RNA-Based Aptamer for the Diagnosis and Targeted Therapy of Glioblastoma

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Aptamers are short segments of nucleic acids that bind to a specific target molecule and are becoming increasingly popular in cancer diagnosis and treatment. Glioblastoma is cancer of the glial cells and it can surround nerve endings in the brain. Integrin $\alpha5\beta1$ receptors are present on the surface of these glial cells and are involved in cell communication with the extracellular matrix. In addition, this receptor plays a major role in cancer cell proliferation, tumor angiogenesis, tumor invasion, and metastasis. In addition, due to their overexpression on cancer cells they are considered as biomarker for glioblastoma and other cancers. Recently, aptamer H02 binding to these integrin receptors has been developed that distinguishes the glioblastoma cells from normal cells. We hypothesize that specific binding of H02 aptamer to the glioblastoma should be due to the structural and electrostatic interactions between the receptor and the aptamer. In this work, we have studied RNA-based aptamers targeting the Integrin $\alpha5\beta1$ receptor of glial cells. Computational tools have been used to predict the 3D structure of these aptamers and their interaction with the Integrin $\alpha5\beta1$ receptor protein. The secondary and tertiary structures of the RNA aptamers were predicted using the Vfold2D and Vfold3D 2.0 web servers. The aptamer-protein docking was performed using HADDOCK software. Based on our docking simulations the aptamers form strong interactions to the binding site of the $\beta1$ component of the receptor. This work will further assist in designing target-specific RNA-based aptamers to aid in the detection of glioblastoma tumor cells and developing novel therapeutic strategies against this disease.

1. INTRODUCTION

Glioblastoma is a type of aggressive brain cancer that forms from star-shaped glial cells called astrocytes.(1) It is also known as glioblastoma multiforme (GBM) because it has a tendency to contain many different types of cells.(2) Glioblastomas are classified as grade IV tumors, according to the World Health Organization (WHO) grading system for brain tumors, meaning that they are extremely malignant and commonly metastasize.(2,3) It is the most common primary malignant brain tumor in adults, and according to the American Brain Tumor Association (ABTA), it represents approximately 14.9% of all primary brain tumors.(4) Standard therapy consists of surgical resection to an extent that is safely feasible, followed by radiotherapy and concomitant chemotherapy with temozolomide.(5) Despite these therapies, patients with GBM rarely live longer than 2 years.(6) While the exact cause of glioblastoma is not fully understood, there are a few known risk factors, including exposure to radiation, genetic predisposition, and older age.(2) Symptoms of glioblastoma can vary depending on the location and size of the tumor, but they may include headaches, seizures, cognitive deficits, personality or behavior changes, nausea, vomiting, and weakness in one side of the body.(7) Patients with glioblastoma generally have a high mortality rate after diagnosis.(7) Even with treatments like surgery, radiation therapy, and chemotherapy, the average survival time for patients is typically around 12 to 15 months.(8) Only a small percentage of patients survive beyond five years.(8) Treatment for this cancer generally involves a combination of the interventions listed above, especially surgery and radiation therapy.(2,9) Some patients may also be eligible for clinical trials investigating new treatments or therapies.(10) Most of these treatment methods, however, do not manage the tumor well, as glioblastoma is highly invasive and resistant, and is therefore hard to manage.(10) Currently, there is new research aimed at better understanding glioblastoma biology, identifying new treatment targets, and developing more effective therapies, including immunotherapy, targeted therapy, and personalized medicine approaches.(11)

The integrin $\alpha5\beta1$ receptor is a member of the integrin family, and is a cell surface protein complex that is essential for signaling and cell adhesion.(12) It functions as a receptor for fibronectin, an extracellular matrix protein involved in cell migration, prolif-

eration, and differentiation.(13,14) It is made up of two subunits, $\alpha 5$ and $\beta 1$.(15) A wide range of cell types, including glioblastoma cancer cells, express the $\alpha 5\beta 1$ integrin, Figure 1a. $\alpha 5\beta 1$ increases cell attachment to the extracellular matrix through its interaction with fibronectin, which leads to cell mobility and tissue remodeling processes necessary for immune response, wound healing, and development.(13,16) A possible therapeutic target for intervention is uncontrollable $\alpha 5\beta 1$ expression or activity, which has been linked to a number of pathological diseases, including angiogenesis, fibrosis, and cancer metastasis.(17) The molecular mechanisms supporting $\alpha 5\beta 1$ function are being thoroughly investigated by researchers, who are also looking for ways to control its activity for therapeutic purposes, especially in tissue engineering and cancer treatment.(17) Therefore, ligands that can interact or inhibit can be used in diagnostic or therapeutic functioning in this disease.(18) To understand the binding interactions between the Integrin $\alpha 5\beta 1$ receptors and RNA based aptamer. The Integrin $\alpha 5\beta 1$ receptors are present on the surface of the glial cells. Synthetic RNA based aptamer can be designed that can not only bind but also inhibit these receptors, hence, can be used for both diagnostic and therapeutic function. Our hypothesis is that RNA based aptamers will interact strongly with the Integrin $\alpha 5\beta 1$ receptors which will help in inhibiting the receptor.

Aptamers are short, single-stranded nucleic acid or peptide molecules that can bind to specific target molecules with high specificity.(19) They are generated through a process called systematic evolution of ligands by exponential enrichment (SELEX), where a large library of random sequences is screened for binding to the target of interest.(20) Aptamers have gained much attention for their potential applications in various fields, including medicine, diagnostics, and therapeutics.(20,21) Due to their ability to recognize and bind to a wide range of targets, including proteins, small molecules, and even whole cells, aptamers have been studied for possible use in targeted drug delivery, disease diagnosis, biomarker detection, and imaging. Their flexibility, stability, consistency, and easily modifiable structure make them possible alternatives to antibodies in many applications. As research into aptamers continues to advance, their utility and impact across many scientific fields are expected to grow, contributing to advancements in biotechnology and healthcare. The application of aptamers in the diagnosis and targeted treatment of glioblastoma is shown in Figure 1 b and c.

Molecular docking is a computational technique used in drug discovery to predict how small molecules, such as potential drugs, interact with a target protein.(22,23) By simulating the binding process between a ligand (the small molecule) and a receptor (the target protein), molecular docking helps researchers understand the strength and geometry of their interaction.(24) This method plays a helpful role in identifying potential drug candidates by evaluating their binding affinity and specificity to the target protein. Through molecular docking, scientists can explore chemical space efficiently, accelerating the drug discovery process.

Recently, Fechter et al. determined the effectiveness of nucleic acid aptamers on therapeutic and diagnostic strategies relating to cancer.(16) A specific SELEX (selective evolution of ligands by exponential enrichment) strategy was used to find which sequences of nucleic acids would be the most effective, and it was found that the H02 aptamer was the most effective. The results of the study were promising, however further experimentation and analysis must be done to determine if these aptamers are a viable solution to eradicating tumors in a human cellular environment. In glioblastoma, the integrin receptors are overexpressed which means that these proteins are produced in higher quantities on the cancer cell surface.(25) Therefore, this receptor can be a great tool for cancer cell detection and in some cases targeted therapy. Recently, aptamer H02 binding to these integrin receptors has been developed that distinguishes the glioblastoma cells from normal cells. We hypothesize that specific binding of H02 aptamer to the glioblastoma should be due to the structural and electrostatic interactions between the receptor and the aptamer. In the current work, we have performed in silico investigation to understand the binding interaction mechanism and also have proposed that H02 aptamer cancer cell detection ability. Our computational results are in agreement to our proposed hypothesis and also to the experimental studies. We suggest that H02 interactions (binding location and electrostatics) to the receptor are different from other receptors. The current study will not only help in developing an aptamer as a cancer cell biomarker but can also aid in developing a therapeutic agent for targeted drug delivery.

2. RESULTS

We hypothesize that the aptamer will bind to a specific binding site of the integrin protein. To predict if this specific site is the druggable site, we have utilized graph neural networks (GNN). Specifically, we have used the GRaph Attention Site Prediction (GrASP) web server.(26) The druggable site is shown in Figure 2a. In this image, the druggable site is shown in yellow and green. It is important to note, that the site is too small for aptamer to access. The electrostatic surface potential (ESP) of the receptor was also computed using the ChimeraX software(27), shown in Figure 2b. The electrostatic surface potential of a protein is a measure of the distribution of electric charges on the protein's surface. It provides insights into how the protein interacts with other molecules, such as ligands, DNA, or other proteins. The positive regions are in blue, negative in red, and neutral in white. ESP shows that the aptamer binding region is mostly neutral in nature as shown in the box in Figure 2b.

The secondary structure of aptamers was predicted using Vfold2D, which produced the 2D image shown in Figure 3 and dot-bracket notation shown in Table 1. The secondary structure of the aptamer reveals its base pair interactions (hydrogen bonds) and is crucial in determining the 3D shape of the aptamer. Dot-bracket notation is a common way to represent the secondary structure of nucleic acids, including aptamers. This notation uses dots, brackets, and other symbols to indicate paired and unpaired bases. There are two reasons these 2D structures are predicted: (1) the 2D images also give a dot-bracket notations of the specific aptamers which helps in determining the 3D structure of these aptamer; and (2) many researchers use these 2D images to predict the similarity/difference between the aptamers based on the hypothesis that similar 2D aptamers should have similar binding patterns. However, in this case all the aptamer secondary structures are fairly different and have different binding patterns.

The molecular docking simulations were predicted using HADDOCK web server and the docked structures are shown in Figure 4. The HADDOCK software has been extensively validated for protein-nucleic acid complexes; hence, was used in this study. The docking was performed in three sampling steps; First 10,000 rigid body docking were performed in which the protein and aptamers are kept rigid. In the second step, 400 semi-flexible refinement docking was performed to add flexibility in

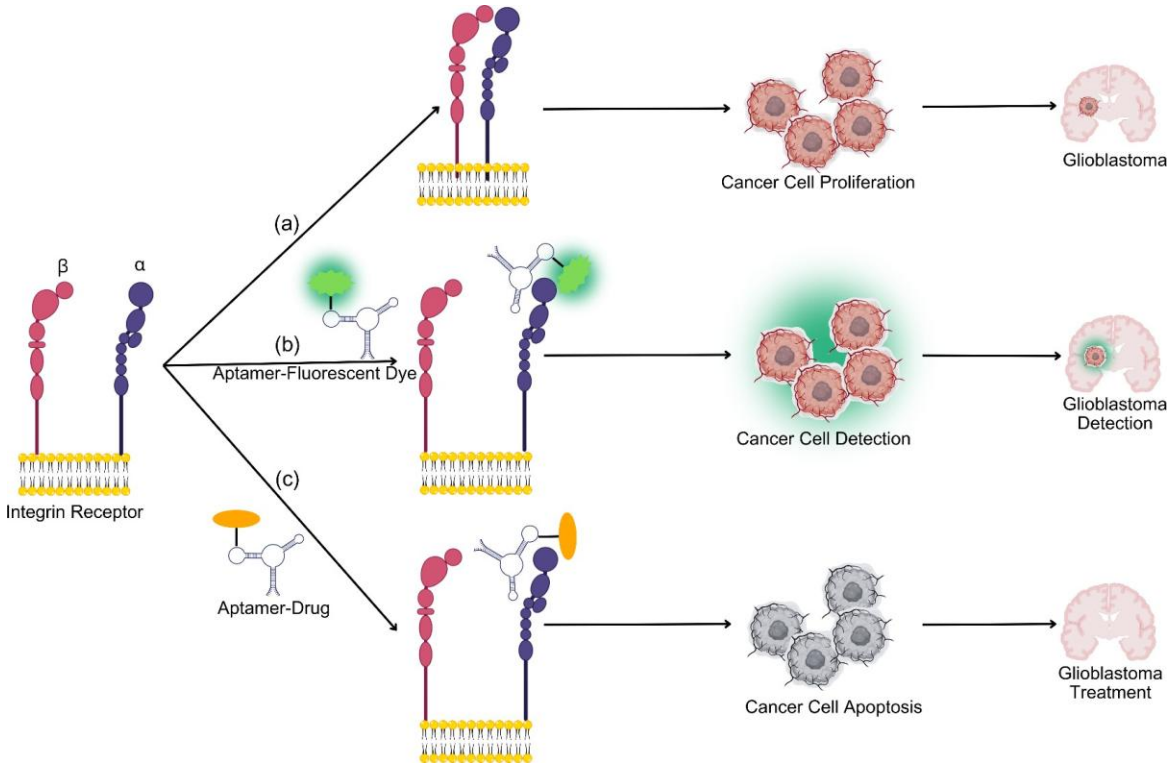

Fig. 1. The research scheme utilized in this paper. (a) Dysregulation of the integrin receptor leads to cancer cell proliferation, contributing to glioblastoma development; (b) fluorescent dye-conjugated aptamers can bind to the alpha subunit of the integrin receptor, serving as potential cancer biomarkers; and (c) Anticancer drug-conjugated aptamers can also bind to the integrin receptor, providing targeted therapy to induce apoptosis in cancer cells.

the protein and aptamer. Finally, the final refinement which included water in the docking process was done. Based on the docking simulations, the aptamer binding pattern was similar in all the aptamers; i.e., they all bind to a specific region of the receptor. The region is shown in Figure 2b and ESP shows that the region is neutral in nature. The α subunit-aptamers interactions were computed using PLIP analysis tools, Figure 5 and Table 2. Based on the analysis, aptamer G11 formed two hydrogen bonds with Asn44 and Arg122 at a distance of 2.62 and 2.72 Å, respectively. In addition, it formed four salt bridges with Lys42, Lys119, Arg122, and Lys369 at a distance of 5.01, 3.86, 5.33, and 3.90 Å, respectively. The H03 aptamer formed one hydrogen bond with Gln156 with a distance of 1.56 Å and three hydrogen bonds with Tyr221 with distances of 2.47, 3.44, and 3.71 Å. Additionally, it formed three salt bridges with Arg122, Arg339, and Lys369 at distances of 5.17, 4.49, and 4.41 Å, respectively. The third aptamer, B03, formed one hydrogen bond with Asn44 at a distance of 2.88 Å, two hydrogen bonds with Thr45 at distances of 3.34 and 2.52 Å, two hydrogen bonds with Gly49 at 3.13 and 2.82 Å, and one hydrogen bond with Lys369 at 2.97 Å. It also formed four salt bridges: two with Lys308 at distances of 5.43 and 3.53 Å, one with Arg339 at a distance of 5.05 Å, and one with Lys369 at a distance of 4.39 Å. Aptamer G11 formed a total of eleven hydrogen bonds with Asp6, Asp6, Ser7, Ser12, Gly13, Ser90, Asp219, Arg245, Ala273, Arg431, and Gln575 at distances of 3.29, 3.29, 3.16, 4.05, 3.18, 3.67, 3.78, 3.86, 2.86, 3.65, 3.29 Å. It also formed four salt bridges: one with Arg65 at a distance of 4.85 Å, two with His91 at distances of 4.84 and 5.22 Å, and one with Arg245 at a distance of 5.18 Å. The final aptamer, H02 formed five hydrogen bonds: one with Asp368 a distance of 3.06 Å, one with Lsy369 at a distance of 2.42 Å, two with Ala397 at distances of 3.40 and 2.21 Å, and one with Arg431 at a distance of 2.61 Å. It also formed salt bridges with Lys308, Lys369, Arg398, and Arg431 at distances of 5.20, 5.03, 4.91, and 5.09 Å, respectively.

3. DISCUSSION

The alpha and beta subunit contains a ligand binding region for extracellular matrix proteins (fibronectin, collagen, laminin), and is primarily located in the interface between the α and β subunits. The β-propeller of the α subunit and the β-I domain of the β subunit create a binding pocket. The interaction between the α and β subunits is crucial for integrin activation and function. Integrins can exist in inactive (bent) and active (extended) conformations. Activation involves a conformational change that increases the affinity for ligands. This is often regulated by inside-out signaling, where intracellular signals induce changes in the integrin's cytoplasmic tails, leading to separation and subsequent conformational changes in the extracellular domains. Biomarkers are chemical indicators that are used for cancer diagnosis and prognosis and in some cases targeted drug delivery. Glioblastoma is the most aggressive and deadly form of brain tumor. Although intensive treatment of this disease is available the prognosis of this disease remains poor. Therefore, there is an urgent need to develop biomarkers for early detection, disease prognosis, and making treatment decisions. Integrin receptor undergoes overexpression in glioblastoma cells and can be an important tool in cancer detection. In addition, the receptor is

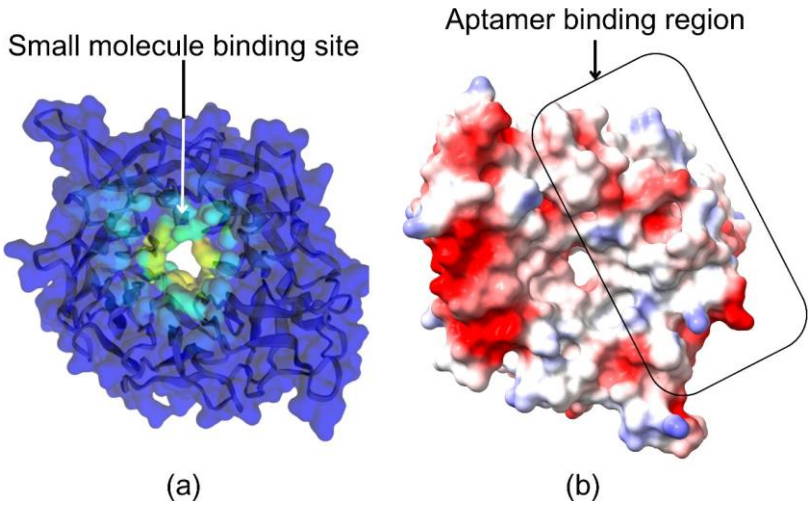

Fig. 2. Druggable site and electrostatic surface potential (ESP) of the receptor. (a) The druggable site was predicted using the GrASP web server and is shown in yellow and green. The druggable site is relatively small, rendering it unaccommodating for aptamer binding. (b) The ESP of the receptor indicates that the aptamer binding region is neutral (white) in nature, while the rest of the region is negative (red). All the aptamers bind to this neutral region.

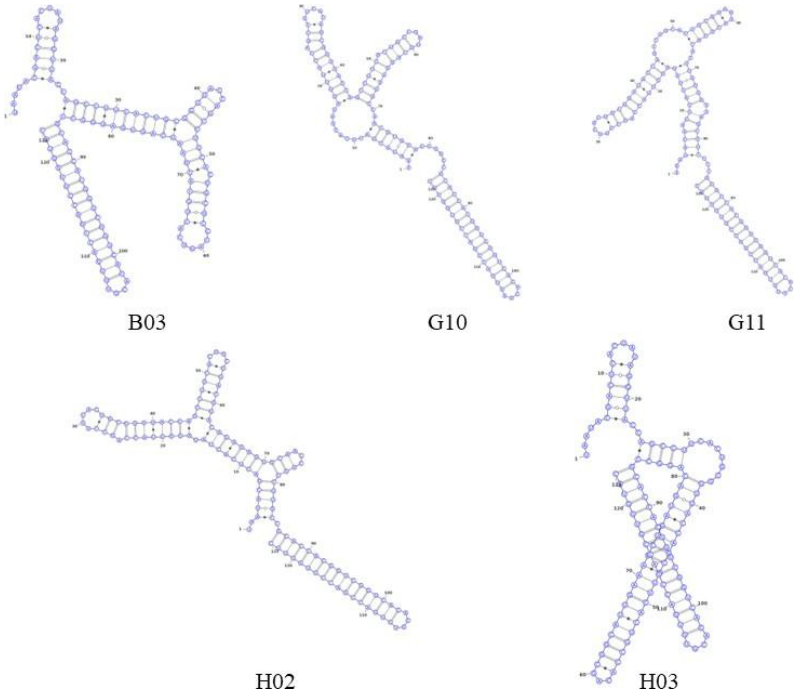

Fig. 3. Secondary (2D) structure of aptamers. This figure illustrates the specific folding patterns formed by the base pair interactions within the aptamer sequences.

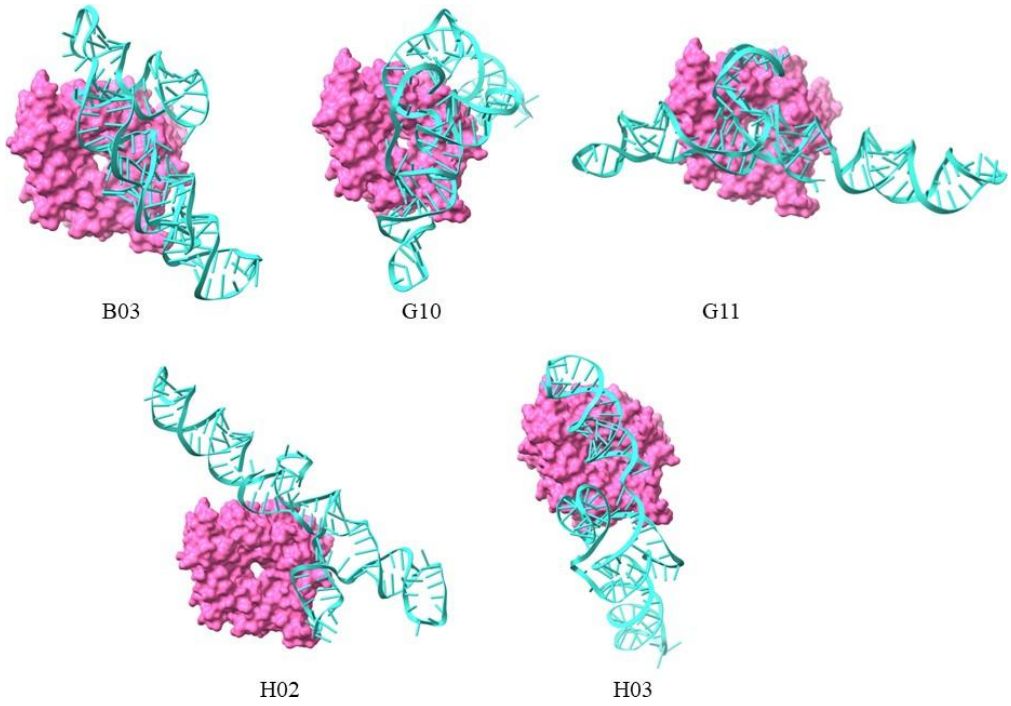

Fig. 4. Protein-aptamer docked structure. The 3D docked structure of the protein and aptamers shows that the aptamers bind to the α subunit of the receptor. Except for H02, all the aptamers completely cover the β subunit binding region, which is unlikely as the α and β subunits interact with each other. We propose that the side binding of the H02 aptamer to the α subunit could be the inhibiting mechanism of this aptamer.

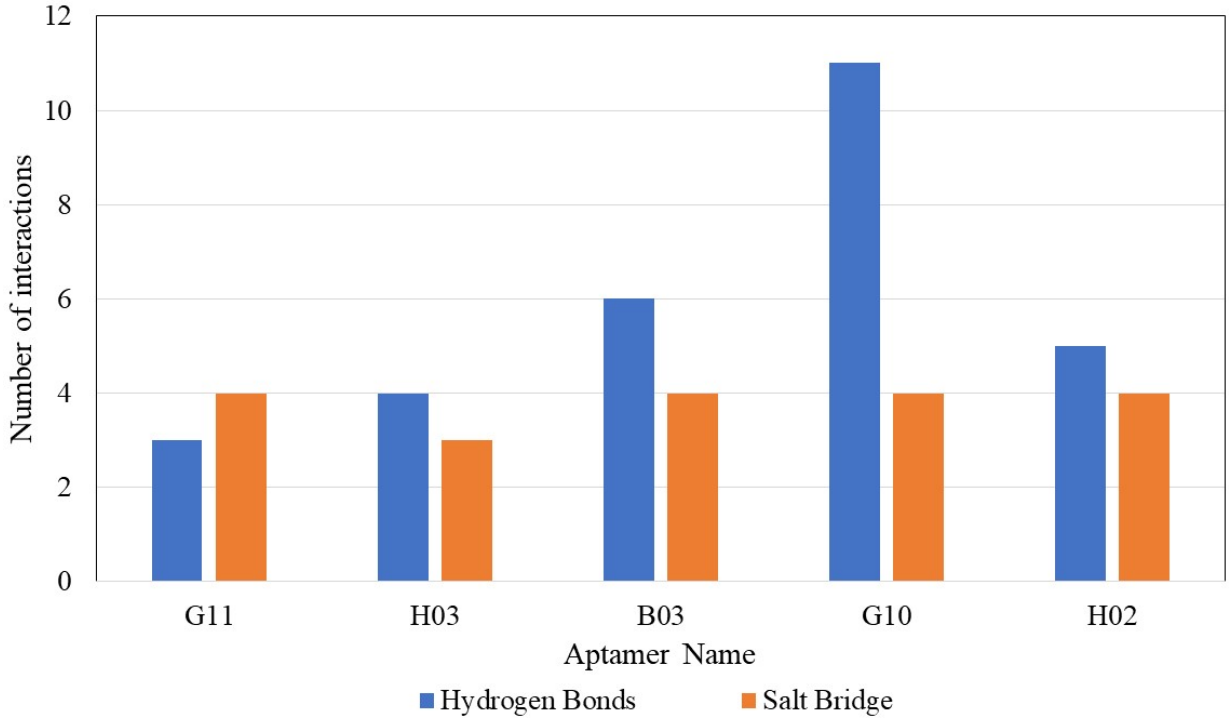

Fig. 5. Bar graph showing the number of interactions formed between integrin and aptamers.

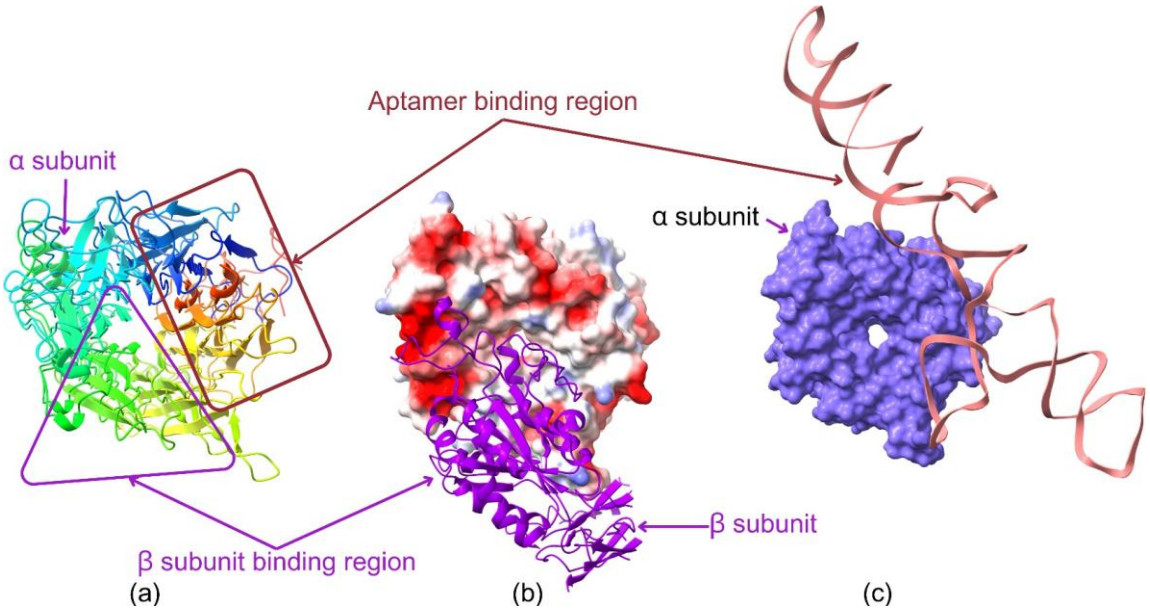

Fig. 6. Aptamer binding region of the receptor.(a) the aptamer binding region is shown in a rectangle, and the subunit binding region is shown in a triangle; (b) the α subunit (in purple) and β subunit (ESP) interactions are shown in this image; and (c) the aptamer is shown in turquoise and β subunit is shown in blue.

present on the cancer cell surface accessibility of drugs to this receptor is easy in comparison to other cancer proteins.

Integrins are composed of two subunits: alpha (α) and beta (β), which come together to form a heterodimer. Based on the X-ray structure, the alpha and beta subunits do not completely bind to each other. Only half of the beta subunit covers the alpha subunit, as shown in the purple triangle in Figures 6a and b. Additionally, the aptamer binding region is shown in Figures 6a and c. It is worth mentioning that only H02 binds to the aptamer binding region and not to the beta subunit binding region. However, all the other aptamers bind to both the alpha and beta subunits. In the context of integrin activation and function, the head region, which includes the ligand-binding domains of the alpha (α) and beta (β) subunits, also undergoes conformational changes. However, in the head region, the separation of the subunits is more about conformational changes that increase the affinity for ligands rather than complete physical separation. Experimentally, only the H02 aptamer was able to bind to the integrin receptor, and therefore we propose that the binding interactions between the H02 aptamer and the alpha subunit could be the guiding reason.

Based on our molecular docking and molecular dynamics simulations we have predicted the integrin inhibition mechanism as follows (Figure 7): 1: Integrin in the inactive state: the integrin-ligand affinity is regulated by the cytoplasmic signals from within the cell, Figure 7a. This process is known as inside-out signaling. The integrins get activated intracellularly from GPCR causing the phosphorylation of the cytoplasmic domain of the β subunit. The inactive state of integrin is characterized by the association of α and β subunit in the receptor. The receptor is in bent conformations as observed in the crystallized structure. 2: Integrin in the active state: in the presence of agonists such as chemokines the activation of integrin takes place which is controlled by GPCR, Figure 7b. During this step the cytoskeletal adaptor protein has been proposed to play a key role in regulating the integrin affinity. In the active state the integrin switches to an upright position and dissociation of the α and β subunit. Cytoplasmic and transmembrane regions which cause the head region to extend outwards in a switchblade-like movement. Conversion from active to inactive takes place in seconds caused by conformational changes in the ligand binding pocket of the headpiece that increases its affinity for ligands. 3: Inhibition of integrin by aptamer: Since there is no physical separation between the α and β subunits, we assume that the active aptamer should bind to a region other than the α and β subunit interface, Figure 7c. Only the H02 aptamer binds to a similar region of the receptor, where the aptamer binding region and the beta subunit binding regions do not overlap. We assume that the receptor inhibition could be due to the blockage of the receptor's flexibility, and the receptor cannot change conformations from bent to erect state.

In the current research work, we have used an in silico approach to study the aptamer-integrin receptor interactions. Specifically, molecular docking simulations were performed to study the receptor-aptamer interactions. Based on the docking simulations, the H02 aptamer binds to the neutral region of the receptor and not to the beta subunit binding regions. Based on this, we have proposed the reason for H02's positive activity compared to other aptamers. In future, molecular dynamics simulations will be performed to further understand the aptamer

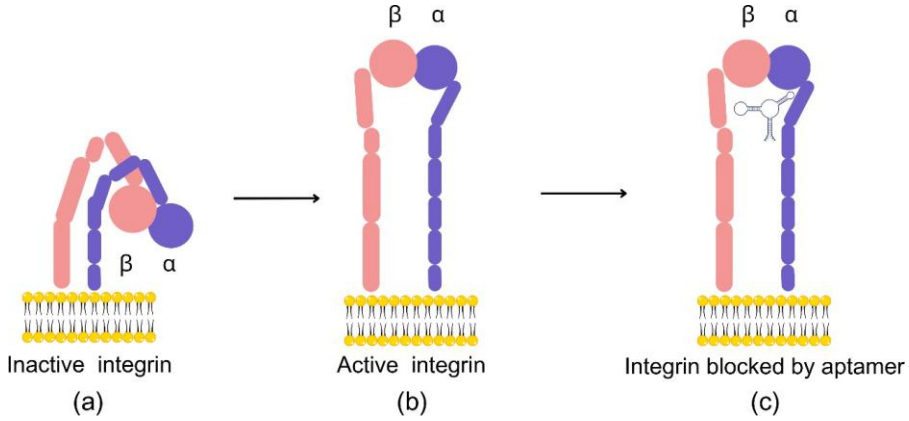

Fig. 7. Proposed inhibition mechanism of integrin receptor.(a) In the inactive state, the α and β subunit of integrin are in an inactive state in the absence of the substrate; (b) upon substrate binding, the two subunit comes into an upright position; and (c) our proposed aptamer-induced integrin inhibition mechanism suggests that the aptamer binds to the α subunit of the integrin receptor, making the two subunits rigid in the upright position. This loss of flexibility prevents the receptor from reacquiring an inactive state.

binding and integrin inhibition mechanism. Our efforts will help in understanding the aptamer binding mechanism and will aid in developing aptamers against the disease.

4. METHOD

The Integrin $\alpha 5\beta 1$ receptors 3D structure was obtained from Protein Data Bank (PDB ID 3VI4).(28) The RNA sequence was obtained from the previous work done by Fechter et. al.(16) The aptamer's primary nucleotide sequence served as the foundation for structural predictions. First, Vfold2D was used to determine the secondary structure of this sequence.(29) After receiving the nucleotide sequence directly, the server predicted the secondary structure and delivered it in dot-bracket notation. Dot-bracket representations outline the three-dimensional structure and purpose of aptamers. When a nucleotide in the aptamer sequence is unpaired, it is represented by a dot, while parentheses are used to represent paired nucleotides that form base pairs; the pairing is shown by matching opening and closing parentheses. For instance, a simple hairpin structure might be shown as ".....(((((.....))))). ", where dots represent unpaired bases and parentheses indicate the paired regions forming the stem of the hairpin. Following the secondary structure prediction, the tertiary structure was modeled using Vfold3D.(30) The resultant 3D structures were seen through molecular visualization tools such as ChimeraX.(27) Since the aptamers contained more than one cluster, the first aptamer which had the lowest energy state was taken for molecular docking simulations. After this, HADDOCK docking software was utilized for docking the aptamer to the integrin receptor.(31) Following the preparation of the input files, the receptor and ligand structures were uploaded to the HADDOCK software. Post-docking, the resulting complexes were analyzed to identify the most probable binding conformation based on the docking scores and interaction analysis. The final binding modes were visualized and further refined for detailed interaction studies using PyMOL.(32) The druggable site of the receptor was obtained by using a graph neural network (GNN) called GrASP.(26) GrASP utilizes a pretrained graph neural network model specifically designed for druggable site prediction. The graph was input into the GNN, which processed the structural information to identify potential binding pockets. Post-prediction, the identified sites were validated against known druggable sites to assess the accuracy of the predictions.

Protein-aptamer interactions. The amino acids and base pair interact through hydrogen bonds and salt bridges. The table shows the amino acids' names, numbers, and interaction distances (in angstroms). This information is crucial for making chemical mutations during various aptamer modification steps.

Table 1.

Aptamer (ID)	Aptamer Sequence and dot-bracket notations
H02	UAAUACGACUCACUAUAGGUUACCAGCCUUCACUGCGGUUACCAGCCUUCACUGCGGACG-GACAGAGAGUGCAACCUGCCGUGCCGCACCACGGUCGGUCACACGUGUGACCGACCGUGGUGC ..((((((((((((.((((((((.(((.....)))))))))))((((((....))))))))))))))))(((...)))))))).((((((((((((((((....)))))))))))))))))
G11	UAAUACGACUCACUAUAGGUUACCAGCCUUCACUGCGGUUACCAGCCUUCACUGCG-GAGUACGCACACUUGGUGUUAGCGUCCCCGCACCACGGUCGGUCACACGUGUGACCGACCGUGGUGC ...((((.((((((((((((((((.(((.....)))))))))))).....((((((...))))))..))))))..))))))...((((((((((((((((....)))))))))))))))))
H03	UAAUACGACUCACUAUAGGUUACCAGCCUUCACUGCGGUUACCAGCCUUCACUGC-CACAGCGGCAUUGAAAGCUGAUGACAGGCCGCACCACGGUCGGUCACACGUGUGACCGACCGUGGUGC .....((((((.....))))))..(((((........(((((((((((((((((((...)))))))))))))))))))))))(((((((((((((((....)))))))))))))))))
B03	UAAUACGACUCACUAUAGGUUACCAGCCUUCACUGCGGUUACCAGCCUUCACUGCGC-CCAGUCACUGCACGAAUCAGUGAUGGCCGCACCACGGUCGGUCACACGUGUGACCGACCGUGGUGC ..............((((((...))))))..(((((((((...[[[[..)))))))))...(((((((((.....)))))))))]]]](((((((((((((((....)))))))))))))))))
G10	UAAUACGACUCACUAUAGGUUACCAGCCUUCACUGCGGUUACCAGCCUUCACUGCC-GAUGCAGGAUGGCCAACGUGUACCUGCCGCACCACGGUCGGUCACACGUGUGACCGACCGUGGUGC .(((((((....[[[[(((((((((.(((.....)))))))))))((((((.((((....))))))))))..)))))))).]]]].((((((((((((((((....))))))))))))))))

Table 2. Aptamer sequences and dot-bracket notations.The dot-bracket notation is a common method for representing the secondary structure of aptamers. In this notation, dots (.) represent unpaired bases, while parentheses ((and)) indicate base pairs. Each pair of parentheses represents a specific base pair, with an opening parenthesis for one base and a closing parenthesis for its complementary pair.

	G11	
Hydrogen Bonds	**Amino acids**	**Distance (Å)**
	Asn44	2.62
	Arg122	2.72
Salt Bridges	Lys42	5.01
	Lys119	3.86
	Arg122	5.33
	Lys369	3.90
	H03	
Hydrogen Bonds	Gln156	3.21
	Tyr221	2.47
	Tyr221	3.44
	Tyr221	3.71
Salt Bridges	Arg122	zzz
	Arg339	4.49
	Lys369	4.41
	B03	
Hydrogen Bonds	Asn44	2.88
	Thr45	3.34
	Thr45	2.52
	Gly49	3.13
	Gly49	2.82
	Lys369	2.97
Salt Bridges	Lys308	5.43
	Lys308	3.53
	Arg339	5.05
	Lys369	4.39
	G10	
Hydrogen Bonds	Asp6	3.29
	Asp6	3.29
	Ser7	3.16
	Ser12	4.05
	Gly13	3.18
	Ser90	3.67

	Asp219	3.78
	Arg245	3.86
	Ala273	2.86
	Arg431	3.65
	Gln575	3.29
Salt Bridges	Arg65	4.85
	His91	4.84
	His91	5.22
	Arg245	5.18
	H02	
Hydrogen Bonds	Asp368	3.06
	Lsy369	2.42
	Ala397	3.40
	Ala397	2.21
	Arg431	2.61
Salt Bridges	Lys308	5.20
	Lys369	5.03
	Arg398	4.91
	Arg431	5.09