identifiers (MID) incorporated into the unmodified primers (Forward primer 1 with MID1 and 454 fusion, 5'-GCC TCC CTC GCG CCA TCA G – AC GAG TGC GT - T GAC GAG CCC AAG TTA CCT-3'; forward primer 2 with MID7 and 454 fusion, 5'-GCC TCC CTC GCG CCA TCA G – CG TGT CTC TA – G ACG AGC CCA AGT TAC CT-3'; forward primer 3 with MID10 and 454 fusion, 5-GCC TCC CTC GCG CCA TCA G – TC TCT ATG CG – T GAC GAG CCC AAG TTA CCT-3'; forward primer 4 with 454 fusion; 5'-GCC TCC CTC GCG CCA TCA G – TG ACG AGC CCA AGT TAC CT-3'; reverse primer with 454 fusion; 5'-GCC TTG CCA GCC CGC TCA G – TG TAG GCA GCG GAG ATT CT-3'). Pools were sequenced by the Genome Sequencing Services Laboratory at the University

of Florida using a Genome Sequencer 20™ System with pyrosequencing 454 strategies.

2.5. *Bioinformatics Analyses*

Sequences were grouped according to their MID sequences for further analysis. The sequences were aligned using MAFFT-6 software[15] and Jalview2.4,[16] and ATV 4.0.5 is used to display and manipulate the phylogenetic tree.[17]

2.6. *Flow Cytometric Analyses*

Flow cytometry was used to monitor the enrichment of pools during the selection. The FITC-labeled ssDNA pool was incubated with $5x10^5$ target cells at a final concentration of 250nM on ice for 30 min. The cells were washed twice with 500μL of washing buffer and resuspended in 300μL of washing buffer. The fluorescence intensity was determined by FACScan cytometer (Becton Dickinson Immunochemistry Systems) by counting 30000 events. The FITC-labeled initial ssDNA library was used as a negative control.

2.7. *K_d Determination*

The binding affinity of aptamers was determined by incubating various concentrations of biotin-labeled aptamer with $5x10^5$ target cells at 4°C for 30 min. Cells were then washed with 500μL of washing buffer twice. The cells with bound aptamers were incubated with 200μL of Streptavidin-PE (Invitrogen, SNN1007) at 4°C for 20 min and washed with 1 mL of washing buffer. The mean florescence intensity of the unselected library was subtracted from that of the aptamer with the target cells to determine the specific binding. The ligand binding analysis function of SigmaPlot (Jandel Scientific) was used to calculate the apparent equilibrium dissociation constant (K_d) of aptamer according to the equation $Y=B_{max}X / (K_d + X)$

2.8. *Effect of Temperature on Binding*

Since the selection was performed at 4°C, some of the aptamers may not bind well at higher temperatures. Because further studies were planned at physiological conditions, the binding studies of aptamers were also performed at 37°C with a 30-min incubation period.

2.9. *Determination of Target Type*

Target cells ($1x10^6$) were washed with 1mL of PBS and then incubated with 2 mL of 0.05% trypsin/0.53 mM EDTA in Hank's balanced salt solution (HBSS) or with 2 mL 0.1mg/mL proteinase K in PBS buffer at 37°C for 3 and for 10 min. DMEM containing 10% FBS was then added to inhibit proteinases. After washing with 2mL of binding buffer, cells were used in the aptamer binding assay as described in the flow-cytometric analysis.

2.10. *Microscopic Studies*

For imaging, 100 pmol of biotin-labeled aptamer was incubated with the cells in 200μL of binding buffer for 30 min. After washing with 1mL of washing buffer, cells were incubated with streptavidin-PE for 20 min. Excess streptavidin-PE was removed using two 2 mL of aliquots of washing buffer. Also Vectashield mounting medium (Vector Laboratories) with 4',6-diamidino-2-phenylindole (DAPI) was used for counterstaining of cellular nuclei. Leica DM600B microscope was used for imaging.

2.11. *Determination of Binding Specificity*

For determination of binding specificity, different cell lines are used for binding affinity. A-172, U87MG, GMBJ1, MCF-7, CEM, RAMOS, H23, HT29, HBE-135, and CAOV-3 cell lines are used for determining binding specificity. The FITC-labeled

ssDNA pool was incubated with $5x10^5$ target cells at a final concentration of 250nM on ice for 30 min. The cells were washed twice with 500µL of washing buffer and resuspended in 300µL of washing buffer. The fluorescence intensity was determined by FACScan cytometer (Becton Dickinson Immunochemistry Systems) by counting 30000 events. The FITC-labeled initial ssDNA library was used as a negative control.

2.12. Optimization of Aptamer Sizes

The secondary structure analysis of aptamers was performed by means of NUPACK analysis algorithms[18] using the Internet tool NUPACK nucleic acid package[19]. Using the secondary structures, possible fragments of aptamers having stem and loop structures were synthesized and screened for their binding affinities.

2.13. Drug Delivery Experiments

2.13.1. Binding of Doxorubicin to Aptamers

Doxorubicin (Dox) is known to intercalate within the DNA strand due to the flat aromatic rings in this molecule. A physical complex between GMT-3, tdo5 aptamers and dox was made by sequential addition of molar ratio of aptamer to dox. Final concentration of 1.5µM dox is incubated with 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, and 0.0781 µM tdo5 and GMT-3 for 30 minutes in washing buffer. Fluorescence was monitored at excitation 480nm and emission was recorded in the interval of 500-750nm using Nanodrop 3300 as flourospectroscopy. The amount of dox that can physically conjugate throughout the intercalation is determined by the quenching of the dox. The maximum emission data were recorded at 595 nm and used in the calculation of binding.

2.13.2. Application of Drug and XTT Cell Viability Assay

A-172 glioblastoma and MCF-7 breast cancer cell lines were used in the study. GMT-3 aptamer has a strong binding affinity on the A-172 and no binding affinity on MCF-7. Tdo-5 was used as a control aptamer and has no binding affinity on both cell lines.

Briefly 100µL aliquot of A-172 and MCF-7 cells ($10x10^3$ cells/well) were seeded in 96 well plates (n=4) and allowed to grow overnight and treated with either GMT-3-dox conjugate (1:1.20 aptamer to dox mole ratio, dox was 10µM), tdo5-dox conjugate(1:1.20 aptamer to dox mole ratio, dox was 10µM), dox alone(10µM) for 45 minutes. After incubation medium with application is discarded and each well is washed with 250µL of washing buffer. After discarding washing buffer each well is supplied with non-FBS and non-phenolic medium and incubated for 8 hours. XTT based *in vitro* toxicology kit is used to determine cell viability. Final concentration of 20%(v/v) XTT+PMSF solution is added to the medium. The absorbance was measured at 450 and 690 nm with a microplate reader. The specific absorbance is calculated using the formula;

Specific absorbance= A_{450nm}(Test) – A_{475nm}(Blank) – A_{690nm}(Test)

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Construction of Library

Success of an aptamer selection is highly dependent on the design of the library. There are some rules to design a suitable library and primers for SELEX. First of all, designed library cannot be too stable as it must be easy to denaturated at 95°C for PCR. Our library; 5′-TGACGAGCCCAAGTTACCT-42N-AGAATCTCCGCTGCCT ACA-3′ had a mean melting temperature of 72.4°C, and it can easily be seen from its secondary structure analysis that it doesn't have stable base pairings (Figure 3.1). This secondary structure analysis ensured us; the 3-D diversity of the library is mainly related with the randomized region and the primer flanks do not have main effect on the 3-D shape.

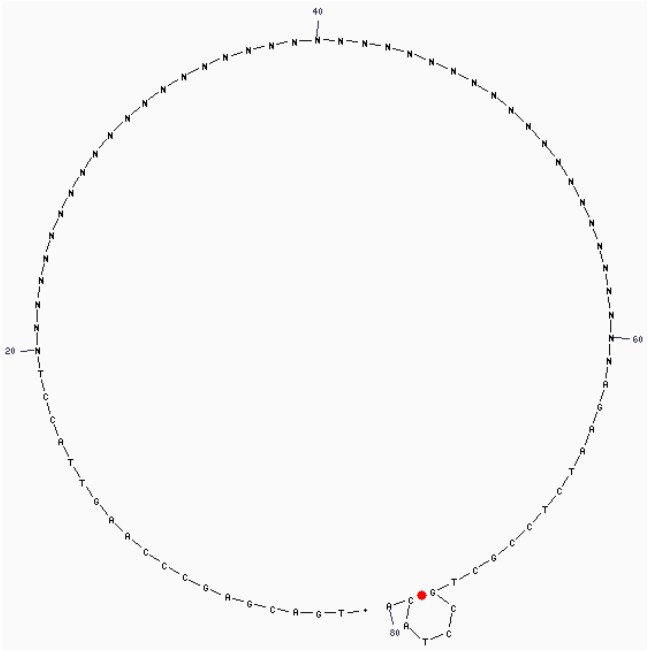


Figure 3.1 Secondary structure analysis of designed library (mfold).

Another rule is primers designed cannot have guanine at 5′ or 3′ ends. Guanine can quench the fluorescent molecules if it is close enough. Since we planned use fluorophore modified aptamers in further applications, we do not want our aptamers to have guanines in their 5′ or 3′ ends. Also deprotection step of synthesis of DNA sequences with guanine in 3′ end is very difficult, unproductive and 17 hours longer than the normal ones. Primers must be 18 to 20 base pairs, must have GC content of 50% or more and anneal to a library around 55°C to 60°C. Our primers 5′-TGACGAGCCCAAGTTACCT-3′ and 5′-TGTAGGCAGCGGAGATTCT-3′ had a GC content of 52.6 %. They had melting temperature of 55.9, respectively. Also specifically each primer must have a hairpin melting temperature lover than 10°C. We had hairpin melting temperatures of -9.7°C and 9.1°C, respectively. A good primer for SELEX mustn't have more than 3 base pairs in their selfdimers,

heterodimers and their ΔG must be lower than 5kcal/mol. Our primers had 2 selfdimer base pairs and ΔG of -36.6kcal/mol and -36.78kcal/mol, respectively. Primers had 3 base pair for their heterodimer and ΔG of -6.21kcal/mol.

After designing, library was synthesized and successfully synthesized DNA library was separated from the truncated sequences (Figure 3.2). Since truncated sequences do not have DMT and only completely synthesized DNA sequence has a DMT group on its 5′ end it was easily purified by reversed-phase ion pairing HPLC.

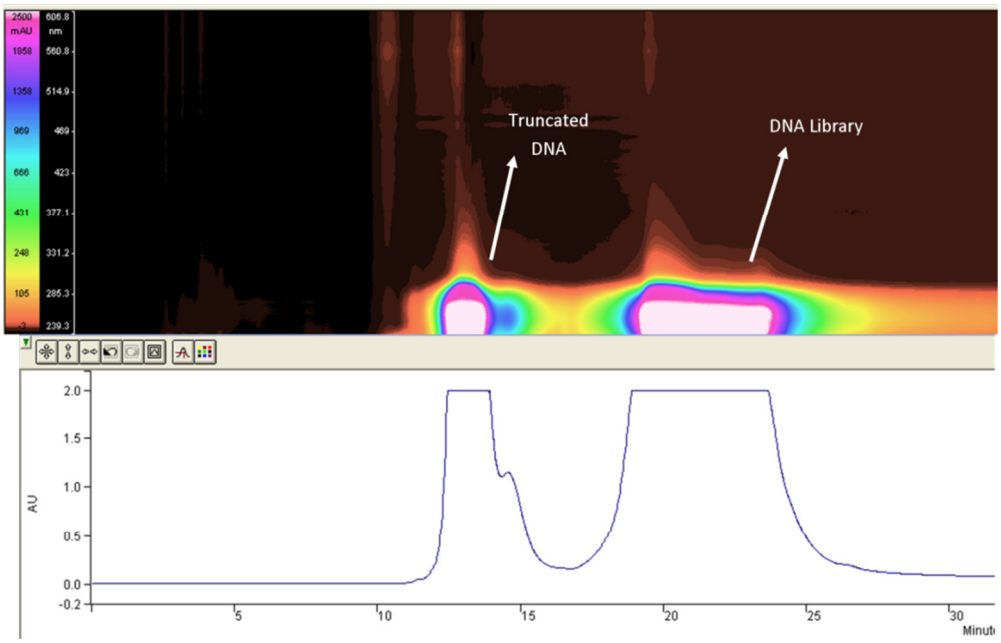


Figure 3.2 HPLC purification results of library

3.2. Enrichment of aptamer candidates

Cell-SELEX was successfully used to select specific for A-172 cell line of glioblastoma multiforme. In our SELEX strategy we didn't use any negative selection round for many reasons. First of all there is no noncancerous cell line of glioblastoma so the only choice is using other cancer cell line. Since glial cells are very specialized and differentiated cells only present in brain and spinal cord except enteric glial cells to use a totally different cell line for differentiation is redundant and null. Also in this study we aimed to select aptamers broadly recognizing the cell membrane proteins (Z Tang et al. 2007). SELEX without negative selection has some pros and cons. One can easily select aptamers more specific and differentiative using negative selection. This handicap can be overcome by screening more candidate aptamers on different cell lines and finding specific aptamers. Common biomarker proteins that are important for cancer can easily be missed using negative selection with other cancer cell lines. Also this can be easily seen in our results; GMT4 which is our best binding aptamer and GMT8 have affinity to a CCRF-CEM leukemia cell line. If we had used CCRF-CEM as a negative selection we would not be able to find these aptamers. Lastly, smooth and undisrupted selection is very important for successive SELEX. By disrupting the SELEX exponential enrichment chain can easily be broken and some enriched sequences can be loosed. So that we have just used A-172 cell line and increased the stringency of SELEX smoothly by decreasing the time of incubation, increasing the washing, and adding FBS for later rounds of SELEX (Table 2.1) without negative selection.

3.8.1. Preparative PCR of SELEX rounds

After first incubation of library with target cells each round is followed by a preparative PCR of eluted binders. This step is one of the most important steps in the SELEX. PCR of random libraries can easily produce nonspecific products that are generally smaller and sometimes double and triple size of the library. These nonspecific PCR products can remain throughout the SELEX and they will enrich themselves for each round. These artifacts in SELEX are called as molecular parasites (Günter Mayer 2009) and can easily ruin selection process in the beginning rounds. Unfortunately since they exponentially increase in number by the rounds, they can be noticed in later rounds and this costs lots of time and money. To protect SELEX these molecular parasites PCR must be optimized for each round and especially in terms of PCR cycles. In figure 3.3 and 3.4 it can be seen that higher number of PCR cycles generally caused nonspecific products. So that in each round PCR was optimized in our SELEX protocol. So the optimized numbers of PCR rounds were 6, 13, 14, 16, 15, 11, 12, 13, 12, 12, 12, 12, 12, 12, 11, and 10 for SELEX round 1 to 16, respectively.

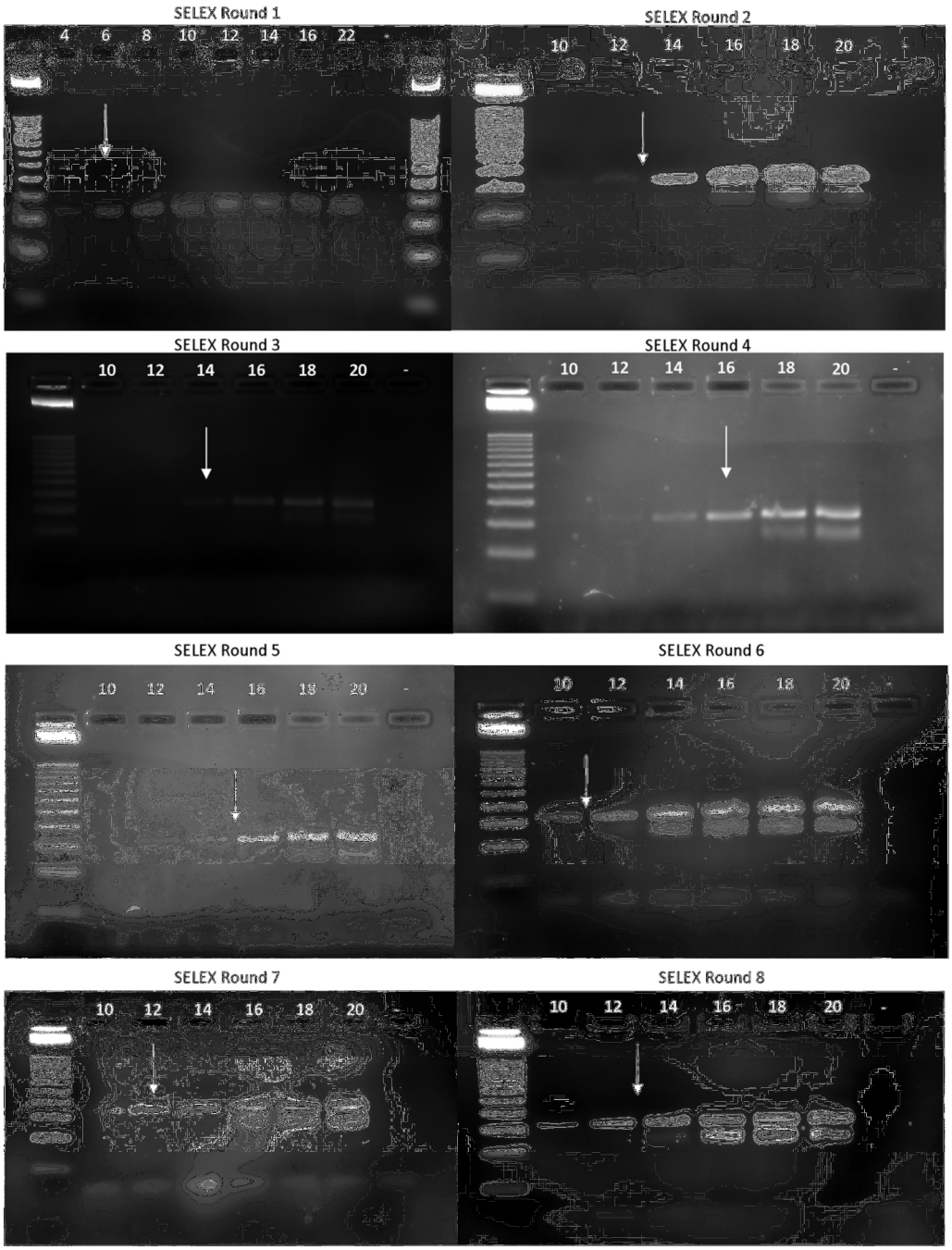


Figure 3.3 PCR cycle optimization of SELEX rounds 1 to 8.

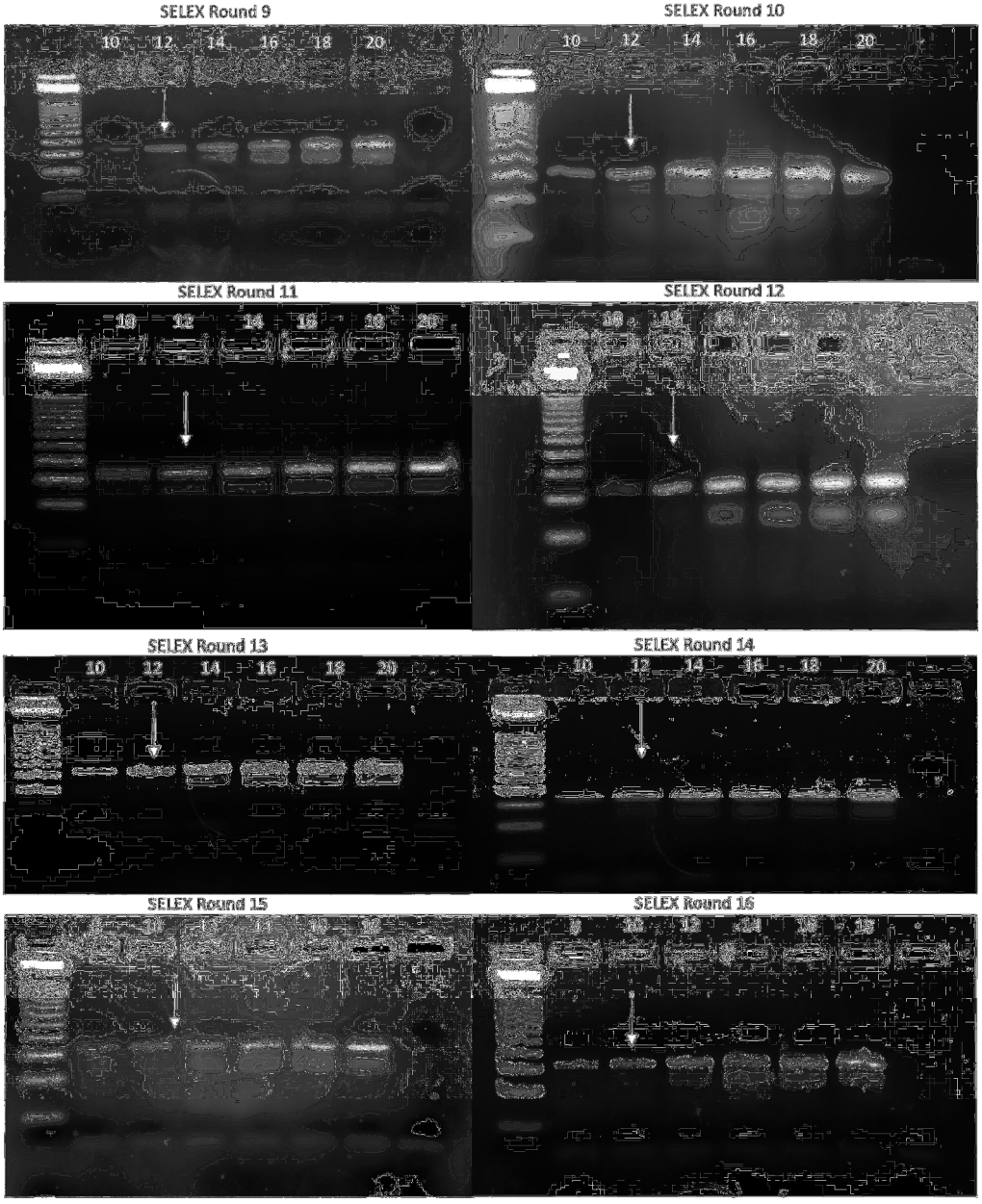


Figure 3.4 PCR cycle optimization of SELEX rounds 9 to 16.

3.8.2. Flow Cytometry

Enrichment of the binding sequences in the SELEX was checked with flow cytometry. As shown in figure 3.5 fluorescence intensities of cells after binding with the fluorescence labeled pool increased considerably and consistently showing the enrichment of binder sequences in the pools. Also in figure 3.6 we can deduce that the mean fluorescence of cells reached to maximum with the increasing number of SELEX rounds. At first 5 rounds since the pools were very young and immature the selection process was desultory so we can see a wavy behavior. After 5^{th} round a consistent increase could be observed in the selection. As it can be also seen from the table 2.1 after 10^{th} round the stringency of the selection increased to its maximum with only 30 minute incubation and 60 second washing with addition of 10%FBS as a competitor. With this higher stringency and exponential selection regime the increase in mean fluorescence was more obvious and keen after 10^{th} round. The last round was decided as 18^{th} round as we reached the triple mean fluorescence of cells alone.

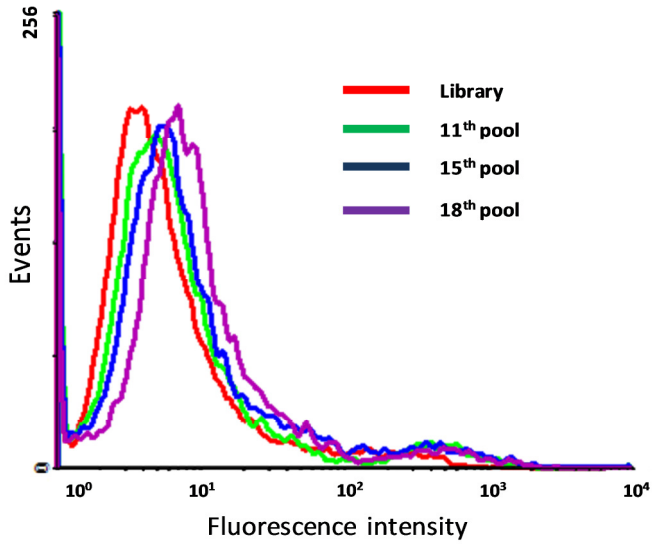


Figure 3.5 Binding assay of selected pools with A-172 cells.

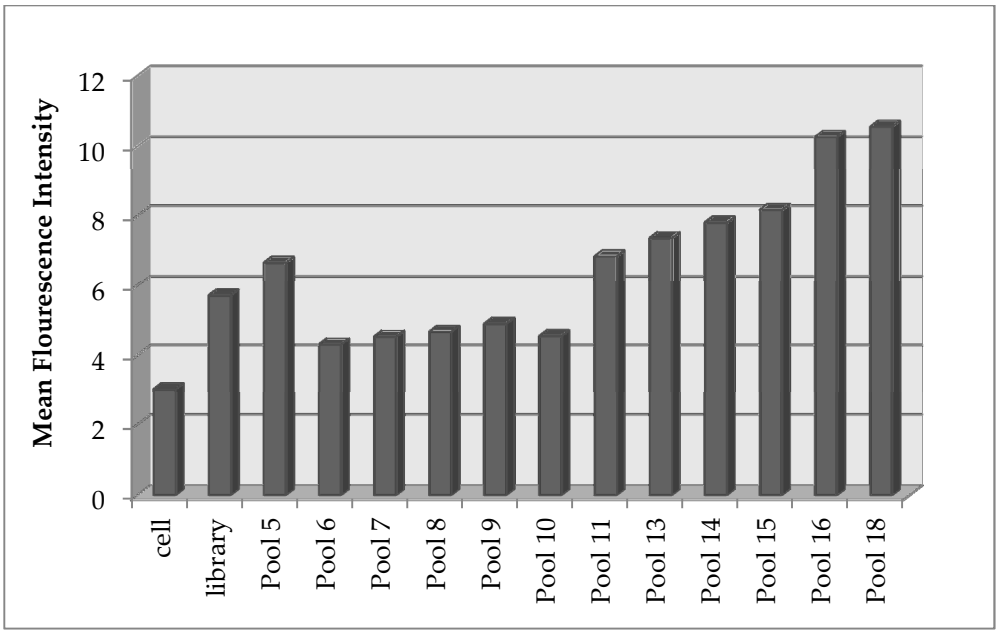


Figure 3.6 Mean fluorescence intensity of bound pools to A-172 cells.

3.3. Sequencing Results

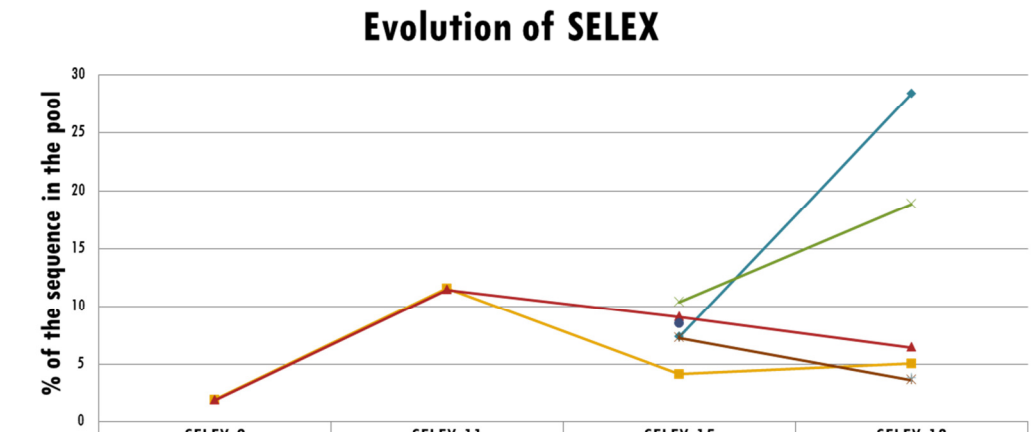
After 18th round of SELEX four representative pools were selected and sequenced using 454 pyrosequencing technologies. Pool 9, 11, 15, and 18 were selected for sequencing to determine the enriched sequences that can be aptamers for target. They were also selected to be able to observe the evolution of sequences in the course of selection. Sequencing retrieved 13094 sequences. Primer regions were removed from each sequence to carry out a healthy multiple alignments. Sequences without primer regions can be seen in a CD in appendix A. After this the sequences were grouped according to their MID barcodes in order to find the sequences of different pool's sequences. Pool 9, 11, 15, and 18 retrieved 912, 6232, 2175, and 3775 sequences, respectively. Although we put in same amounts of each pool in to the sequencing solution, pool 9 gave us less and pool 11 gave us more numbers of sequences than expected. This can be due to the quality of PCR products of each pool. Also since it was a chance event to place each sequence on beads in pyrosequencing, it was acceptable. Since there were millions of copies from each pool the chance of getting well-arranged polls for 13094 sequence was fairly low in probability. Whatever the ratios of pools, it was much more reliable than the sequencing results of SELEX in the literature. With classical cloning and Sanger sequencing research groups generally get sequences about hundreds per SELEX and just for only last pool (Shangguan et al. 2008) (Cerchia et al. 2009). So here we first integrate 454 sequencing to SELEX and get more reliable sequencing results. This also gave us the advantage of learning inner dynamics of SELEX. After grouping the pools and removing the primer and the MID barcode regions each pool was aligned using MAFFT-6 (Katoh et al. 2002). Also ATV 4.05 (Zmasek and Eddy 2001) was used for displaying and manipulating phylogenetic trees. Sequences, alignments, and phylogenetic trees of each pool can be found on

Appendix A. Representative sequences of big groups were selected mainly using trees and alignment tables, and named as GMT1 to 9 as an aptamer candidate.

3.3.1. Evolution of sequences

SELEX is an abbreviation for Systematic Evolution of Ligands by Exponential Enrichment. So in fact it is just an evolution that is fast and directed for a purpose. In our case this purpose was the selection of sequences which were good binders for our target A-172 cell line. In figure 3.7 we can see the rough trees of unaligned sequences. In pool 9, tree was showing a diverse speciation with small numbers and types families with similar or same sequences. Also in figure 3.8 we saw the most grouped sequences were only holding the 2 percent of the whole pool. In pool 9, SELEX was still immature and only some groups of sequences was just beginning to be enhanced in number. It can be seen in pool 11 enrichment of these sequences reached to 12 and 11%, respectively. In pool 15, 47% of the whole pool was in enriched same sequence groups. This number reached to 66% in 18th pool sequence results. Enrichment was so obvious that GMT1 was compromising 28% of whole pool which means 1070 exactly same sequence. If we look the Figure 3.7 in detail some sequences like GMT2 and GMT3 enhanced up to appoint and survived throughout the course of SELEX. Most probably these sequences had a target that is highly expressed on the surface of the cell so that they can easily be enriched because of their high probability of binding. After a while with the enrichment of other sequences with other targets, they somehow maximized and limited in to the number of cell for remaining rounds. Some sequences suddenly appeared at the 15th or 18th rounds of SELEX. These sequences were probably had lower amounts of target on the cell surface. It takes some SELEX rounds in order to enrich these sequences enough. Since these sequencing result were just representative from millions of sequences, it was very low in probability to catch these sequences in previous rounds because of unenrichment. We have to consider also that our target

was a living cell, which can change expression of many proteins depending on the environmental and intrinsic factors. So some targets on the cell for aptamer candidates can easily increase or decrease in number or can be modified biologically like phosphorylation. The reason of incoherent increase or decrease of the ratios of some candidate sequences could be these changes.



	SELEX-9	SELEX-11	SELEX-15	SELEX-18
GMT1			7.31	28.32
GMT2	1.97	11.54	4.14	5.09
GMT3	1.86	11.46	9.1	6.46
GMT4			10.39	18.83
GMT5			7.26	3.63
GMT6			8.51	
GMT7				3.74

Figure 3.7 Evolution of aptamers throughout the SELEX process.

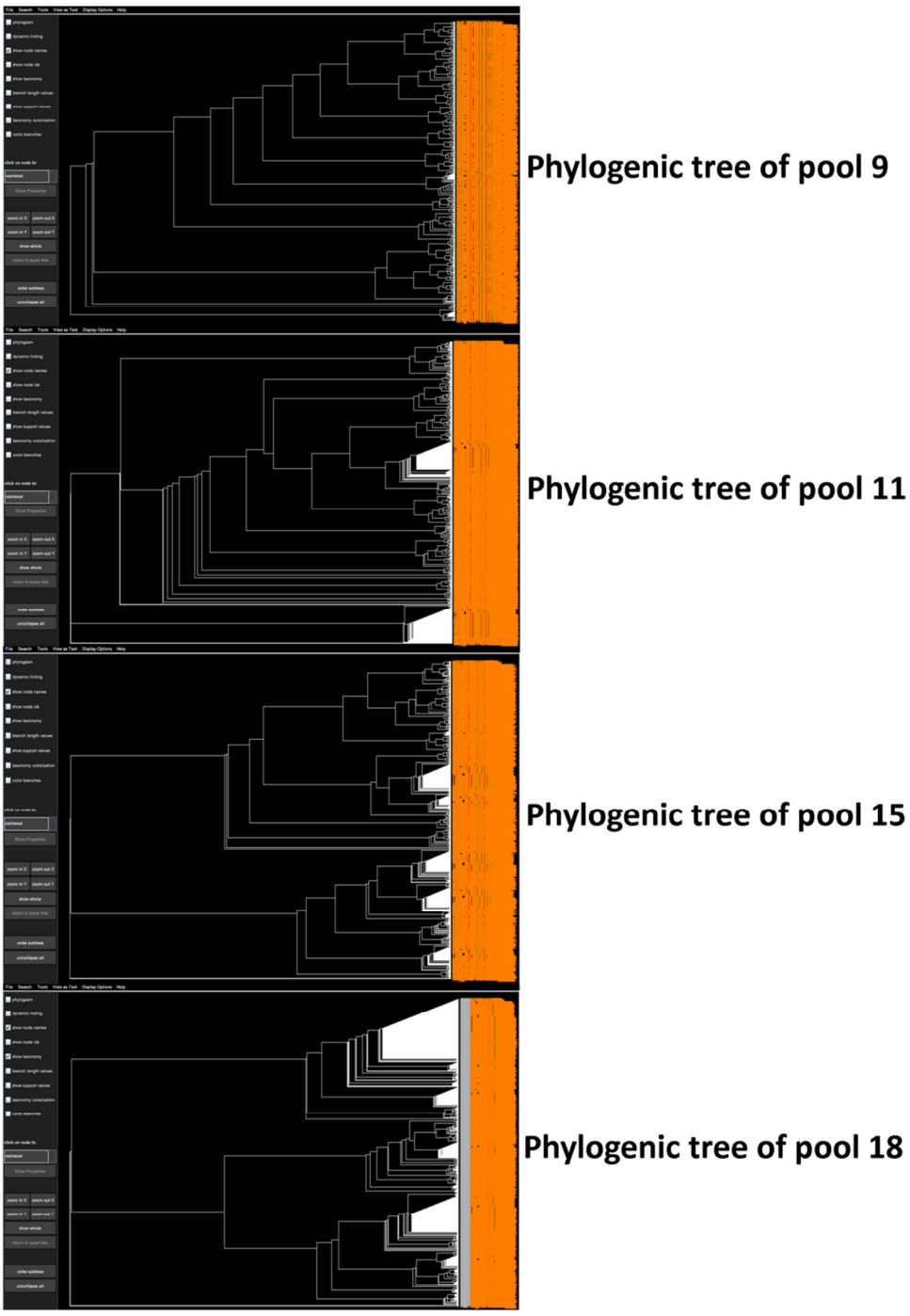


Figure 3.8 Dendogram for visual classification of similarity among sequences of different rounds of selection.

3.4. *Synthesis of aptamers*

After selecting candidate aptamers from pools they were synthesized and separated from the truncated sequences. Since truncated sequences do not have DMT and only completely synthesized DNA sequence has a DMT group on its 5′ end it was easily purified by reversed-phase ion pairing HPLC. GMT1 to GMT7 were as highly populated sequences and 2 rarer sequences (GMT8 and GMT 9) were synthesized and firstly screened in the characterization experiments. Only GMT3, GMT4, GMT5, GMT8, and GMT 9 gave promising binding results in first experiments and they were produced in the mass amount for characterization. HPLC results of produced aptamers can be seen in figure 3.9.

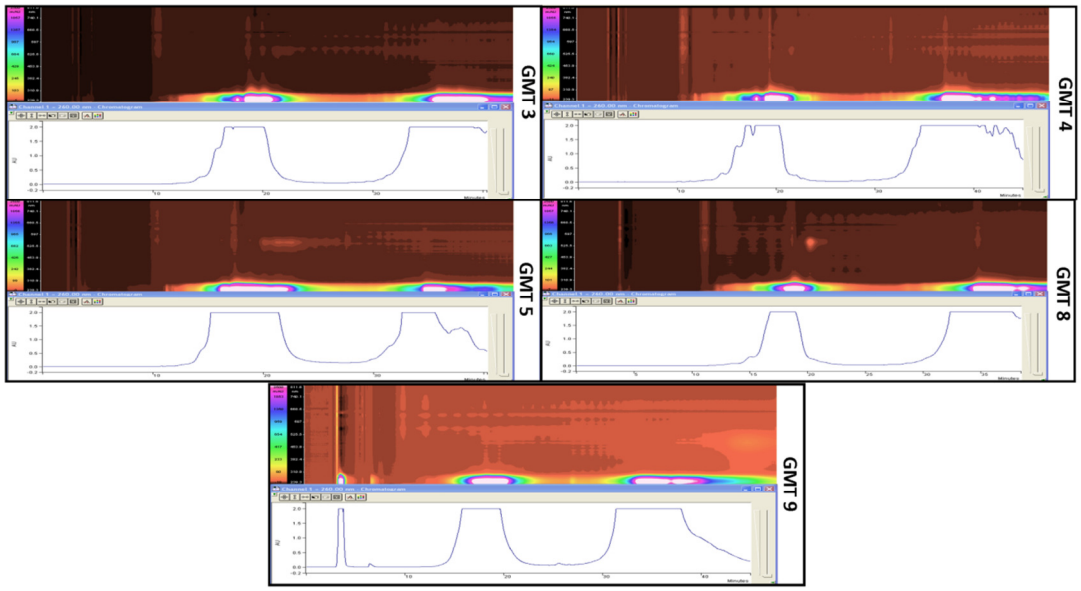


Figure 3.9 HPLC results of synthesized aptamers

3.5. Characterization of aptamers

A representative set of nine abundant sequences was chosen for testing their binding abilities to target. GMT-1 to GMT-9 was firstly checked for their binding affinities using flow cytometry and best binders were further selected for characterization. In preliminary binding test with flow cytometry GMT-1, GMT-2, GMT-6, and GMT-7 were designated as weak binders and eliminated for further studies. GMT-3, GMT-4, GMT-5, GMT-8, and GMT-9 were decided as promising aptamer candidates and further characterizations were carried out by these sequences.

3.5.1. Weak and nonbinding aptamers

Our SELEX was designed for selection of binding sequences to the target cell. Preliminary flow cytometry analysis showed us that some of the sequences that is populated throughout the SELEX and enriched in the number with ongoing selection tours were not binding good enough to our target cell line. As it can be seen from figure 3.10, GMT-1, GMT-2, and GMT-7 had very week binding. They had almost same binding scheme with library. Even GMT-6 had binding affinity lower than the library. The first reason for the selection of these sequences can be contaminates in the SELEX. As we mentioned in the introduction part SELEX has ability to select aptamers for even ions and small organic molecules. So any material or chemical used the SELEX is a potential target for our library and they can easily survive throughout the selection process because of their usage in all step. Even the chemicals of the petri plates and tubes made of can be a target and survive throughout the SELEX.

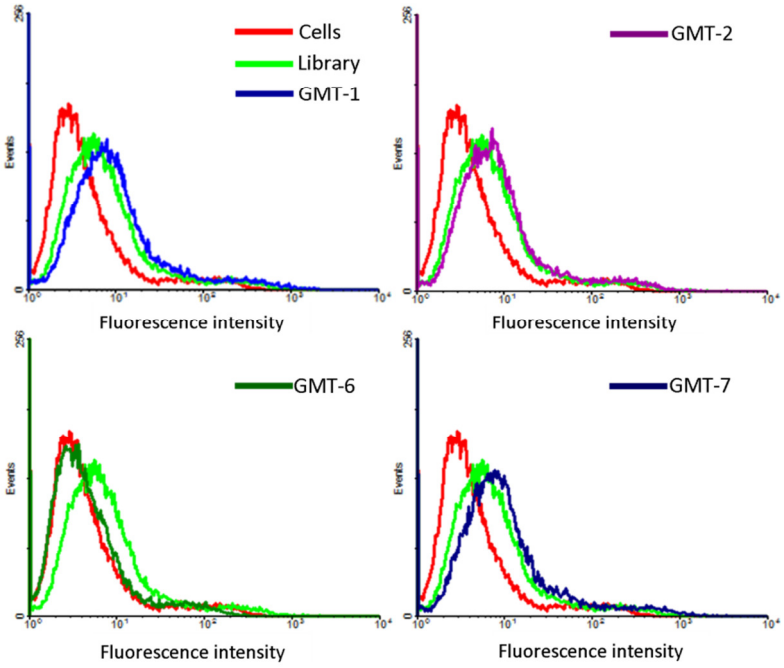


Figure 3.10 Fluorescence intensities of GMT-1, GMT-2, GMT-6, and GMT-7.

Contaminations could be reason for the selection of weak sequences like GMT-2 and GMT-7 with low populated sequences but GMT-1 with 28.32% and GMT-6 with 8.51% of all pool was fairly big populations and there must be other explanations for their enrichment. After the analysis of selected sequences in NUPACK (Dirks et al. 2007) we found the reason of enrichment of GMT-1 and GMT-6. If we placed same amount of GMT-1 and GMT-4 in the same environment 88.96% of the sequences would be hybridized, and just 11.01% of GMT-1 and 11.03% of GMT-4 would be in their non-hybridized free form. Also from figure 3.11 it can be seen that hybridized form was not affecting their 3-D shape too much. Probably their binding sites were still able to bind their targets. Gibbs free energy the hybridized form of GMT-1 and GMT-4 was far more favorable than free forms. GMT-4 had the best K_d throughout our aptamers. So during the SELEX GMT-4

carried out GMT-1 in hybridized form since it was more favorable to stay hybridized. When we synthesized and used by them alone GMT-4 was still binding but GMT-1 had no affinity on the target. Also same story was true for GMT-5 and GMT-6. 96.07% stayed in hybridized form while abundance of the free forms was only 3.93%. Again it can be seen from figure 3.12 hybridizing was not affecting the 3-D shape of binder sequence.

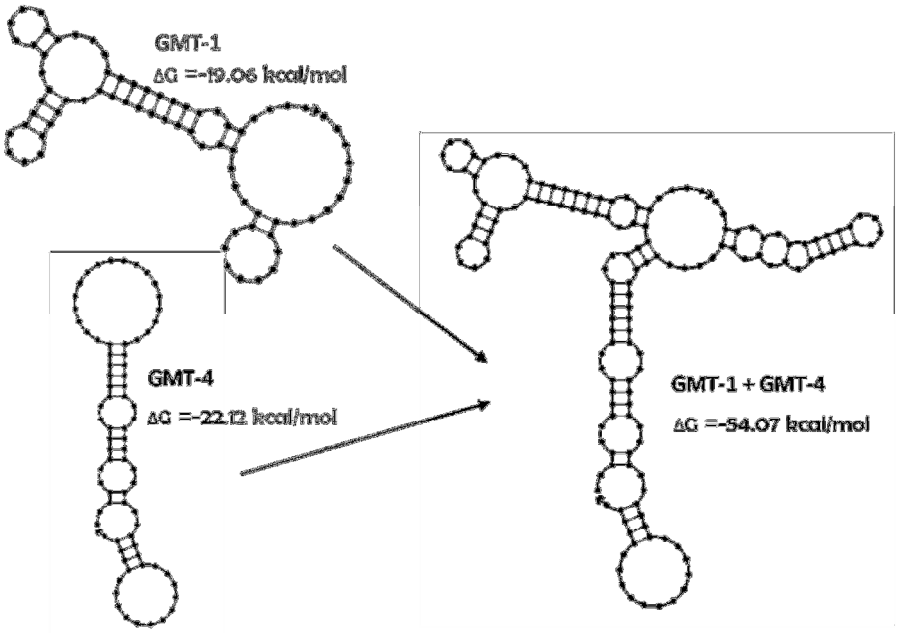


Figure 3.11 Hybridization of GMT-1 and GMT-4

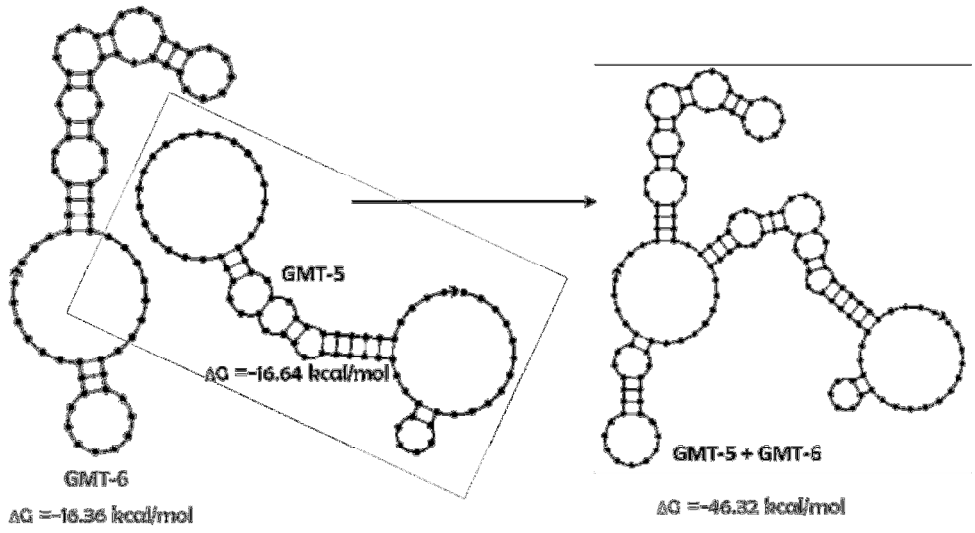


Figure 3.12 Hybridization of GMT-5 and GMT-6

3.5.2. Binding at different temperatures

As implied in material methods all selection and binding experiments were carried out at 4°C. Although it is very important to be able to use aptamer at room or body temperature, to carry out SELEX at elevated temperatures is very difficult. At high temperatures 3-D forming factors like dimers and hairpins loosen and it becomes low in probability to have characteristic shape that can fit and bind.

Luckily all of our selected aptamers had obvious binding at both 4 and 37°C (Figure 3.13). This property was because of their high melting temperatures and factors holding their 3-D shapes. This gave us many advantages for the future use of our aptamers. First of all they will be able to bind their target easily *in vivo*. So that they can be used in any purposes in vivo like imaging the cancer cells. Also internalization of aptamers in to cells is only possible at higher temperatures. It is impossible to internalize any bound aptamers at 4°C because of the rigidity of the cell membrane. This property made aptamers a candidate for drug delivery vector.

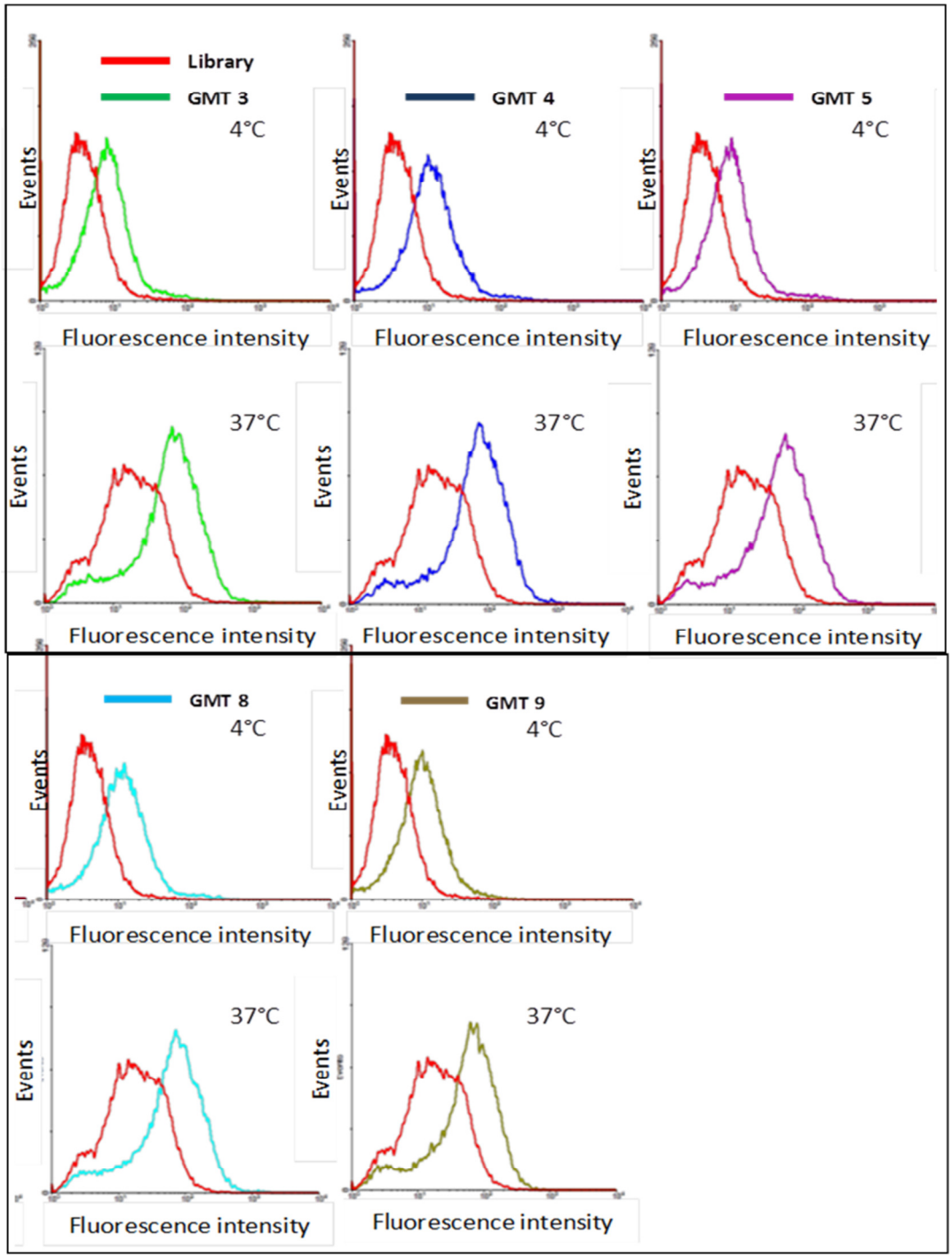
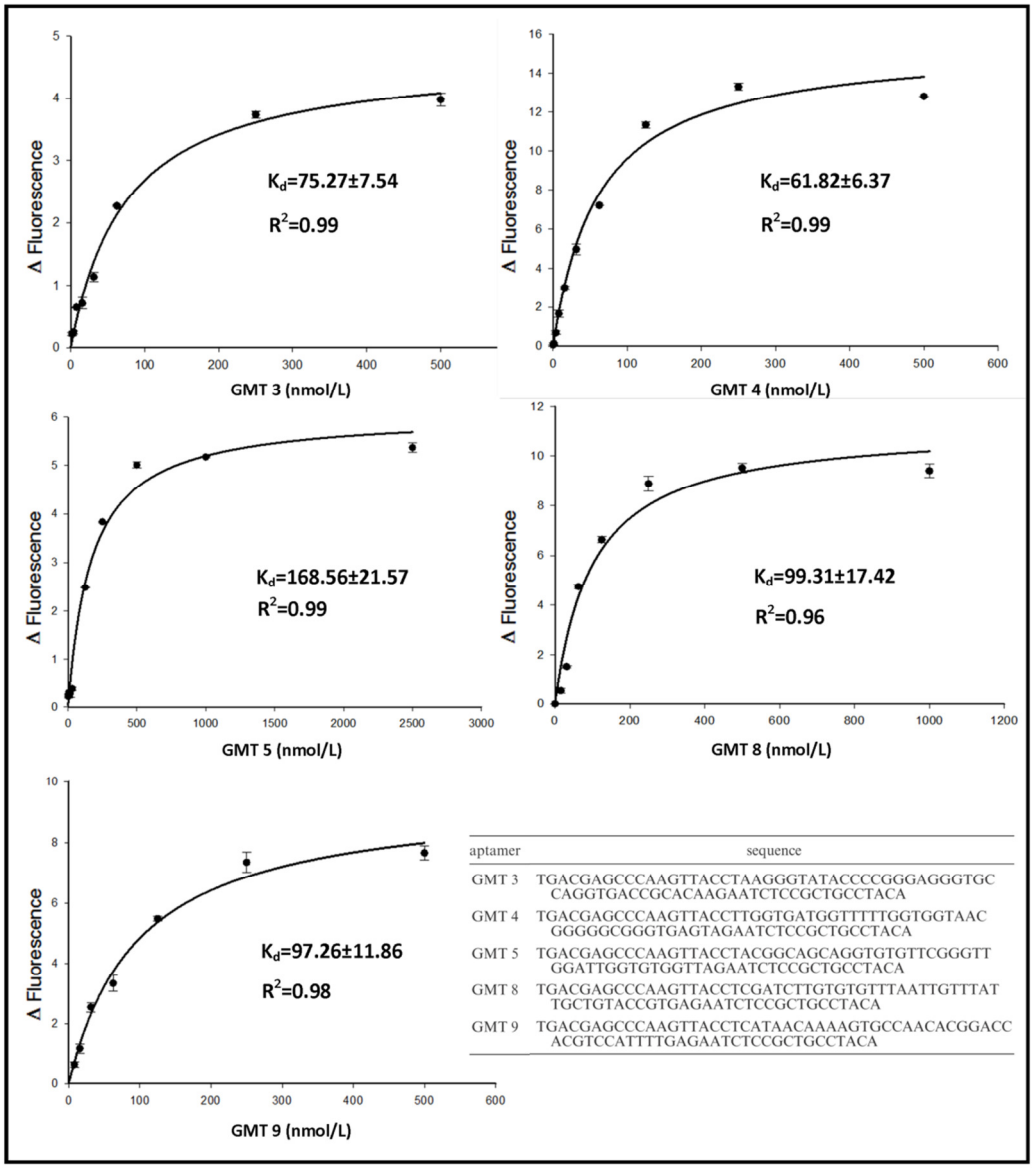


Figure 3.13 Binding of candidate aptamers at 4°C and 37°C.

3.5.3. Determination of binding affinity

As shown in figure 3.14 all aptamers showed high binding affinities with low dissociation constants in nanomolar range. GMT-4 was the best binder with an apparent K_d of 61.82nmol/L. GMT-3, GMT-9, GMT-8, and GMT-5 were following GMT-4 with 75.27nmol/L, 97.26nmol/L, 99.31nmol/L, and 168.56nmol/L of K_d. If we compare it mainly most of the aptamers aiming for living cells had K_d around same nanomolar ranges.



aptamer	sequence
GMT 3	TGACGAGCCCAAGTTACCTAAGGGTATACCCCGGGAGGGTGC CAGGTGACCGCACAAGAATCTCCGCTGCCTACA
GMT 4	TGACGAGCCCAAGTTACCTTGGTGATGGTTTTTGGTGGTAAC GGGGGCGGGTGAGTAGAATCTCCGCTGCCTACA
GMT 5	TGACGAGCCCAAGTTACCTACGGCAGCAGGTGTGTTCGGGTT GGATTGGTGTGGTTAGAATCTCCGCTGCCTACA
GMT 8	TGACGAGCCCAAGTTACCTCGATCTTGTGTGTTTAATTGTTTAT TGCTGTACCGTGAGAATCTCCGCTGCCTACA
GMT 9	TGACGAGCCCAAGTTACCTCATAACAAAAGTGCCAACACGGACC ACGTCCATTTTGAGAATCTCCGCTGCCTACA

Figure 3.14 Sequences and binding affinities of selected aptamers to A-172 cells.

3.5.4. Determination of binding specificity

To find the specificity of aptamers they were also tested with many cell lines from different origins (Table 3.1). GMT-3, GMT-5, and GMT-9 didn't showed binding affinity on cell lines from different origins. GMT-4 and GMT-8 interestingly showed high affinity for CEM cells which is an acute lymphoblastic leukemia. This clearly showed that glioblastoma and leukemia have same molecular recognition sites on their surface for these two aptamers. This may also show that leukemia and glioblastoma can share some same genetic background. Although these two aptamers showed affinity on the CEM they did not show any affinity on RAMOS which is an also leukemia type. This was because of their cellular origins CEM is a T lymphoblast but RAMOS is a B lymphoblast.

Also it was clear from the results that all aptamers have specificity on all glioblastoma cell lines examined. Although SELEX was carried out on the A-172 cell line, GMT-3, GMT-4, and GMT-5 had higher binding affinities for GMBJ1 which is a primary culture cell line. This showed that GMBJ1 was expressing more of these molecular recognition sites than A-172.

Table 3.1 Binding of aptamers to cell lines from different origins.

	Origin									
	Glioblastoma			**Breast**	**Leukemia**		**Lung**	**Colon**	**Epithelial**	**Ovary**
Aptamer	**A-172**	**U87MG**	**GMBJ1**	**MCF-7**	**CEM**	**RAMOS**	**H23**	**HT29**	**HBE-135**	**CAOV-3**
GMT 3	+	+	++	-	-	-	-	-	-	-
GMT 4	++	+	+++	-	+++	-	-	-	-	-
GMT 5	+	+	+++	-	-	-	-	-	-	-
GMT 8	+++	++	+++	-	+++	-	-	-	-	-
GMT 9	++	-	++	-	-	-	-	-	-	-

The fluorescence intensity of the unselected library is used as background binding, and binding efficiencies are expressed as relative to the background binding: high binding (more than 4 fold) is represented as (+++) , middle binding (3-4 fold) is represented as (++), low binding (2-3 fold) is represented as (+), and no binding (less than 2-fold) is represented as (-)

While designing the SELEX without negative selection step we were curious about the specificities of aptamers that we would find. We were expecting some aptamers with affinity to common proteins or molecules that can be found on many cell lines. But results are showing that a long run SELEX with smooth and stringent selection is resulting in very specific aptamers for cells. In our result it can be seen that all aptamers were binding to all glioblastoma cell lines monitored, but not binding other cell lines except CEM. Most importantly none of them was binding to HBE-135 which is a noncancerous epithelial cell line. This showed us that our aptamers can differentiate between cancerous and noncancerous cells. For more convenient proof we have to use brain tissues with healthy and cancerous parts.

3.5.5. Determination of target type

In order to determine if the target of our aptamers on the cell surface is protein or closely accompanied by proteins we repeated binding experiment using 2 different proteinases. To digest the surface proteins of the cells they were incubated with trypsin and proteinase K and then the binding experiments were repeated using aptamers with digested cells. If we examine the result in figure 3.15; all aptamers loosed it's binding after treatment with proteinase K. Proteinase K is broad spectrum serine proteinase and it's cleavage site is the peptide bond adjacent to carboxyl group of aliphatic and aromatic amino acids. Since it is a broad spectrum protease it removed all proteins and protein related molecules like glycoproteins. So that we can say that our aptamers most probably have protein targets or targets closely accompanied by proteins.

If we look for the results of trypsin cleavage; we can see that GMT-3 and GMT-5 did not lose their binding affinity to cells. Trypsin cuts the peptide bond specifically at the carboxyl end of the basic amino acids arginine and lysine. So it is not a broad spectrum protease as proteinase K. Binding of GMT-3 and GMT-5 with trypsin treated cells indicates that binding entities of these aptamers are resistant to trypsin cleavage.

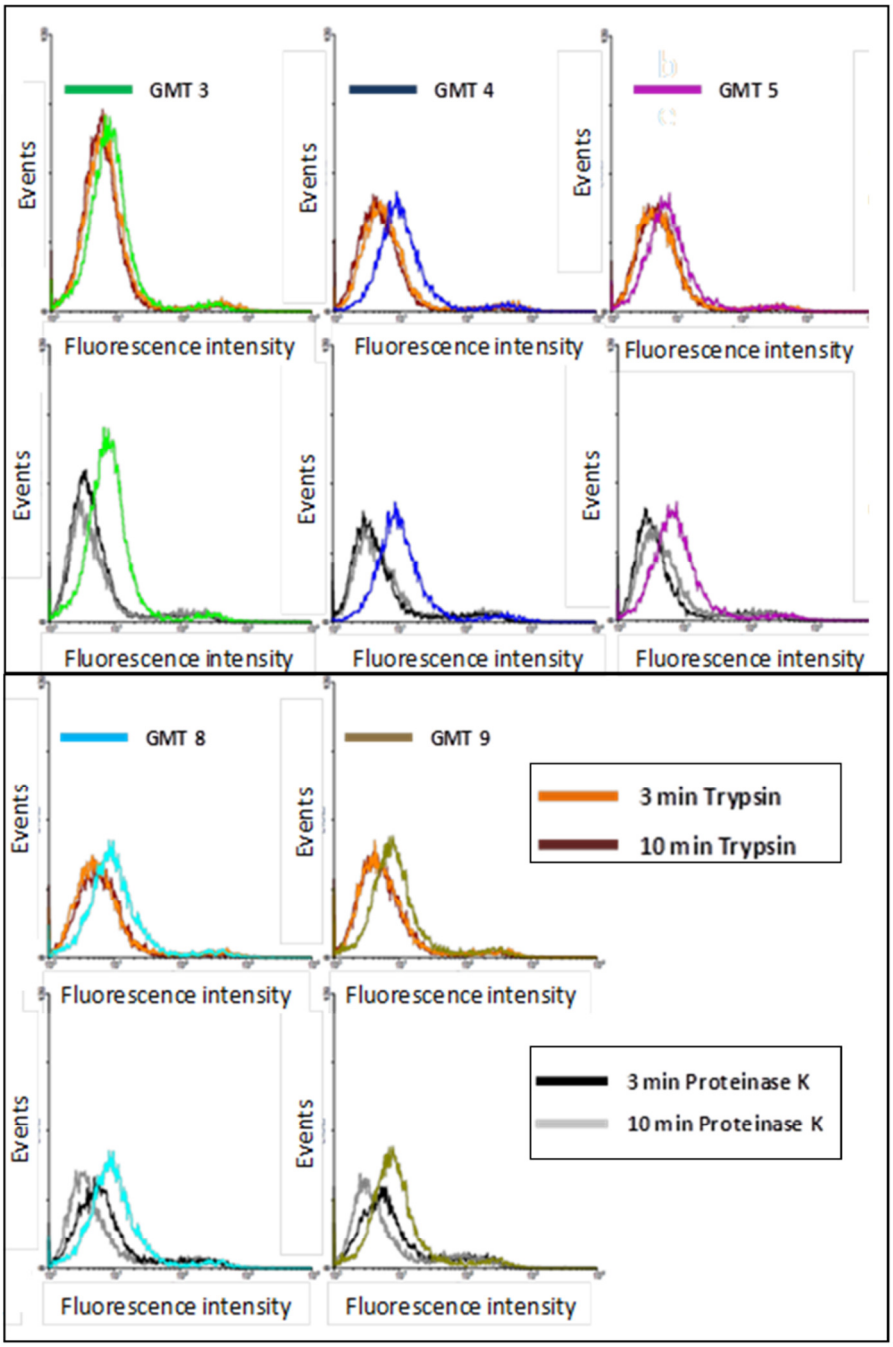


Figure 3.15 Binding of aptamers to trypsin and proteinase K treated A-172 cells for 3 and 10 minutes.

3.5.6. *3-D Structures of aptamers*

Identity shading and 3-D schemes of selected aptamers can be seen in figure 3.16. All size optimization studies are based carried out in the light of these structures.

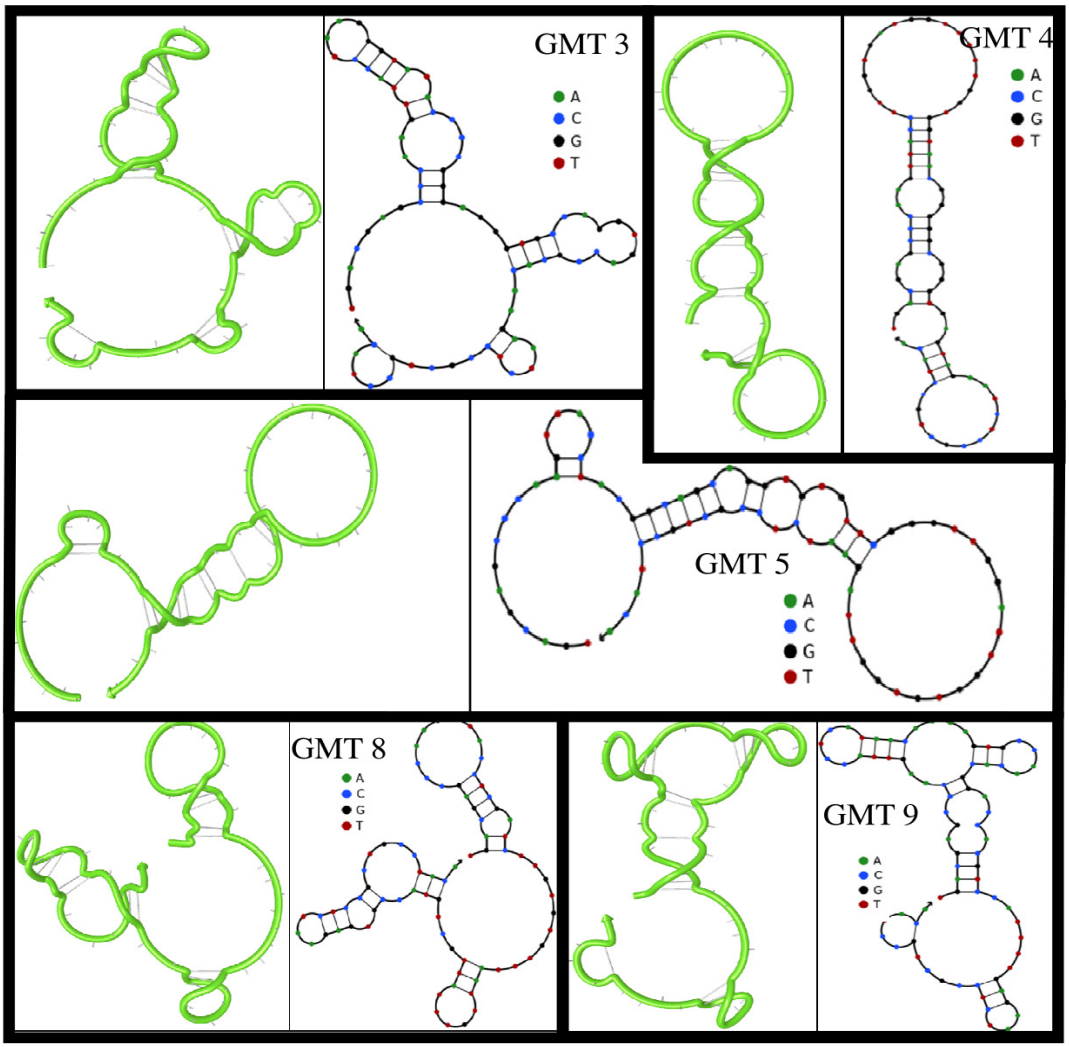


Figure 3.16 2-D structures of selected aptamers.

3.6. *Optimization of aptamer sequences*

SELEX was carried out using 80-base library with 40 random bases to be able to have enough diversity. So our aptamers were around 80 bases. Generally not all nucleotides are necessary for aptamers to bind their target. Generally a part of the aptamer which has a characteristic shape like hairpins is the sequence only interacting with the target. So that optimization of an aptamer size is an important process. Shortening the size of aptamers increases their tissue penetration. Also their stability generally increases with the removal of bulky flanks that do not have effect on binding. Another obvious advantage of shortening aptamers are lowering the costs and increasing the synthesis efficiency. For this purpose we used calculated 3-D shapes of aptamers and synthesized the different parts of aptamers.

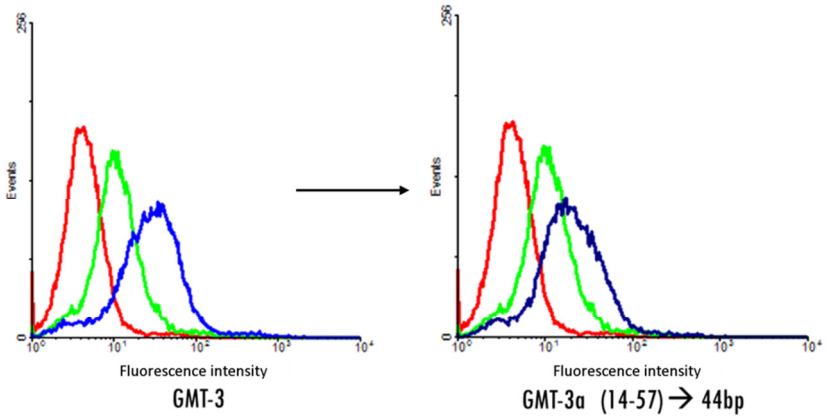


Figure 3.17 Binding of GMT-3 and GMT-3a to A-172 cells.

In figure 3.17 we can see that shortened GMT-3a was still binding to its target but with small decrease in binding. Also in figure 3.18 we examined 2 shortened sequence of GMT-4; GMT-4a includes the 35th to 75th base, and GMT-4b includes 43rd to 71st base of whole aptamer. Although GMT-4b was shorter and GMT-4a comprises GMT-4b, GMT-4b was binding better to its target than GMT-4a. So the bases from 35 to 43 were not needed and also decreased the binding stability. GMT-4b was a nice optimized aptamer with only 28 bases and good affinity.

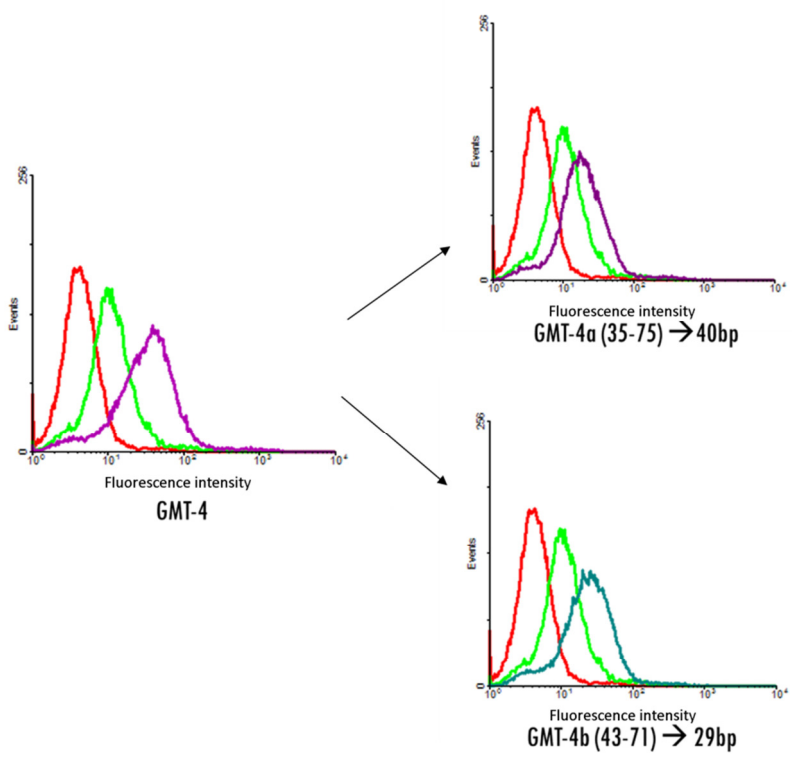


Figure 3.18Binding of GMT-4, GMT-4a, and GMT-4b to A-172 cells.

GMT-8 was just divided in to two parts for optimization study from the 27th base. The first 27 bases called GMT-8a had no binding affinity, but the remaining part, GMT-8b were still binding to its target (Figure 3.19). For GMT-9 we tried again two different parts of the whole aptamer. Although GMT-9a was holding the most of the parts of original aptamer with 50 bases it did not have any binding on target. GMT-9b was also not binding to its target (Figure 3.20). It was obvious that GMT-9 was interacting with its target using the shape formed by all its nucleotides.

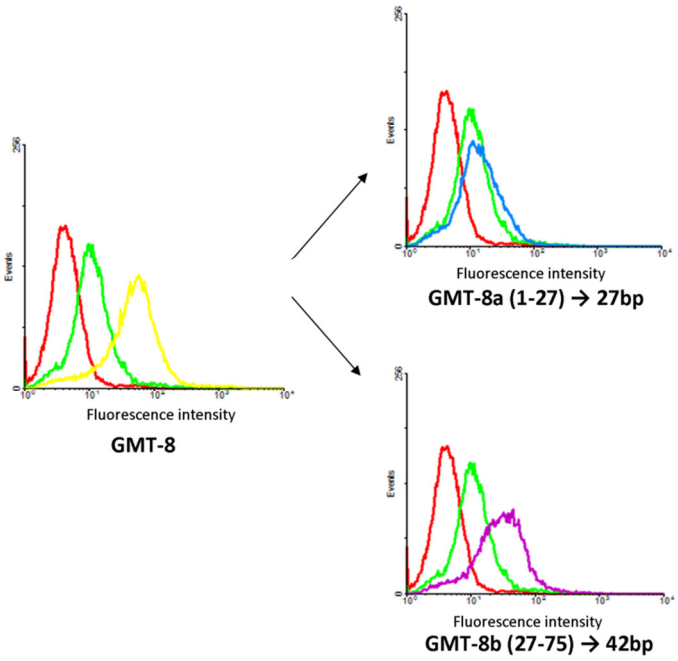


Figure 3.19 Binding of GMT-8, GMT-8a, and GMT-8b to A-172 cells.

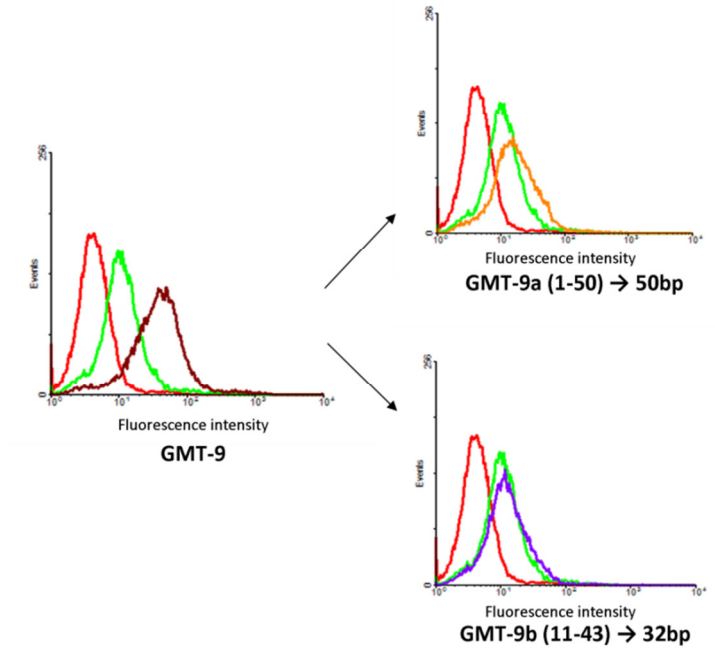


Figure 3.20 Binding of GMT-9, GMT-9a, and GMT-9b to A-172 cells.

3.7. Hybrid Aptamer

After optimization of the aptamer sizes, the best optimized small aptamers were chosen for hybridized aptamer study. GMT-3a and GMT-4b were suitable for this aim. They were small, have good binding and structures were conformable for consolidating them together. To not to lose their original structures many sorts of additions of nucleic acids were tried for the joining points. A thymine and three adenine addition theoretically saved the original shapes of the each aptamer. As it can be seen form figure 3.21 GMT-3a was binding to our cell with mean fluorescence of 21.97, GMT-4b was binding with mean fluorescence of 23.46 but GMT 3-4 hybrid was binding better with mean fluorescence of 30.18.

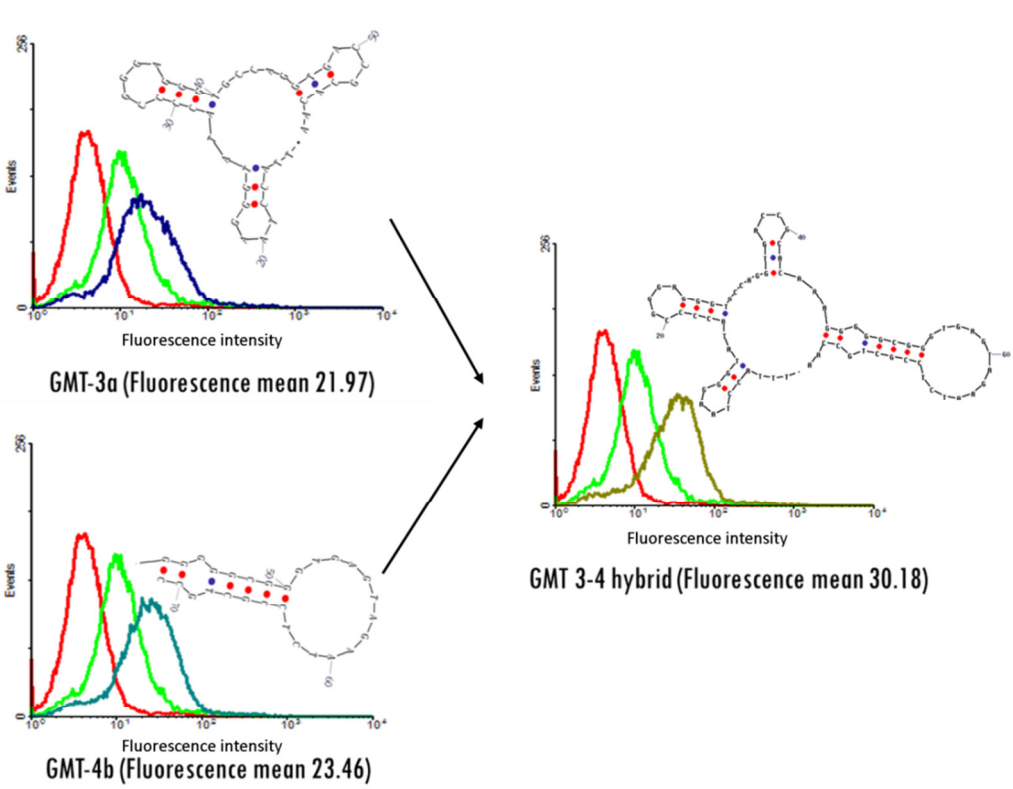


Figure 3.21 Binding of GMT-3a, GMT-4b, and GMT 3-4 hybrid.

3.8. Applications of aptamers

3.8.1. Cell imaging with aptamers

Selected aptamers firstly used as recognition elements for glioblastoma multiforme. In figure 3.22, 3.23, and 3.24 binding of selected aptamers to A-172 cell line was studied using fluorescence microscopy. It can be easily seen that library bound cells had very faint fluorescence considering to the aptamer bound cells. Interestingly GMT-3 and GMT-4 was binding on the main body of the glial cells (Figure 3.23) but GMT-5, GMT-8, and GMT-9 was binding to the arms of the glial cells. This showed that GMT-5, GMT-8, and GMT-9 have a target molecule that mainly expressed in the arms and GMT-3 and GMT-4 have target molecule mainly expressed in the main body. Also GMT-3 and GMT-4 may have targets that can be easily internalized after binding.

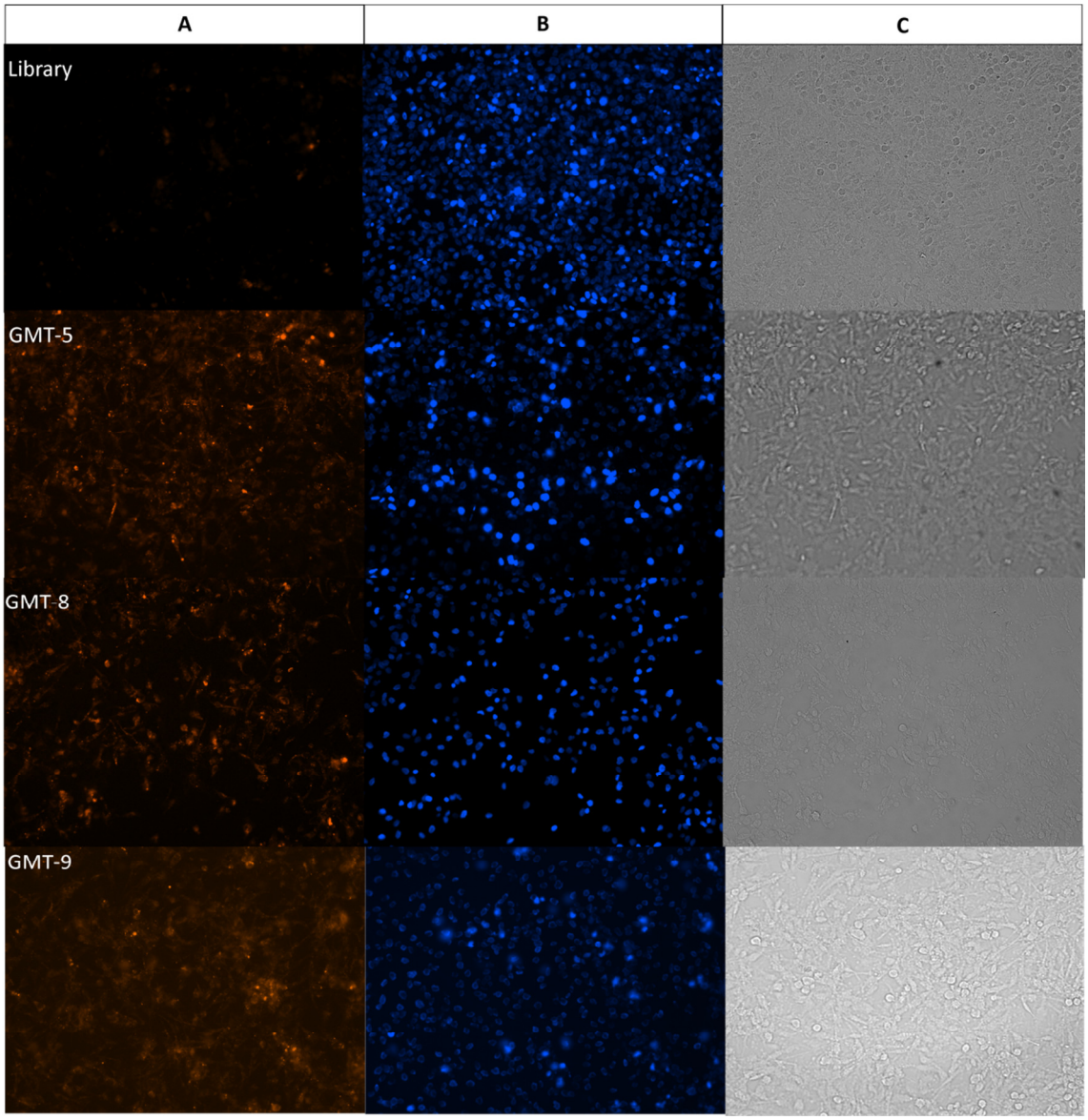


Figure 3.22 Microscopy images (10X) of library, GMT-5, GMT-8, and GMT-9. (A)fluorescence image, (B) DAPI image, (C) optical image.

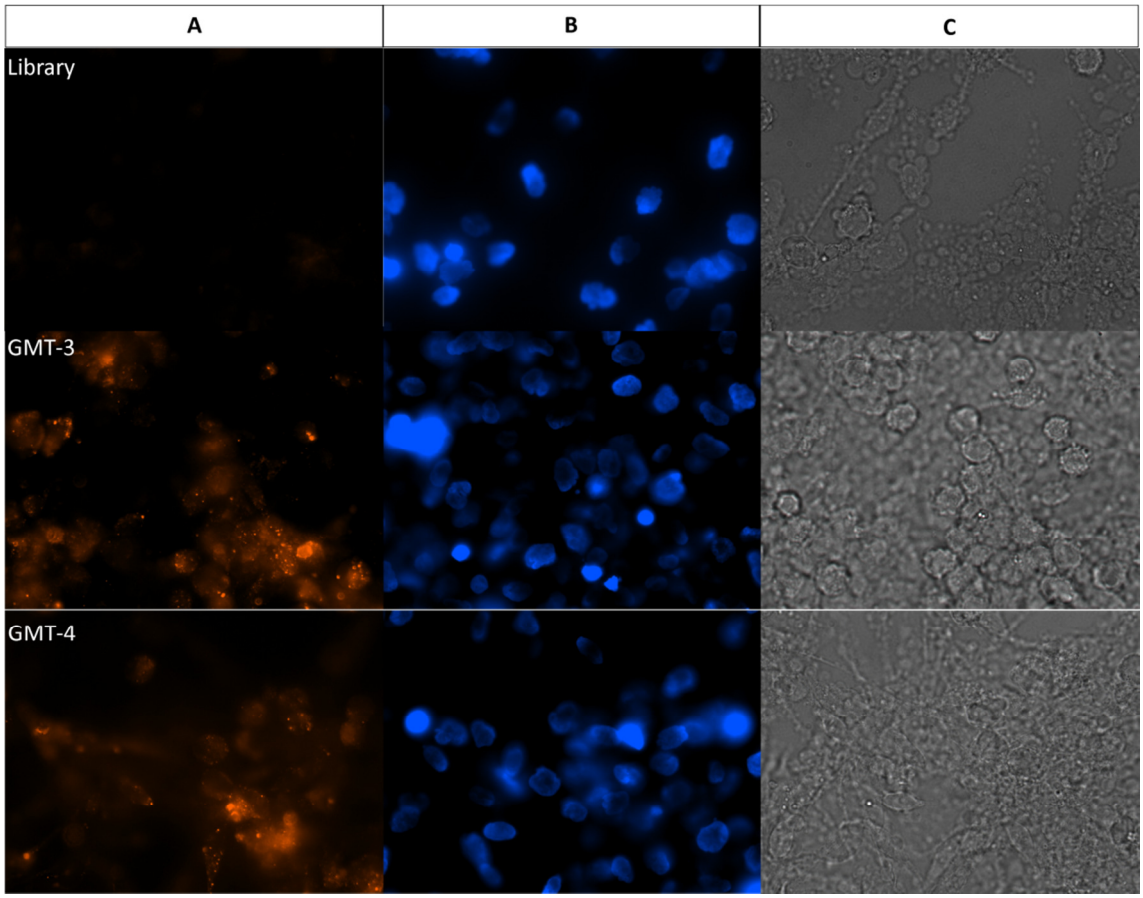


Figure 3.23 Microscopy images (63X) of library, GMT-3, and GMT-4. (A) fluorescence image, (B) DAPI image, (C) optical image.

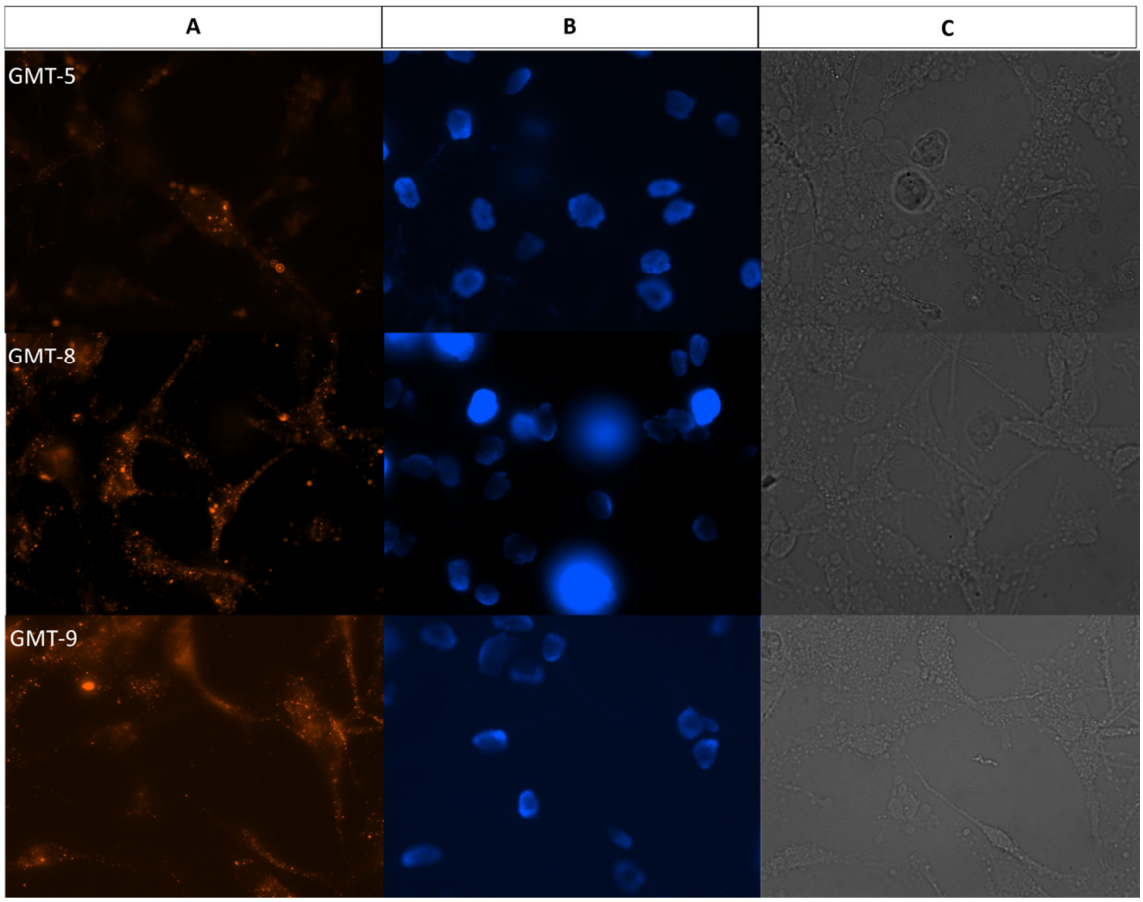


Figure 3.24 Microscopy images (63X) of GMT-5, GMT-8, and GMT-9. (A) fluorescence image, (B) DAPI image, (C) optical image.

3.8.2. Drug delivery using aptamers

3.8.2.1. Binding of dox to aptamers

Dox is a well-known anticancer drug which is known to intercalate within DNA strand due to the presence of flat aromatic rings in this molecule(Bagalkot et al. 2006). Dox intercalates into double stranded regions and form a physical noncovalent interaction(Min et al. 2011).

Binding of dox to different aptamers were designated using the fluorescence of dox molecule. Binding of dox to aptamers causes a quenching because of the close interaction of aromatic ring(Bagalkot et al. 2006). In figure 3.25 we see spectra of fluorescence quenching as a function of increasing aptamer concentration. In table 3.2 we see the maximum absorption at 595nm. We used these data to form quenching of tdo-5 and gmt-3 (Figure 3.26, 3.27). It is obvious that 1.25μM GMT-3 and 2.5μM of tdo5 is quenching the 1.5μM dox at max value. So that we used 1:1.20 molar ratio of GMT-3:dox and 1:0.6 molar ratio of tdo5:dox.

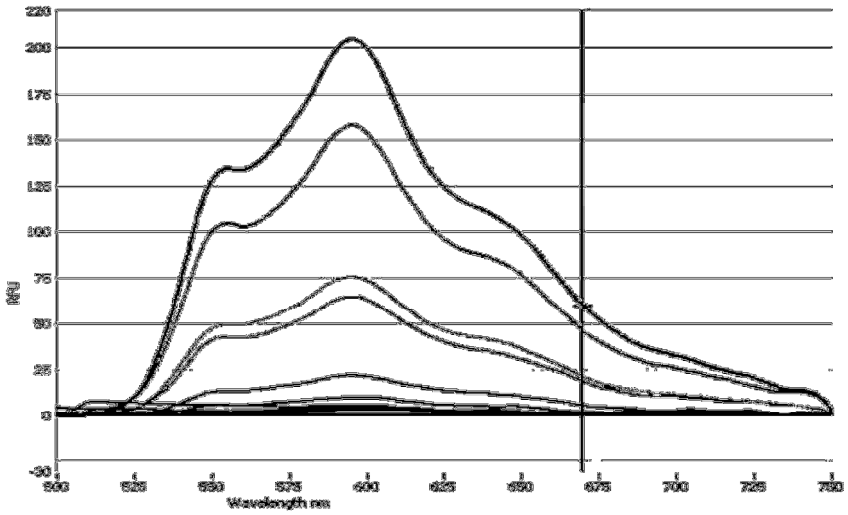


Figure 3. 25 Fluorescence spectra of doxorubucin solution (1.5μM) with increasing molar ratios of the GMT-3 (from bottom to top 10.00, 5.00, 2.50, 1.25, 0.62, 0.31, 0.15, and 0.07 μM).

Table 3. 2 Absorbance of GMT-3 and tdo5 with doxorubicin at 595nm

GMT-3 Concentration (μM)	RFU	tdo5 Concentration (μM)	RFU
0	204.90	0	306.60
0.03	157.50	0.03	249.50
0.07	75.40	0.07	234.00
0.15	64.80	0.15	191.75
0.31	22.00	0.31	114.25
0.62	10.10	0.62	90.90
1.25	3.90	1.25	63.30
2.50	3.60	2.50	37.60
5.00	3.30	5.00	14.80
10.00	5.50	10.00	12.55

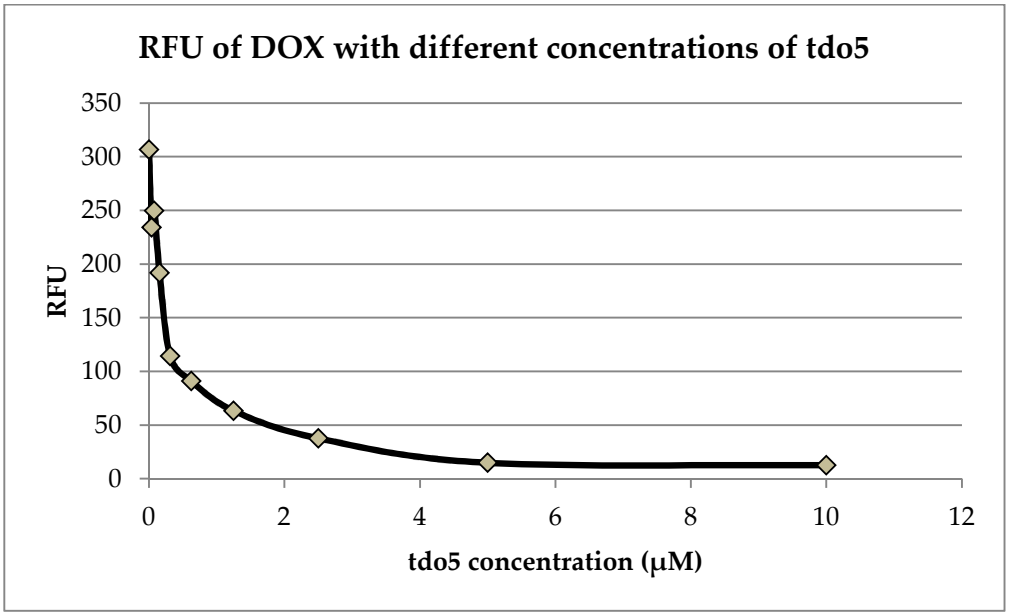


Figure 3. 26 RFU of DOX with different concentrations of tdo5

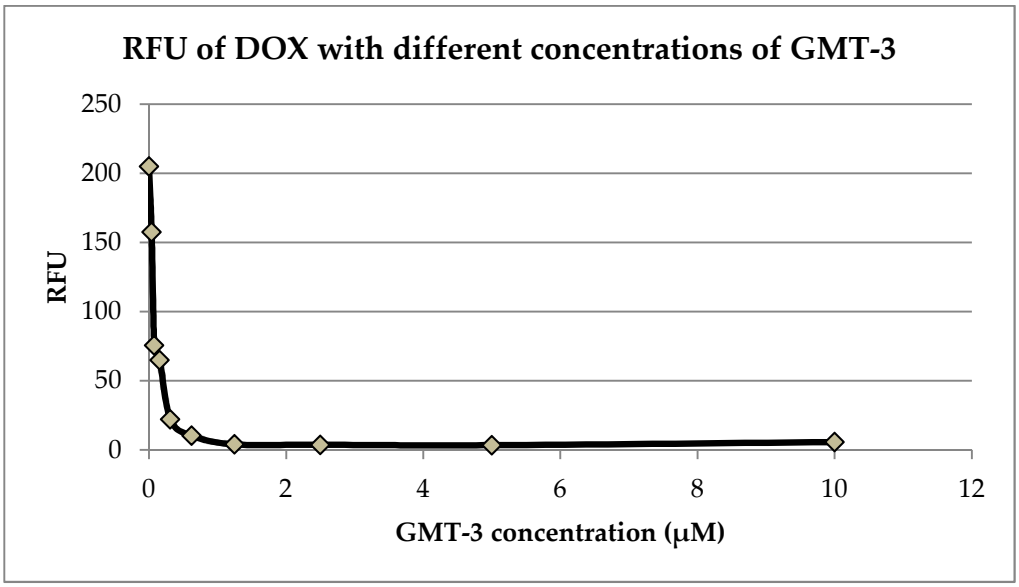


Figure 3. 27 RFU of DOX with different concentrations of GMT-3

3.8.2.2. Specific growth inhibition of doxorubicin bound aptamers

We next examined whether the targeted delivery of the aptamer-dox physical conjugate to specific cells results in enhanced cellular cytotoxicity compared to untreated cells. Same dose of dox is loaded on the aptamers and applied on the target cell line A-172 and target cell line MCF-7. The preliminary data demonstrated that GMT-3-dox conjugate caused more inhibition on growth for target cells (A-172) than the control cell line (MCF-7). The same dose of only dox application was causing cytotoxicity in both cell lines. Also control aptamer (tdo5) dox conjugate was not causing cytotoxicity in both cell lines. These results showed that GMT-3 is a promising aptamer to use in targeted drug delivery and can carry its cargo to its specific target but repeat of experiment is compulsory to prove it statistically.

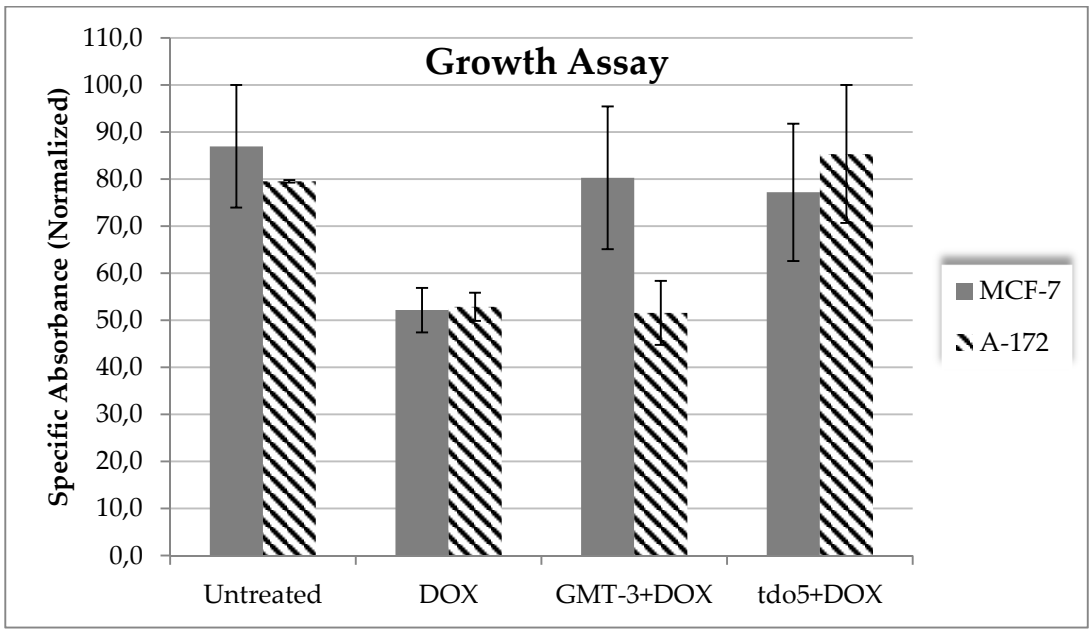


Figure 3. 28 Specific absorbances of A-172 and MCF-7 cells applied with DOX, GMT-3+DOX, and tdo5+DOX

CHAPTER 4

CONCLUSION

In this report, multiple rounds of cell-SELEX without negative selection were applied to obtain molecular probes for glioblastoma multiforme. A 454 pyrosequencing technology was used to monitor SELEX to select aptamers from the pool sequences. Bioinformatics was used extensively to be able to handle the large amount of data in the study. By using new sequencing technology and bioinformatics, more representative sequences were easily selected from the pools. Also evolution of sequences throughout the SELEX can easily be observed by bioinformatics tools. Selected aptamers showed high affinity to different glioblastoma cell lines of A-172, U87MG, GMBJ1 and showed little or no affinity to other cancer cell lines of MCF-7, CEM, RAMOS, H23, HT29, HBE-135, and CAOV-3. Most of the selected aptamers had Kd values in the nanomolar range and were very specific. It is clear that usage of new sequencing technologies with the help of bioinformatics can provide early and detailed information for progress of SELEX by displaying the intensity of selected sequences in the earlier rounds.

Selected aptamers were used as tools in some applications. First of all they were used as recognition elements for their specific target in microscopy. They displayed a distinct binding on their target with observable microscopy images. Also a preliminary experiment is carried out with a selected aptamer to be used as drug delivery vehicle. Aptamer loaded with doxorubicin showed more growth inhibition

on target cells than control cell line. Also control aptamer didn't showed growth inhibition on any cell lines.

Based on these studies we plan to improve drug delivery using aptamer functionalized drug carriers. Final goal of the study is the usage of these functionalized carriers both as recognition and drug delivery elements in live animal models.

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APPENDIX A

SEQUENCING DATA

CURRICULUM VITAE

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Surname, Name: Bayraç, Abdullah Tahir
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EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biotechnology	2004
BS	METU Biology	2002
High School	Karaman High School, Karaman	1997

WORK EXPERIENCE

Year	Place	Enrollment
2003-2011	METU Department of Biotechnology	Research Assistant
2009-2010	Chemistry Dept., Florida University	Visiting Scholor
2001-2002	Institute of Oncology, Hacettepe Uni.	Internship

PUBLICATIONS

1. Bayrac AT, Sefah K, Parekh P, Bayrac C, Gulbakan B, Oktem HA, Tan W, (2011) In Vitro Selection of DNA Aptamers to Glioblastoma Multiforme, ACS Chemical Neuroscience 2 (3) p. 175-181

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3. Öktem HA, Eyidogan F, Demirbas D, Bayraç AT, Öz MT, Özgür E, Selçuk F, Yücel M "Antioxidant Responses of Lentil to Cold and Drought Stress" (2008) Journal of Plant Biochemistry and Biotechnology Volume 17-1