In-Silico Based Multi Epitope Vaccine Construction against Glioblastoma (GBM): A Comparative Study

by

Tauhidul Islam

Student ID: 21146062

A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy

School of Pharmacy

BRAC University

February 2026

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing a degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name and Signature:

_____

Tauhidul Islam

Student Id: 21146062

Approval

The thesis titled “In-Silico Based Multi Epitope Vaccine Construction against Glioblastoma (GBM): A Comparative Study” submitted by Tauhidul Islam (ID: 21146062) of Summer, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

Supervised By:

Supervisor:

Mohammad Kawsar Sharif Siam
Senior Lecturer
School of Pharmacy BRAC University

Approved By:

Chairperson:

Dr. Shamind Neelotpol, PhD
Professor and Chairperson
School of Pharmacy
BRAC University

Ethics Statement

This thesis was completed in full compliance with required ethical standards, with no involvement of any human participants or animal trials. Research integrity and ethical principles were strictly maintained throughout the study.

Abstract

The most aggressive and fatal malignant primary tumor of the central nervous system with rapid proliferation, extensive invasion, and frequent recurrence even following standard treatment is glioblastoma (GBM).The existing management approaches such as maximum surgical resection, radiotherapy, and temozolomide chemotherapy only offer limited survival benefits due to heterogeneity in tumors, resistance to treatments, evasion of immunity, and lack of ability of drug to penetrate all parts of the body via the blood brain barrier.Hence, there is an urgent need to develop effective immunotherapy plans like therapeutic vaccination to enhance the outcomes and prevent recurrence.

The proposed work provides an in silico multi-epitope vaccine design plan based on immunoinformatics against glioblastoma.The comparative analyses of calculations were carried out using seven chosen glioblastoma-related proteins.The screening and prioritization of the antigenic proteins were followed by prediction of cytotoxic T-lymphocyte (CTL), helper T-lymphocyte (HTL), and B-cell epitopes.Toxicity, allergenicity, and the antigenicity of the predicted epitopes were filtered to ensure the predicted epitopes are safe and effective antigens.Appropriate linkers and a component of adjuvants were used to reconstitute epitopes of interest into a multi-epitope vaccine construct to enhance the immunogenicity.The vaccine candidate that was designed was tested using physicochemical property analysis, structural modeling and validation, receptor-binding testing of the vaccine candidate using molecular docking, and immune simulation to estimate the potential of the immune response to the vaccine.

In general, this work offers a cost-efficient computational vaccine design framework and also provides a promising GBM vaccine candidate that can be subjected to experimental validation in the future.

Keywords

Glioblastoma, immunoinformatics, multi-epitope vaccine, CTL epitope, HTL epitope, docking, immune simulation.

Acknowledgement

I would like to take this opportunity to express my heartfelt gratitude to my supervisor, Mohammad Kawsar Sharif Siam, Senior Lecturer, School of Pharmacy, BRAC University, in his consistent supervision and guidance during my course of this study.His patience, encouragement and valuable feedback played a crucial role in ensuring the quality of this work and was critical in ensuring the completion of this thesis successfully.

Table of Content

List of Tables

List of Figures

List of Acronyms

BBB	Blood-Brain Barrier
CTL	Cytotoxic T- Lymphocyte
GBM	Glioblastoma
GRAVY	The Grand Average of Hydropathicity
HTL	Helper T-Lymphocyte
MHC	Major Histocompatibility complex
TLR	Toll-Like Receptor

Chapter 1
Introduction

Glioblastoma (GBM) is the most malignant and deadly primary tumor of the central nervous system (CNS), and exhibits fast-growing cellular growth, infiltrative spread to surrounding brain tissue, abundant new blood vessel formation and inevitable recurrence following aggressive multimodal therapy (Weller et al., 2017). Glioblastoma is defined in the most recent World Health Organization (WHO) classification of brain tumors as a WHO grade IV diffuse astrocytic glioma and is most frequently encountered in adults as IDH-wildtype glioblastoma, which has a much worse prognosis than low-grade gliomas (Weller et al., 2017). Glioblastoma is not communicable (cannot be spread from person to person) like a food-borne disease (e.g. salmonellosis), and it results from a series of cellular transformations and tumor-promoting molecular changes in the brain.

While glioblastoma can occur at any age, including in children (0-17 years), it most commonly affects adults (≥18 years) and occurs most commonly in middle-aged adults (40-64 years) and the elderly (≥65 years) (Neagu & Reardon, 2015). We know that there are distinct age-related patterns in disease outcome, with younger adults (18-39 years) experiencing fewer treatment-related complications and improved survival rates compared to those aged ≥60-65 years. By contrast, older patients have a more aggressive disease course and reduced survival, potentially because of higher incidence of comorbidities and reduced tolerance to the disease (Neagu & Reardon, 2015). These findings demonstrate that glioblastoma is not only a highly aggressive disease but also more challenging to treat in the elderly.

Epidemiologically, glioblastoma is the most frequent malignant primary brain tumor in adults and a significant contributor to global neurological cancer-related morbidity. Despite the rapid progress in the field of neuro-oncology, clinical outcomes are still bleak with a median survival reported to be around 14-16 months with current standard-of-care treatment and poor long-term survival (beyond five years) (Weller et al., 2017; Neagu & Reardon, 2015). This bleak outlook highlights the need for better treatment approaches than the standard of care.

Glioblastoma has a complex and mostly sporadic cause, without a clear, lifestyle-related, preventable cause. Tumor initiation and development involve multiple genetic changes,

abnormal growth signaling, DNA repair deficiencies, and microenvironmental adaptations. Key molecular characteristics of altered growth signaling, evasion of immune destruction, and resistance to cell death are critical to disease development. As such, glioblastoma is considered to be a complex disease of the tumor rather than a preventable disease, and the value of molecular and immunological therapeutic approaches is highlighted.

Glioblastoma patients typically exhibit progressive neurological signs and symptoms resulting from mass effect and increased intracranial pressure. These can include headache, seizures, nausea and vomiting, weakness, dysphasia (speech impairment), memory loss and cognitive dysfunction. Initial symptoms are often vague and may be misdiagnosed as benign neurological disease, delaying diagnosis until the patient experiences severe neurological symptoms. There is anecdotal evidence that a delay between the start of symptoms and diagnosis is common in brain tumors and may have a negative impact on prognosis.

Despite advances in surgical and medical therapies, glioblastoma is a highly aggressive disease. The current standard of care is maximum safe surgery, followed by radiation and chemotherapy (temozolomide). But it is difficult to eradicate all tumor cells as glioblastoma extensively invades into normal brain tissue outside the radiologically visible tumor (Weller et al., 2017). This means that most patients experience disease recurrence and survival rates are only modestly improved even with intensive treatment.

A key barrier to the successful treatment of glioblastoma is the blood–brain barrier (BBB), which limits the entry of many systemic anti-cancer drugs into the brain. While the BBB can be disrupted by the presence of the tumor to form a blood–tumor barrier (BTB), this is still not uniform, resulting in variable drug delivery and inconsistent drug coverage of the tumor (Upton et al., 2022). This enables cancer cells to persist and recurrently re-grow after therapy.

On a molecular level, glioblastoma growth is impacted by various clinically significant biomarkers. Characteristics such as IDH mutational status, promoter methylation of the gene for O6-methylguanine-DNA methyltransferase (MGMT), and pathway deregulation of oncogenic signaling are vital for classification, treatment and outcome (Weller et al., 2017). These features contribute to therapeutic resistance, and evidence the need for precision-based therapy.

Besides the physical barriers, glioblastoma creates an immunosuppressive tumor microenvironment. GBM is considered an immunologically "cold" tumor with low levels of cytotoxic T-cell infiltration and high levels of immune suppression (Weller et al., 2017). Moreover, the standard use of corticosteroids to control peritumoral edema also suppresses immune responses, making immunotherapies less effective. Thus, the development of approaches that overcome immune suppression and promote strong tumor-specific immune responses are greatly needed.

The use of cancer immunotherapy, especially vaccines, has shown potential in overcoming these issues. Therapeutic cancer vaccines seek to induce immune recognition of tumor antigens and induce the killing of cancer cells. Of the different vaccine platforms, dendritic cell vaccines have been extensively studied in glioblastoma due to their capacity to induce antigen-specific T-cell responses (Olin et al., 2014). While clinical trials have proven safety and immunogenicity, they have yet to produce consistent clinical improvement, suggesting that antigen selection and vaccine design need to be improved (Mineharu et al., 2011).

As with infectious diseases, glioblastoma is highly resistant to treatment. Temozolomide resistance, radiotherapy resistance and evolution after repeated therapeutic intervention contribute to therapeutic failure and relapse. Such resistance mechanisms justify the need for immune-based therapies to eliminate resistant residual tumor cells.

In developing nations like Bangladesh, access to timely diagnosis, sophisticated neuroimaging, and the cost of treatment and new immunotherapies may compound glioblastoma treatment difficulties. Designing vaccines using computational approaches could help minimise costs during initial research and create a platform for potential future translational work in these countries.

Conventional vaccine design is primarily based on extensive experimentation and clinical trials. However, immunoinformatics allows quick in silico analysis of several tumor antigens and their immunogenic epitopes. This allows in silico assessment of antigenicity, allergenicity, toxicity, physicochemical characteristics, structural stability and binding to immune receptors before experimental validation (Kiel et al., 2025). This approach is useful for glioblastoma due to its molecular diversity.

Thus, there is a need to develop a successful therapeutic vaccine against glioblastoma to enhance survival and decrease recurrence. In this research, an immunoinformatics-based in silico multi-epitope vaccine construction plan against glioblastoma is suggested. Five glioblastoma-associated proteins will be studied with the help of computational analysis, and estimated CTL, HTL, and B-cell epitopes will be analyzed to create an optimized multi-epitope vaccine candidate that will induce a robust and safe anti-tumor immunity.

Chapter 2

Methodology

The in-silico multi-epitope vaccine against glioblastoma (GBM) was designed in the form of a systematic computational workflow including the antigen selection, epitope prediction, vaccine construct assembly, and a thorough immunological and structural validation.

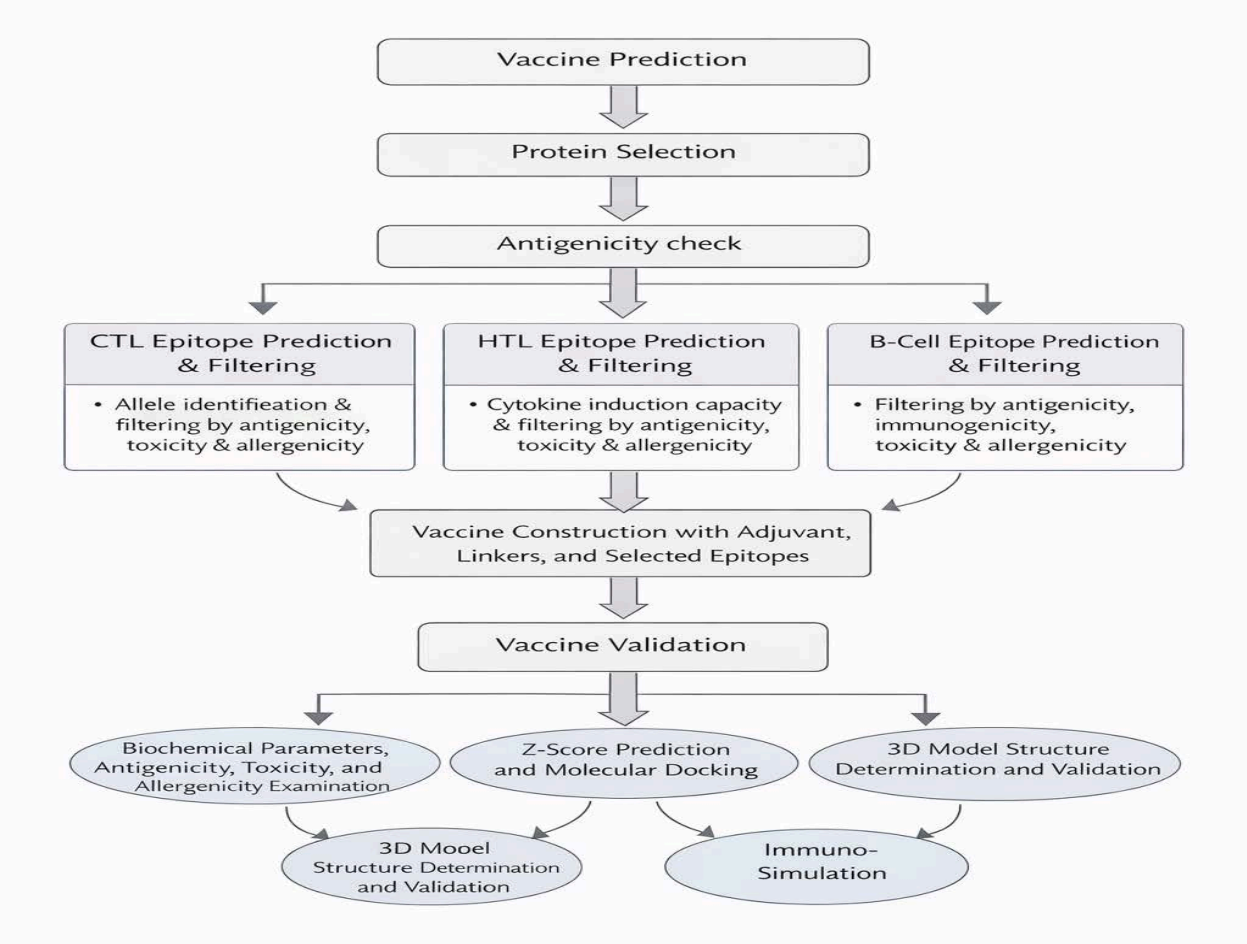


Figure 1: Summarizes the complete bioinformatics workflow employed for the rational design and in-silico evaluation of the proposed GBM vaccine candidate

2.1 Identification of Suitable Candidate Protein Sequences

Appropriate glioblastoma-associated sequences of target proteins that have the ability to trigger an immunological response were picked using publicly available bioinformatics databases. Glioblastoma multiforme-relevant protein sequences were obtained in FASTA format via the NCBI Protein database and the UniProt Knowledgebase and were limited to tumor-associated proteins that have been reported to be overexpressed in GBM tissues (Pearson et al., 2016; Bateman et al., 2023).

The choice of these proteins was due to reported relevance to the biology and immunotherapeutic targeting of glioblastoma. Protein sequences of appropriate length (400-600 residues) were chosen to make sure that it is suitable as a vaccine design and downstream immunoinformatics analysis. The retrieved sequences were then tested on antigenic potential on VaxiJen v2.0 server, which predicts antigenicity using an alignment independent approach, based on physicochemical properties. To perform tumor-specific screening, the tumor parameter was adjusted to tumor and a threshold value of 0.5 was used. This cutoff was chosen because it is the most popular and empirically validated cutoff used in cancer immunoinformatics research to provide the best tradeoff between sensitivity and specificity to detect immunogenic tumor-associated proteins (Pearson et al., 2016).

2.2 Identification and Selection of CTL-Specific Epitopes and Associated Alleles

It was also predicted in cytotoxic T lymphocyte (CTL) epitopes by using NetCTL 1.2 to find out peptide sequences that will generate cell-mediated immune responses against glioblastoma-associated antigens. NetCTL combines the binding affinity of the MHC class I, the cleavage activity of the proteasomes and the transporter associated with antigen processing (TAP) scores to compute high CTL recognition potential peptides. The sequences of the proteins were submitted in FASTA format and were filtered through a representative collection of human HLA class I alleles to cover the population. The high-confidence epitopes were selected with a threshold score of 0.75 which is a criterion that has been widely used in cancer vaccine design projects to select the high-confidence epitopes with high immunogenicity and reduce false-positive prediction. The pairs of epitopes/alleles identified were further developed to be used in the multi-epitope vaccine development (Celis, 1994; Vaccines, 2015).

2.3 Analysis of CTL Epitopes and Associated HLA Alleles

The predicted cytotoxic T lymphocyte (CTL) epitopes in high-affinity human leukocyte antigen (HLA) class I alleles were detected under the established MHC class I binding prediction methods which analyse the interactions between peptides and HLA alleles over a wide range of alleles. The predicted CTL antigens were put in FASTA format and binding affinity level was done with selection of 9-mer length peptides which is highly known to be efficient in MHC class I presentation and CTL activation.

The strength of peptide-HLA binding was determined by the binding affinity (BA) scores and epitopes were paired by strength of prediction. Strong and weak binders threshold of 0.5 and 2.0, respectively, were put in place in line with widely used immunoinformatics thresholds. To screen out predicted peptides, ranking was done according to the binding scores and only those were selected that were grouped as strong binders to assure a greater likelihood of antigen presentation and CTL-mediated activation of the immune response.

To be immunologically appropriate as well as safe, the chosen CTL epitopes were further assessed on the basis of antigenicity, allergenicity and toxicity. The determination of antigenicity was carried out based on a validated alignment-independent antigenicity prediction methods commonly used in cancer vaccines, with a threshold value of 0.5 to only accept peptides that have a greater immunogenic potential (Vaccines, 2015). Allergenicity screening was carried out with standard methods of computational classification that kept out of the peptide pool peptides that had the potential to be allergens, whereas toxicity screening was done with peptide toxicity prediction strategies reported in the immunoinformatics-based vaccine development literature to remove peptide sequences with the potential to be cytotoxic (Vaccines, 2015).

The final selection to be incorporated in the multi-epitope vaccine construct was CTL epitopes that had high HLA binding affinity, good antigenicity and no allergenicity or toxicity.

2.4 Identification and Selection of HTL-Specific Epitopes (Helper T-cell Lymphocytes)

The epitope prediction of the Helper T lymphocyte (HTL) was done to determine peptide sequences that could be able to trigger an immune response mediated by the CD4 -T cells thus playing a crucial role in the coordination of antitumor immunity by the release of cytokines, activation of cytotoxic T-cells and antibody mediated immunity. The role of the HTL in glioblastoma is especially crucial in maintaining immune surveillance and providing therapeutic vaccines with a better effect (Celis, 1994).

The protein sequences of the targets were entered into NetMHCIIpan platform as FASTA sequences. The length of peptide used was 15 amino acids which were chosen according to the binding affinities of MHC class II molecules. To prevent the absence of a wide population coverage, 20 HLA class II alleles were examined per prediction run and the process was repeated to cover all the relevant alleles (Vaccines, 2015).

Epitopes were selected based on percentile rank cut-offs which were calculated using predicted score on binding affinity. The classification of peptides was based on the strength of the binding, as a strong (SB) and weak (WB) binding, and a stringent cutoff of 1% rank was used to define strong binding. The binding affinity scores indicate the affinity of the peptide and the MHC and affinity scores that would be considered as strong binders were preferred and such peptides have high chances of inducing successful HTL responses. High-confidence HTL epitopes were then chosen to be tested further with immunological assays and incorporated into the multi-epitope vaccine design paradigm.

2.5 Screening and Ranking of Predicted HTL Epitope Candidates

After prediction of the binding of the MHC II, the immunological activity of candidate HTL epitopes was further tested by means of cytokine induction profiling. Successful helper T-cell responses can be characterized by the release of essential cytokines, such as interferon-gamma (IFN- gamma), interleukin-4 (IL-4), and interleukin-10 (IL-10) that collectively have roles in controlling antigen presentation, the activation of macrophages, B-cell growth, and immune homeostasis in the tumor microenvironment (Celis, 1994).

Computationally evaluated predicted HTL epitopes were tested to induce IFN-g, IL-4 and IL-10 using the cytokine prediction servers with default parameters. The priorities included IFN- 7

poses because of their role in the enhanced antigens presentation and the stimulation of antitumor immunity, whereas IL-4 and IL-10 induction were considered to guarantee the balance between immune modulation and the absence of excessive inflammatory reactions (Reynisson et al., 2020).

Also, antigenicity, allergenicity, and toxicity of all HTL epitopes were assessed with an aim to make sure of immunogenicity safety and appropriateness to include into the multi-epitope vaccine construct. High-confidence candidates were chosen to be included in the design of glioblastoma vaccines based on HTL epitopes, which demonstrated a strong binding affinity in the MHC class II, desirable cytokine induction profiles, antigenic potential, and absence of allergenic or toxic properties.

2.6 Identification and Computational Assessment of B cell Epitope candidates

B-cell epitopes are important in developing humoral immune responses by the production of antibodies, which play an important role in providing immunological protection in the long term. Linear B- cell epitopes are a continuous amino-acid sequence on antigenic proteins which is picked directly by a B-cell receptor. In the current research, the prediction of linear B-cell epitopes was conducted with the help of the Immune Epitope Database (IEDB) B-cell epitope prediction tools, which offer experimentally validated computation framework of antibody-targeted epitope mapping (Vita et al., 2019).

After the prediction of B-cell epitopes, all shortlisted B-cell epitopes were filtered based on antigenicity, allergenicity and toxicity according to the same computational screening criteria and servers as used in analyses of CTL and HTL epitopes, allowing a methodological comparison across immune pathway analyses. B-cell epitopes with desirable antigenic characteristics and non-allergenic and non-toxic were only maintained to be incorporated in downstream multi-epitope vaccine approaches, thus facilitating safe and efficient humoral immune response against glioblastoma.

2.7 Assembly of the Vaccine Construct with Optimized Epitope Linkers Screening of the Vaccine Construct for Antigenicity and Safety Parameters (Allergenicity & Toxicity)

Multi-epitope vaccines would not be possible without appropriate linker peptides, which help to assemble various immunogenic components in their correct order into one protein sequence. In this research, several linker sequences were strategically placed in order to connect epitopes that are separated and to make sure that the space is appropriately laid out. The EAAAK linker was incorporated in order to separate cytotoxic T lymphocyte (CTL) epitopes to render its structure rigid and the GPGPG linker between helper T lymphocyte (HTL) epitopes to enable its efficient processing into antigens. The KK linker was also used to conjugate B-cell epitopes to create flexibility and availability to the antibody. These linkers are used to minimize undesirable aggregation between many neighboring epitopes, enable proper folding of the vaccine construct and maintain the functional integrity of each immune component, thus augmenting the immunogenic properties of the multi-epitope vaccine, as a whole.

Figure 2: Design and Assembly of the Vaccine Construct Incorporating Epitope Linkers

After constructing the multi-epitope vaccine construct, it was assessed by its immunological suitability and safety profile using known silico screening strategies. The antigenicity of the overall vaccine sequence was evaluated by using a physicochemical property-based computational model that would ensure that the vaccine was capable of triggering an immune reaction. They used a threshold of 0.5, which is widely used in tumor-associated vaccine research to achieve an optimal sensitivity-specificity balance in the screening of antigens (Nezafat et al., 2016).

The allergenicity of the vaccine construct was then tested with a curated allergen prediction site which matches query sequences with experimentally validated allergen data. The vaccine sequence was entered in FASTA format with default values to make sure the removal of allergenic determinants that might cause adverse immune responses was done (Sanchez-Trincado et al., 2017).

In the name of biosafety, toxicity analysis was conducted with the help of a peptide toxicity prediction framework that compares sequences similarity with an existing toxic peptide profile. This was a screening step to remove possible harmful motifs that may jeopardize the vaccine safety or restrain its translational applicability (Potocnakova et al., 2016).

Only the vaccine constructs that are likely to be antigenic, non-allergenic and non-toxic were chosen to undergo further structural validation and immunological evaluation to assure the appropriateness of the proposed glioblastoma vaccine candidate to downstream computational and experimental analysis.

2.8 Physicochemical Property Assessment of the Constructed Multi-Epitope Vaccine

Physicochemical properties of the constructed multi-epitope vaccine construct were determined by evaluating the parameters relating to protein stability, solubility, and functionality using Expasy ProtParam tool (Gasteiger et al., 2005). The properties that were analyzed were molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, grand average of hydropathicity (GRAVY), extinction coefficient and estimated half-life.

The N-terminal residue was used to estimate protein half-life, and offered stability predictions in mammalian cells, the Escherichia coli, and the yeast expression systems (Wilkins et al., 1999). In vitro stability was determined using the instability index where lower values (below 40) imagined a stable protein structure. Hydropathic was determined using a GRAVY score with negative values indicating hydrophilicity and enhanced solubility. The aliphatic index was considered to be a measure of thermostability, as an extension of the contribution of aliphatic side chains to structural rigidity. The coefficient of extinction was determined in order to estimate the qualities of light absorption that would be used to determine the quantity of the protein.

These physicochemical analyses are fundamental in designing glioblastoma vaccines because soluble and stable constructs that remain structurally stable are important in the efficient antigen presentation and the long-term immune activation within tumor microenvironment.

2.9 Computational Generation of the 3D Structure of the Vaccine Candidate

The conformational integrity and spatial organization of the constructed multi-epitope vaccine was assessed by performing the three-dimensional structural modeling of the vaccine in question. The completed amino acid sequence of the vaccine was uploaded to the Phyre2 web server, which is a homology-based protein modeling system and aspires to predict tertiary structures through fold recognition algorithms and profile-profile matching algorithms (Kelley et al., 2015). The sequence of the vaccine was also given in plain FASTA format and modeling was performed with default parameters.

After submission, the Phyre2 server produced predicted structural models, confidence and coverage score, which indicates the confidence of the predicted folds. The estimated Protein Data Bank (PDB) files were then recaptured and seen in the PyMOL molecular visualization software to check the overall pattern of folds and structural stability of the vaccine construct. This type of structural modeling was necessary to ensure that the planned multi-epitope vaccine assumes a stable three dimensional conformation that could be further subjected to structural validation and further immunological interaction (Bhattacharya et al., 2016).

3.0 Validation of the Vaccine Model Using Ramachandran Plot Assessment

The quality of the predicted three-dimensional vaccine model was determined by the analysis of the Ramachandran plots, which are one of the commonly used methods to determine the quality of the backbone conformation in proteins modeled computationally (Sahay et al., 2020). This was done to validate the distribution of backbone dihedral angles and to ascertain that the modeled vaccine construct is in energy favorable conformations that were in agreement with known protein geometry. The created refined PDB structure at the homology modeling phase was evaluated with the SWISS-MODEL structural evaluation platform that offers residue-level analysis of phi (phi), psi (psi) torsion angle and characterizes regions as favored, allowed, and disallowed (Studer et al., 2020). The percentage of amino acid residues in preferential and accessible sites is a measure of good folding behavior, stability, and model fidelity.Ramachandran plot validation is an essential quality-control measure in vaccine development based on immunoinformatics, since it allows to guarantee the biological plausibility and compatibility of the predicted protein structure with downstream molecular docking, receptor interaction, and immune simulation studies (Sahay et al., 2020; Studer et al., 2020).

3.1 Z-Score Based Evaluation of the Predicted 3D Vaccine Structure

Z-score analysis was used to determine the structural reliability of the overall quality of the predicted three-dimensional structure of the vaccine construct. The optimized PDB model has been tested using the ProSA-web interface that is used to estimate the quality of a model using the total energy profile of the predicted structure compared to reference datasets based on experimentally determined protein structures. The resultant Z-score is the amount of deviation of the modeled structure against native proteins of similar size and gives a quantitative assessment of the overall accuracy of structure. A Z-score within the range of structurally validated protein models suggests that the quality of the folding is good and the structure is energetically stable to allow the vaccine construct to be used in further molecular docking and immune simulation studies (Bhattacharya et al., 2007; Prajapat et al., 2014; Sahay et al., 2020).

3.2 Receptor-Vaccine Interaction Analysis Through Molecular Docking

Molecular docking was used to study the interaction of the designed multi-epitope glioblastoma vaccine construct to determine the interaction with the host innate immune receptors, particularly, Toll-like receptors (TLRs) which are an essential part of the induction of the anti-tumor immune response. TLRs play a central role in antigen presentation and dendritic cell activation and similarly the induction of downstream adaptive immunity and are therefore of interest as the targets of therapeutic cancer vaccines (Akira and Takeda, 2004).

The three-dimensional fold of the selected human TLR were retrieved in the RCSB Protein Data Bank in PDB format and the predicted vaccine structure obtained via the homology modeling were used as the ligand. Protein docking was performed using the ClusPro server, which is a docking algorithm developed based on the fast fourier transform (FFT) to sample receptor-ligand conformations and cluster docked poses, and is based on the score of interaction energy (Comeau et al., 2004). Different docked complexes were received and ranked according to the size of the cluster and binding energy with the strongest complex taken to undergo further studies. The docking analysis provides an idea on the binding strength, and immunostimulatory capacity of the vaccine construct to justify its applicability to the case of glioblastoma immunotherapy.

3.3 In-Silico Immune Simulation and Response Prediction of the Vaccine Candidate

To overcome the immunogenicity of the developed multi-epitope glioblastoma vaccine in-silico, the simulation of immune responses was carried out on the C-ImmSim server, which is a validated agent-based model to analyze the dynamics of immunogenicity (Castiglione et al., 2012). It is a computer system that simulates key biological activities in the human immune system, including humoral, cellular and innate immune reactions, and offers time-dependent graphical displays of the immune cell subsets and cytokine responses. The time points 1, 84 and 168 that model a four weeks interval were gated with three virtual doses to recapitulate a standard vaccination regimen, with each step of the simulation an 8 hour interaction as commonly employed in immunomodulatory-based vaccine experiments (Singh et al., 2021). The other parameters were maintained within default settings so as to render it reproducible. The immune phenotypes resulting were assessed to ascertain the capacity of the vaccine construct in inducing durable immune response that is relevant to glioblastoma immunotherapy.

3.4 Core Components of the Research Methodology

vaccine design strategy involves systematic screening, epitope predicting and other computational studies using available online bioinformatics tools. This approach enables rapid screening and testing first viability of potential vaccine candidates early and minimizes time, cost and experiment constraints. However, even though the efficacy of immunoinformatics-based methods in candidate prioritization and their theoreticality are high, they are limited to computational forecasting and a lack of information concerning the actual vaccine efficacy and safety in detail. Consequently, even though the provided study aims at proposing a viable multi-epitope therapeutic vaccine candidate in the case of glioblastoma, further in-vitro, in vivo and clinical research is required to establish its immunogenicity, safety and efficacy in the therapy process, prior to its possible use in a clinical practice.

Chapter 3

Results

3.1Collection of Candidate Proteins and Antigenicity Assessment

This study has chosen five glioblastoma-associated protein sequences that were obtained in FASTA and in UniProt Knowledgebase and NCBI Protein database. These proteins were selected because they have been reported to be of interest in glioblastoma biology and because they may be useful as immunogenic targets. After the retrieval of the sequence, antigenic potential of each protein was determined by the VaxiJen server. Antigenicity testing is a very important process in vaccine development as it defines the chances of a protein to cause an immune reaction when it is identified by the host immune system. Proteins, which were predicted to be antigens, were only retained to further analyses of epitope prediction and vaccine construction. The protein sequences chosen by UniProt and NCBI have detailed information as shown below:

Protein 1

Protein Name: Fascin

Gene: FSCN1

Amino acids: 493

Organism: Homo Sapiens (Human)

Antigenicity: 0.6379

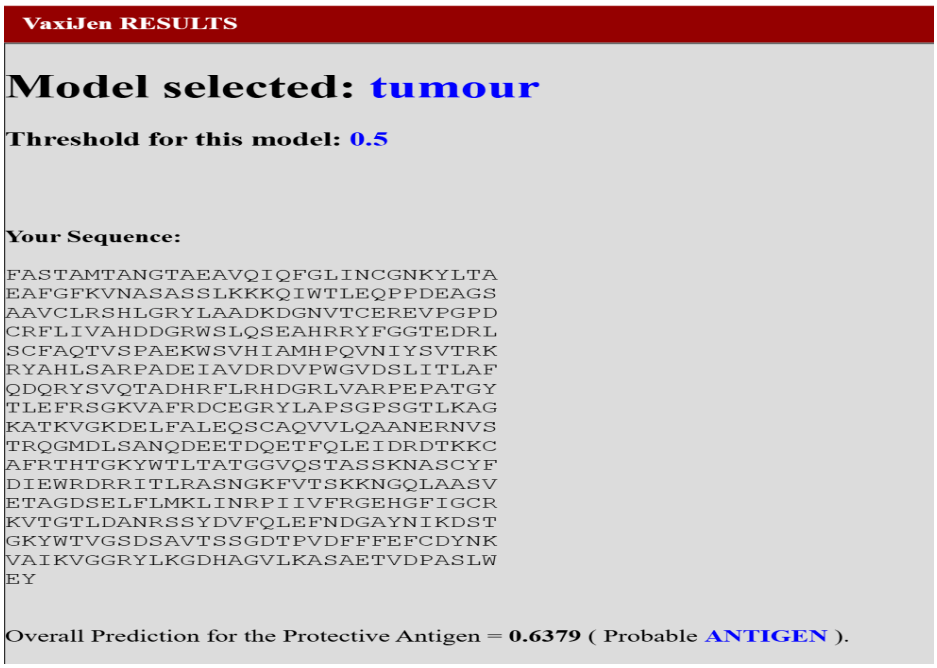


Figure 3: Evaluation of Antigenicity for Protein 1

Protein 2

Protein Name: Dihydropyrimidinase-related protein 2

Gene: DPYSL2

Amino Acids: 572

Organism: Homo Sapiens (Human)

Antigenicity: 0.5867

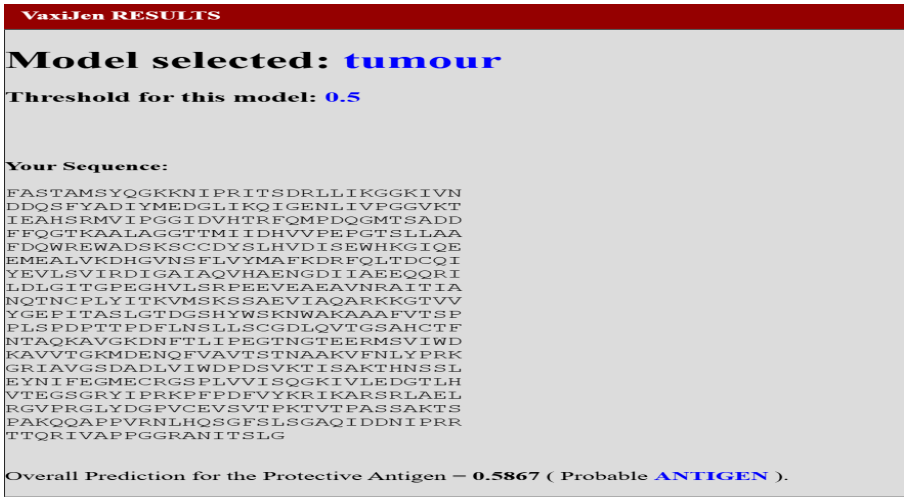


Figure 4: Evaluation of Antigenicity for Protein 2

Protein 3

Protein Name: Actin-related protein 3

Gene: ACTR3

Amino Acids: 418

Organism: Homo Sapiens (Human)

Antigenicity: 0.4267

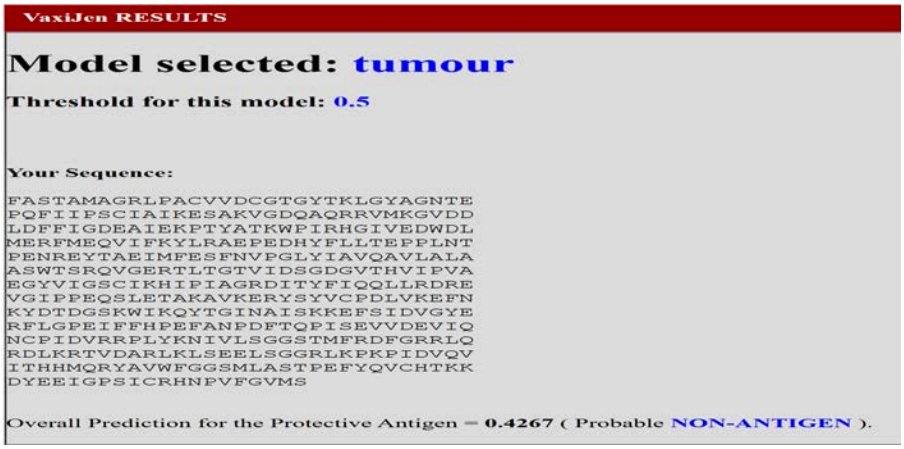


Figure 5: Evaluation of Antigenicity for Protein 3

Protein 4

Protein Name: Dihydropyrimidinase-related protein 5

Gene: DPYSL5

Amino Acids: 564

Organism: Homo Sapiens (Human)

Antigenicity: 0.5274

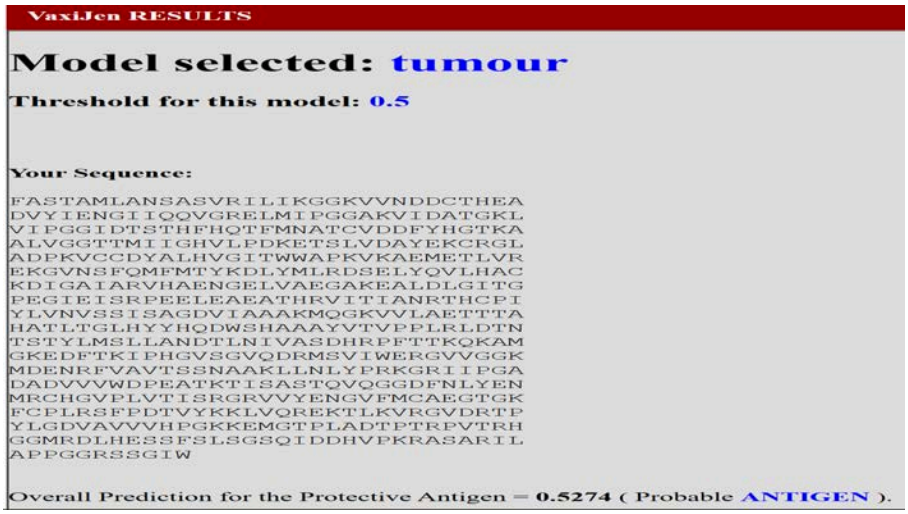


Figure 6: Evaluation of Antigenicity for Protein 4

Protein 5

Protein Name: Coronin-2B

Gene: CORO2B

Amino Acids: 480

Organism: Homo Sapiens (Human)

Antigenicity: 0.6374

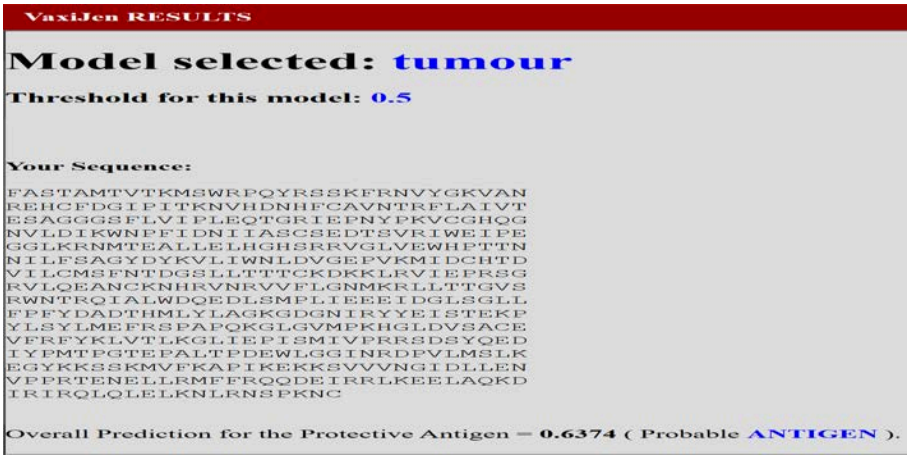


Figure 7: Evaluation of Antigenicity for Protein 5

Table 1:Protein Based on Antigenicity scores

Protein no.	Antigenicity Score	Comments
Protein 01	0.6379	High antigenicity, strong potential
Protein 02	0.5867	Highest Antigenicity, Good Potential
Protein 03	0.4267	Low antigenicity, less preferred
Protein 04	0.5274	Highest Antigenicity, Good Potential
Protein 05	0.6374	Highest Antigenicity, Good Potential

Antigenicity analysis showed that the Protein 1 (FSCN1) and Protein 5 (CORO2B) had the highest scores and were a top-priority candidate. Protein 2 (DPYSL2) and 4 (DPYSL5) were moderately antigenic and were given high priority whereas Protein 3 (ACTR3) was relatively lowly antigenic and was of low priority in the further analysis.

3.2 Selection and Screening CTL Allele

Following the process described by Pearson et al., in the selection of CTL (Cytotoxic Lymphocytes) epitopes, Net CTL1.2 was employed with the threshold set at 0.75. The identified CTL epitopes were found by the use of MHC-I supertype A1 which was required to create an effective vaccine. Through the CTL epitopes selection process, 14, 11, 13, 15 and 18 epitopes of protein no. 1, 2, 3,4, and 5 respectively that have potential to generate strong immune responses as revealed below in Figure - were identified in this study. In the study, a table of the combined scores with the CTL epitopes is also provided and Table 2 gives a full list of the data.

Table 2: CTL epitopes of the 5 proteins with their respective combined scores

Protein no. 1		**Protein no. 2**		**Protein no. 3**	
CTL epitopes	**Combined Scores**	**CTL epitopes**	**Combined Scores**	**CTL epitopes**	**Combined Scores**
ASSKNASCY	3.1150	KTHNSSLEY	3.1940	FMEQVIFKY	2.9390
TLDANRSSY	2.7170	NAAKVFNLY	3.0410	MLASTPEFY	3.0620
QLEFNDGAY	2.7080	LTDCQIYEV	0.0720	ESFNVPGLY	2.7680
TLAFQDQRY	2.9490	HVTEGSGRY	2.9840	GTGYTKLGY	2.5540
AMHPQVNIY	3.2290	IVNDDQSFY	3.0930	PIDVRRPLY	2.3900
ETDQETFQL	0.3860	SLGTDGSHY	2.9880	VITHHMQRY	3.0300
NIKDSTGKY	3.0360	NSSLEYNIF	2.5850	DTDGSKWIK	-0.1010
LQSEAHRRY	3.0540	DQSFYADIY	2.5410	HVIPVAEGY	3.0510
SYDVFQLEF	2.7300	HVDISEWHK	0.3840	SIDVGYERF	2.4350
VAIKVGGRY	3.2090	VTGSAHCTF	2.3630	CVVDCGTGY	3.2540
TADHRFLRH	-0.7290	VTSTNAAKV	0.5050	PIAGRDITY	2.5990
ETAGDSELF	2.3730			DLVKEFNKY	2.5610
GTEDRLSCF	2.1600			YTAEIMFES	-2.4370
VTSKKNGQL	0.8860				

Protein no. 4		Protein no. 5	
CTL epitopes	Combined Scores	CTL epitopes	Combined Scores
RLDTNTSTY	3.0600	STEKPYLSY	3.4472
NSFQMFMTY	3.1610	RSSKFRNVY	2.2465
NAAKLLNLY	3.0850	LSGLLFPFY	1.7690
HATLTGLHY	3.0040	YDADTHMLY	1.3207
ATLTGLHYY	3.0420	MTEALLELH	1.2375
YMLRDSELY	3.0920	SACEVFRFY	1.2066
NATCVDDFY	3.0140	DADTHMLYL	1.1031
TSTHFHQTF	2.3790	HTDVILCMS	1.0797
NTSTYLMSL	0.9760	RSDSYQEDI	1.0718
MFMTYKDLY	3.1550	QTGRIEPNY	0.9639
STQVQGGDF	2.6880	NTDGSLLTT	0.9410
DTNTSTYLM	-0.0790	RTENELLRM	0.8765
GIDTSTHFH	-0.7920	DTSVRIWEI	0.8713
TISRGRVVY	3.0750	VSACEVFRF	0.8383
VQGGDFNLY	2.8500	VLMSLKEGY	0.8059
		VLDIKWNPF	0.7860
		ILFSAGYDY	0.7727
		CSEDTSVRI	0.7589

There were high-affinity MHC class I alleles that were identified using the NetMHCpan 4.1 platform and were used to further analyze the predicted CTL epitopes. The chosen alleles are reported in Table 3, and they are the dominant supertypes based on the CTL prediction of epitopes produced with NetCTL 1.2, as shown in Table 2. Epitopes were grouped into two groups, strong and weak binders, according to their binding affinity score; strong binders with a binding score of 0.500 or less, and weak binders (binding score of 2.000 or more). This classification approach allowed the prioritization of CTL epitopes with better MHC class I binding potential, and thus, the selection of the most immunologically relevant ones to include them in the glioblastoma vaccine construct.

Table 3: NetMHCpan 4.1 Analysis of Epitopes: Sequence Length, EL Score, Percentile Rank, and MHC-I Binding Affinity

Protein no:1				
Binding Allele	**Sequence no.**	**% Rank EL**	**% Rank BA**	**Aff(nM)**
HLA-A*03:01	AMHPQVNIY	0.459	1.214	533.91
HLA-A*26:01	NIKDSTGKY	0.027	0.085	181.07
HLA-A*26:01	ETAGDSELF	0.049	0.045	86.14
HLA-A*01:01	ETDQETFQL	0.262	0.514	1163.20

Protein no:2				
Binding Allele	**Sequence no.**	**% Rank EL**	**% Rank BA**	**Aff(nM)**
HLA-A*01:01	NAAKVFNLY	0.258	0.323	609.46
HLA-A*01:01	HVTEGSGRY	0.358	0.913	2619.05

Protein no:3				
Binding Allele	**Sequence no.**	**% Rank EL**	**% Rank BA**	**Aff(nM)**
HLA-A*01:01	ESFNVPGLY	0.143	0.162	225.91

Protein no:4				
Binding Allele	**Sequence no.**	**% Rank EL**	**% Rank BA**	**Aff(nM)**
HLA-A*01:01	RLDTNTSTY	0.040	0.107	133.86
HLA-A*01:01	NSFQMFMTY	0.365	0.252	415.23
HLA-A*26:01	DTNTSTYLM	0.133	0.123	254.38
HLA-B*58:01	TSTHFHQTF	0.137	0.459	92.75
HLA-A*01:01	NAAKLLNLY	0.331	0.446	943.57

Protein no:5				
Binding Allele	**Sequence no.**	**% Rank EL**	**% Rank BA**	**Aff(nM)**
HLA-A*01:01	YDADTHMLY	0.129	0.162	226.07
HLA-A*01:01	QTGRIEPNY	0.366	1.023	3062.04
HLA-A*26:01	SACEVFRFY	0.402	0.759	2476.75
HLA-B*58:01	RSSKFRNVY	0.347	0.523	112.93

The predicted CTL epitopes were subsequently tested on antigenicity, allergenicity and toxicity to determine their fitness to be included in the vaccine after confirmation of a high MHC class I binding affinity by the NetMHCpan 4.1 server. Antigenicity was analyzed by use of the VaxiJen v2.0 server and allergenicity and toxicity were analyzed by AllerTOP v2.0 and ToxinPred respectively. The screening process identified four antigenic epitopes of CTLs in the proteins mentioned in Table 1, and two antigenic epitopes in each protein presented in Table 2 and Table 3. Also, five antigenic and immunologically safe CTL epitopes were found on the protein discussed in Table 4. The chosen epitopes were all found to be immunologically safe as they were predicted to be non-toxic and non-allergenic. The CTL epitopes that passed all the screening questions were then prioritized to be included in the preparation of the multi-epitope glioblastoma vaccine.

Table 4: Selection of Selected CTL Epitopes to screen against Antigenicity, Allergenicity and Toxic Effects

No.	Selected CTL Epitopes	Antigenicity	Allergenicity	Toxicity
Protein no:1	AMHPQVNIY	Antigen	Non-Allergen	Non-Toxin
	NIKDSTGKY	Antigen	Non-Allergen	Non-Toxin
	ETAGDSELF	Antigen	Non-Allergen	Non-Toxin
	ETDQETFQL	Antigen	Non-Allergen	Non-Toxin
Protein no:2	NAAKVFNLY	Antigen	Non-Allergen	Non-Toxin
	HVTEGSGRY	Antigen	Non-Allergen	Non-Toxin
Protein no:3	ESFNVPGLY	Antigen	Non-Allergen	Non-Toxin
Protein no:4	RLDTNTSTY	Antigen	Non-Allergen	Non-Toxin
	NSFQMFMTY	Antigen	Non-Allergen	Non-Toxin

	DTNTSTYLM	Antigen	Non-Allergen	Non-Toxin
	TSTHFHQTF	Antigen	Non-Allergen	Non-Toxin
	NAAKLLNLY	Antigen	Non-Allergen	Non-Toxin
Protein no:5	RSSKFRNVY	Antigen	Non-Allergen	Non-Toxin
	SACEVFRFY	Antigen	Non-Allergen	Non-Toxin
	QTGRIEPNY	Antigen	Non-Allergen	Non-Toxin
	YDADTHMLY	Antigen	Non-Allergen	Non-Toxin

3.3 Selection and Screening of HTL Epitopes

Predicting helper T-lymphocyte (HTL) epitopes with high binding affinity was done using NetMHC-IIpan 4.0 server. The shortlisted HTLs were further examined based on their immunomodulatory effect in the form of cytokine induction by looking at the profile of IFN-7, IL-4 and IL-10 production using specific prediction servers. The epitopes that met the cytokine stimulation criteria were further filtered to measure antigenicity, allergenicity and toxicity with the help of the already available immunoinformatics tools. The different layers of this filtering approach were used to select immunologically relevant and biologically safe HTL epitopes that could be incorporated into glioblastoma multi-epitope vaccine construct.

Table 5: Anticipated Patterns of HTL Epitopes and Cytokine-Inducing Profiles across Selected Proteins

Protein No: 1			
HTL Epitopes	IFNγ	IL-4 Prediction	IL-10 Prediction
TRKRYAHLSARPADE	Positive	IL-4 Inducer	IL-10 Inducer
RKRYAHLSARPADEI	Positive	IL-4 Inducer	IL-10 Inducer
VTRKRYAHLSARPAD	Positive	IL-4 Inducer	IL-10 Inducer

Protein No: 2			
HTL Epitopes	IFNγ	IL-4 Prediction	IL-10 Prediction
AAKVFNLYPRKGRIA	Positive	IL-4 Inducer	IL-10 Inducer

Protein No: 3			
HTL Epitopes	IFNγ	IL-4 Prediction	IL-10 Prediction
No Epitopes	NA	NA	NA

Protein No: 4			
HTL Epitopes	IFNγ	IL-4 Prediction	IL-10 Prediction
NDTLNIVASDHRPFT	Positive	IL-4 Inducer	IL-10 Inducer
DTLNIVASDHRPFTT	Positive	IL-4 Inducer	IL-10 Inducer
EGIEISRPEELEAEA	Positive	IL-4 Inducer	IL-10 Inducer
TGPEGIEISRPEELE	Positive	IL-4 Inducer	IL-10 Inducer

Protein No: 5			
HTL Epitopes	IFNγ	IL-4 Prediction	IL-10 Prediction
No Epitopes	NA	NA	NA

Table 6: Screening of Selected HTL Epitopes as antigenicity, allergenicity and toxic effects

No.	Selected HTL Epitopes	Antigenicity	Allergenicity	Toxicity
Protein no:1	TRKRYAHLSARPADE	Antigen	Non-Allergen	Non-Toxin
	RKRYAHLSARPADEI	Antigen	Non-Allergen	Non-Toxin
	VTRKRYAHLSARPAD	Antigen	Non-Allergen	Non-Toxin
Protein no:2	AAKVFNLYPRKGRIA	Antigen	Non-Allergen	Non-Toxin
Protein no:3	No Epitopes	N/A	N/A	N/A
Protein no:4	NDTLNIVASDHRPFT	Antigen	Non-Allergen	Non-Toxin
	DTLNIVASDHRPFTT	Antigen	Non-Allergen	Non-Toxin
	EGIEISRPEELEAEA	Antigen	Non-Allergen	Non-Toxin
	TGPEGIEISRPEELE	Antigen	Non-Allergen	Non-Toxin
Protein no:5	No Epitopes	N/A	N/A	N/A

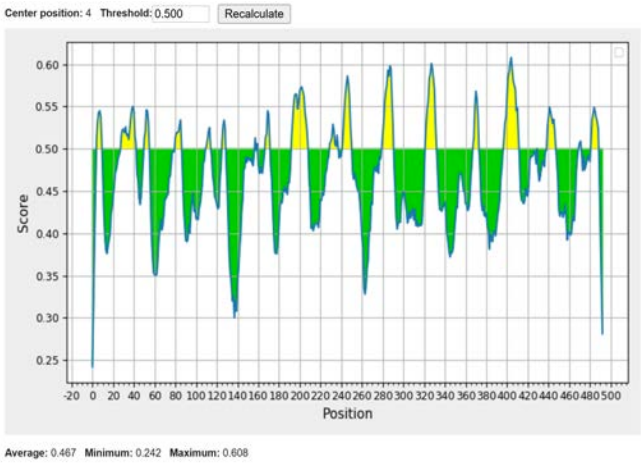
3.4 Computational Prediction of B-cell Epitopes

The IEDB Analysis Resource B-cell epitope prediction platform was used to predict linear B-cell epitopes to determine the areas of peptides that could potentially cause antibody-mediated immune response. The algorithm used in the prediction process was the BepiPred Linear Epitope Prediction 2.0 algorithm because this prediction technique has a higher predictive accuracy and is based on machine-learning. The outputs of the prediction were plotted in the form of a residue position based scoring profile and a threshold of 0.5 was used to identify the likely regions of the epitope.

The residues whose score was lower than the set cutoff were deemed to have a lower probability of being functional B-cell epitopes and those that were above the cutoff were interpreted as better-confidence epitope candidates. The peptide fragments that exceeded the threshold were then chosen to be further processed in the immunological test. Shortlisted epitopes were then screened again to obtain biological fitness to vaccine design by validated computational assessment tools to ensure antigenicity, allergenicity, and toxicity.

Only epitopes with positive antigenic properties and which have been predicted to be non-allergenic and non-toxic were included to be incorporated into the final vaccine construct. This hierarchical filtering approach enhances the strength of the epitope selection strategy and provides the rational development of an immunologically capable multi-epitope vaccine candidate.

Protein 1

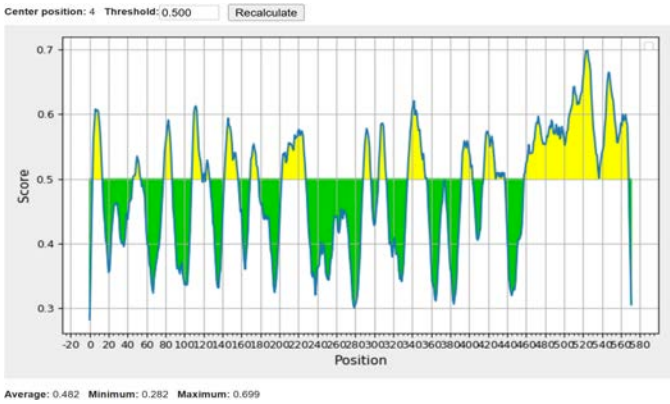


Predicted peptides:

No.	Start	End	Peptide	Length
1	5	10	GTAEAV	6
2	29	43	FGFKVNASASSLKKK	15
3	51	55	PPDEA	5
4	81	87	EREVPGP	7
5	110	115	RYFGGT	6
6	126	129	VSPA	4
7	158	158	R	1
8	160	160	A	1
9	168	172	DVPWG	5
10	193	209	HRFLRHDGRLVARPEPA	17
11	230	238	YLAPSGPSG	9
12	242	251	AGKATKVGKD	10
13	281	291	LSANQDEETDQ	11
14	322	333	GVQSTASSKNAS	12
15	368	373	ETAGDS	6
16	398	412	RKVTGTLDANRSSYD	15
17	440	448	AVTSSGDTP	9
18	470	472	LKG	3
19	482	489	AETVDPAS	8

Figure 8 (a)

Protein 2

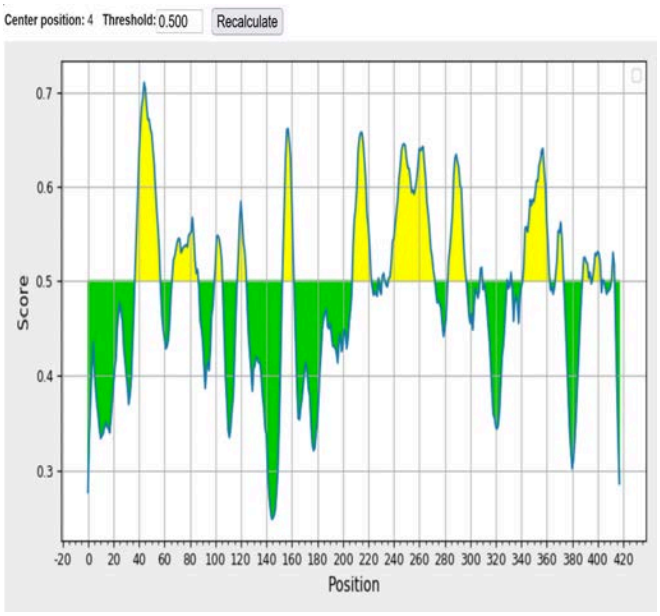


Predicted peptides:

No.	Start	End	Peptide	Length
1	5	14	GKXNIPRITS	10
2	47	55	ENLIVPGGV	9
3	79	87	PDQGHTSAD	9
4	109	119	VPEPGTSLLAA	11
5	121	122	DQ	2
6	124	127	REWA	4
7	143	157	EWHKGIQEEMEALVK	15
8	171	179	KDRFQLTDC	9
9	204	228	IIAEEQQRILDLGITGPEGHVLSRP	25
10	290	297	YWSKNWAK	8
11	306	313	PLSPDPTT	8
12	337	356	NTAQKAVGKDNFTLIPEGTN	20
13	393	404	NLYPRKGRIAVG	12
14	417	428	VKTISAKTHNSS	12
15	430	438	EYNIFEGME	9
16	459	569	LHVTEGSGRYIPRKPFPDFVYKRIKARSRLAELRGVPRGLYDGPVCEVSVTPKTVTPASSAKTSPAKQQAPPVRNLHQSGFSLSGAQIDDNIPRRTTQRIVAPPGGRANIT	111

Figure 8 (b)

Protein 3

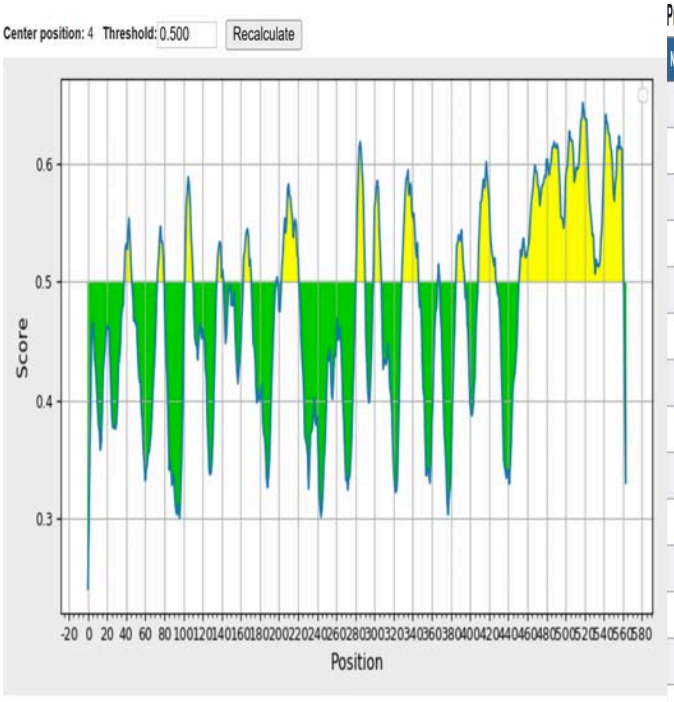


Predicted peptides:

No.	Start	End	Peptide	Length
1	38	57	KESAKVGDQAQRRVMKGVDD	20
2	67	87	IEKPTYATKWPIRHGIVEDWD	21
3	101	106	LRAEPE	6
4	119	125	TPENREY	7
5	154	162	TSRQVGERT	9
6	209	223	RDREVGIPPEQSLET	15
7	229	229	E	1
8	232	234	SYV	3
9	237	273	DLVKEFNKYDTDGSKWIKQYTGINAISKKEFSIDVGY	37
10	284	297	HPEFANPDFTQPIS	14
11	309	310	ID	2
12	330	330	D	1
13	333	333	R	1
14	343	363	VDARLKLSEELSGGRLKPKPI	21
15	368	374	ITHHMQR	7
16	389	395	EFYQVCH	7
17	397	403	KKDYEEI	7
18	405	405	P	1
19	412	414	PVF	3

Figure 8 (c)

Protein 4

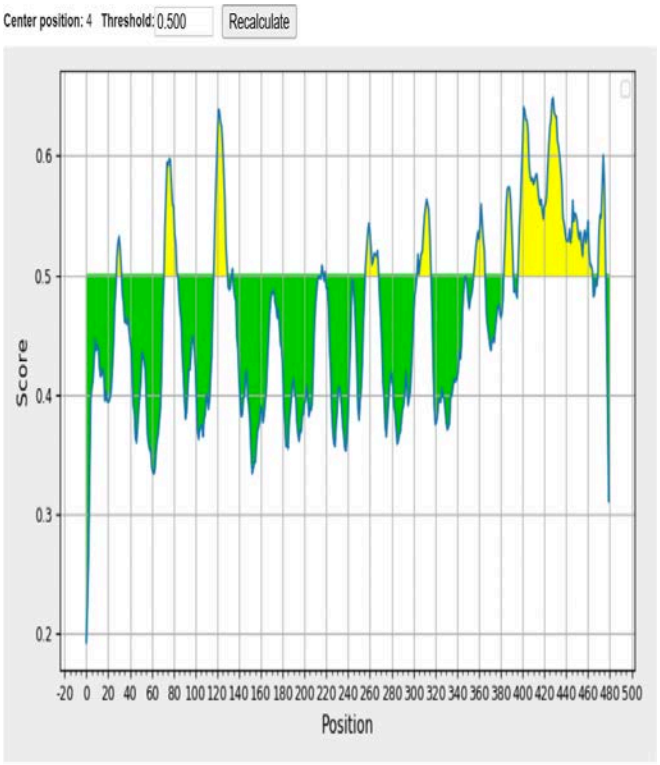


Predicted peptides:

No.	Start	End	Peptide	Length
1	39	46	GRELMIPG	8
2	74	80	MNATCVD	7
3	103	111	PDKETSLVD	9
4	136	142	MWAPKVK	7
5	164	172	KDLYMLRDS	9
6	198	199	VA	2
7	204	221	EALDLGITGPEGIEISRP	18
8	282	291	HYYHQDMGHA	10
9	300	308	LRLDTNTST	9
10	330	347	TTKQKAMGKEDFTKIPHG	18
11	368	369	MD	2
12	387	395	LYPRNGRII	9
13	410	428	TKTISASTQVQGGDFNLYE	19
14	453	561	NCAEGTGKFCPLRSFPDTVYKKLVQREKTLKVRGVDRTPYLGDVAVVHPGKKEMGTPLADTPTRPVTRHGGMRDLHESSFSLSGSQIDDHVPKRASARILAPPGGRSS	109

Figure 8 (d)

Protein 5



Predicted peptides:

No.	Start	End	Peptide	Length
1	29	33	HCFDG	5
2	73	84	TGRIEPWYPKVC	12
3	118	130	PEGGLKRNMTEAL	13
4	135	135	G	1
5	217	218	EA	2
6	220	220	C	1
7	257	269	DLSMPLIEEEIDG	13
8	305	317	PYLSYLMEFRSPA	13
9	357	366	VPRRSDSYQE	10
10	384	392	WLGGINRDP	9
11	397	465	LKEGYKKSSKMVFKAPIKEKKSVVVNGIDLLEMVPPRTENELLRMFFRQQDEIRRLKEELAQKDIRIRQ	69
12	471	477	KNLRNSP	7

Figure 8 (e)

The IEDB Analysis Resource was used to analyze the data with the BepiPred Linear Epitope Prediction 2.0 algorithm resulting in the discovery of several candidate peptides in the selected proteins. Protein 1, 2, 3, 4 and 5 were predicted to have 11, 8, 9, 7 and 7 peptides respectively as possible B-cell epitopes. These simulated peptide fragments are linear parts that have increased likelihood of being part of antibody mediated immune recognition.

The selection process was further refined to remove peptide sequences with extremely short residue lengths, to increase the biological relevance of the selection process. These short segments are not usually capable of being maintained in stable structural conformations, or of causing significant immunological responses. This resulted in a selective retention of only peptides that pass reasonable length and prediction criteria, to undergo further antigenicity and safety assessments.

Table 7: Screening of Selected B-Cell Epitopes as antigenicity, allergenicity and toxic effects

No.	Selected B-cell Epitopes	Antigenicity	Allergenicity	Toxicity
Protein no:1	AGKATKVGKD	Antigen	Non-Allergen	Non-Toxin
	GVQSTASSKNAS	Antigen	Non-Allergen	Non-Toxin
	AETVDPAS	Antigen	Non-Allergen	Non-Toxin
	AVTSSGDTP	Antigen	Non-Allergen	Non-Toxin
Protein no:2	NTAQKAVGKDNFTLIPEGTN	Antigen	Non-Allergen	Non-Toxin
	GKKNIPRITS	Antigen	Non-Allergen	Non-Toxin
	KDRFQLTDC	Antigen	Non-Allergen	Non-Toxin
Protein no:3	TSRQVGERT	Antigen	Non-Allergen	Non-Toxin
	HPEFANPDFTQPIS	Antigen	Non-Allergen	Non-Toxin
	EFYQVCH	Antigen	Non-Allergen	Non-Toxin
	KKDYEEI	Antigen	Non-Allergen	Non-Toxin
	VDARLKLSEELSGGRLKPKPI	Antigen	Non-Allergen	Non-Toxin
Protein no:4	KDLYMLRDS	Antigen	Non-Allergen	Non-Toxin
	TTKQKAMGKEDFTKIPHG	Antigen	Non-Allergen	Non-Toxin
Protein no:5	PEGGLKRNMTEAL	Antigen	Non-Allergen	Non-Toxin
	PYLSYLMEFRSPA	Antigen	Non-Allergen	Non-Toxin
	KNLRNSP	Antigen	Non-Allergen	Non-Toxin

3.5 Design of the Vaccine Construct Using Appropriate Linker Sequences and In-Silico Evaluation of Antigenicity, Allergenicity, and Toxicity Profiles

Depending on the peptide linkers used, the immunogenic epitopes predicted by different bioinformatics tools were combined into vaccine constructs. In order to achieve adequate separation and structural stability of epitope segments, special linkers were used, such as EAAAK with CTL epitopes, GPGPG with HTL epitopes and KK with B-cell epitopes. These linkers enhance the proper folding and enhance the independent immunological functionality of each epitope of the construct. According to this plan, five multi-epitope vaccine constructs were produced, one of them being based on each of the chosen protein sequences.

Construction of vaccine 01 (Protein 01):

MTANGTAEAVQIQFGLINCGNKYLTAEAFGFKVNASASSLKKKQIWTLEQPPDEAGSAAVCLRSHLGRY
LAADKDGNVTCEREVPGPDCRFLIVAHDDGRWSLQSEAHRRYFGGTEDRLSCFAQTVSPAEKWSVHIA
MHPQVNIYSVTRKRYAHLSARPADEIAVDRDVPWGVDSLITLAFQDQRYSVQTADHRFLRHDGRLVARP
EPATGYTLEFRSGKVAFRDCEGRYLAPSGPSGTLKAGKATKVGKDELFALEQSCAQVVLQAANERNVST
RQGMDLSANQDEETDQETFQLEIDRDTKKCAFRTHTGKYWTLTATGGVQSTASSKNASCYFDIEWRDR
RITLRASNGKFVTSKKNGQLAASVETAGDSELFLMKLINRPIIVFRGEHGFIGCRKVTGTLDANRSSYDV
FQLEFNDGAYNIKDSTGKYWTVGSDSAVTSSGDTPVDFFFEFCDYNKVAIKVGGRYLKGDHAGVLKAS
AETVDPASLWEYEAAAKETAGDSELFGPGPGTRKRYAHLSARPADEKKAGKATKVGKDKKAVTSSGDT
PKKGVQSTASSKNASKKAETVDPAS

Construction of vaccine 02 (Protein 02):

MSYQGKKNIPRITSDRLLIKGGKIVNDDQSFYADIYMEDGLIKQIGENLIVPGGVKTIEAHSRMVIPGGI
DVHTRFQMPDQGMTSADDFFQGTKAALAGGTTMIIDHVVPEPGTSLLAAFDQWREWADSKSCCDYS
LHVDISEWHKGIQEEMEALVKDHGVNSFLVYMAFKDRFQLTDCQIYEVLSVIRDIGAIAQVHAENGDII
AEEQQRILDLGITGPEGHVLSRPEEVEAEAVNRAITIANQTNCPLYITKVMSKSSAEVIAQARKKGTVVYG
EPITASLGTDGSHYWSKNWAKAAAFVTSPPLSPDPTTPDFLNSLLSCGDLQVTGSAHCTFNTAQKAVGK
DNFTLIPEGTNGTEERMSVIWDKAVVTGKMDENQFVAVTSTNAAKVFNLYPRKGRIAVGSDADLVIWD
PDSVKTISAKTHNSSLEYNIFEGMECRGSPLVVISQGKIVLEDGTLHVTEGSGRYIPRKPFPDFVYKRIKA
RSRLAELRGVPRGLYDGPVCEVSVTPKTVTPASSAKTSPAKQQAPPVRNLHQSGFSLSGAQIDDNIPRRT
TQRIVAPPGGRANITSLGEAAAKNAAKVFNLYEAAAKHVTEGSGRYGPGPGAAKVFNLYPRKGRIAKKN
TAQKAVGKDNFTLIPEGTNKKGKKNIPRITSKKKDRFQLTDC

Construction of vaccine 03 (Protein 03):

MAGRLPACVVDCGTGYTKLGYAGNTEPQFIIPSCIAIKESAKVGDQAQRRVMKGVDDLDFFIGDEAIEK
PTYATKWPIRHGIVEDWDLMERFMEQVIFKYLRAEPEDHYFLLTEPPLNTPENREYTAEIMFESFNVPG
LYIAVQAVLALAASWTSRQVGERTLTGTVIDSGDGVTHVIPVAEGYVIGSCIKHIPIAGRDITYFIQQLLRD
REVGIPPEQSLETAKAVKERYSYVCPDLVKEFNKYDTDGSKWIKQYTGINAISKKEFSIDVGYERFLGPEI
FFHPEFANPDFTQPISEVVDEVIQNCPIDVRRPLYKNIVLSGGSTMFRDFGRRLQRDLKRTVDARLKLSE
ELSGGRLKPKPIDVQVITHHMQRYAVWFGGSMLASTPEFYQVCHTKKDYEEIGPSICRHNPVFGVMSE
AAAKESFNVPGLYEAAAKCVVDCGTGYKKEFYQVCHKKTSRQVGERTKKVDARLKLSEELSGGRLKPK
PIKKKKDYEEIKKHPEFANPDFTQPIS

Construction of vaccine 04 (Protein 04):

MLANSASVRILIKGGKVVNDDCTHEADVYIENGIIQQVGRELMIPGGAKVIDATGKLVIPGGIDTSTHFH
QTFMNATCVDDFYHGTKAALVGGTTMIIGHVLPDKETSLVDAYEKCRGLADPKVCCDYALHVGITWW
APKVKAEMETLVREKGVNSFQMFMTYKDLYMLRDSELYQVLHACKDIGAIARVHAENGELVAEGAKEA
LDLGITGPEGIEISRPEELEAEATHRVITIANRTHCPIYLVNVSSISAGDVIAAAKMQGKVVLAETTTAHAT
LTGLHYYHQDWSHAAAYVTVPPLRLDTNTSTYLMSLLANDTLNIVASDHRPFTTKQKAMGKEDFTKIPH
GVSGVQDRMSVIWERGVVGGKMDENRFVAVTSSNAAKLLNLYPRKGRIIPGADADVVVWDPEATKTIS
ASTQVQGGDFNLYENMRCHGVPLVTISRGRVVYENGVFMCAEGTGKFCPLRSFPDTVYKKLVQREKTL
KVRGVDRTPYLGDVAVVVHPGKKEMGTPLADTPTRPVTRHGGMRDLHESSFSLSGSQIDDHVPKRAS
ARILAPPGGRSSGIWEAAAKNSFQMFMTYEAAAKTSTHFHQTFEAAAKDTNTSTYLMGPGPGNDTLNI
VASDHRPFTGPGPGDTLNIVASDHRPFTTKKTTKQKAMGKEDFTKIPHGKKKDLYMLRDS

Construction of vaccine 05 (Protein 05):

MTVTKMSWRPQYRSSKFRNVYGKVANREHCFDGIPITKNVHDNHFCAVNTRFLAIVTESAGGGSFLVIP
LEQTGRIEPNYPKVCGHQGNVLDIKWNPFIDNIIASCSEDTSVRIWEIPEGGLKRNMTEALLELHGHSR
RVGLVEWHPTTNNILFSAGYDYKVLIWNLDVGEPVKMIDCHTDVILCMSFNTDGSLLTTTCKDKKLRVI
EPRSGRVLQEANCKNHRVNRVVFLGNMKRLLTTGVSRWNTRQIALWDQEDLSMPLIEEEIDGLSGLLF
PFYDADTHMLYLAGKGDGNIRYYEISTEKPYLSYLMEFRSPAPQKGLGVMPKHGLDVSACEVFRFYKL
VTLKGLIEPISMIVPRRSDSYQEDIYPMTPGTEPALTPDEWLGGINRDPVLMSLKEGYKKSSKMVFKAPI
KEKKSVVVNGIDLLENVPPRTENELLRMFFRQQDEIRRLKEELAQKDIRIRQLQLELKNLRNSPKNCEA
AAKRSSKFRNVYEAAAKSACEVFRFYEAAAKQTGRIEPNYEAAAKYDADTHMLYKKKNLRNSPKKPYLS
YLMEFRSPAKKPEGGLKRNMTEAL

After constructing the multi-epitope vaccine candidates, the different constructs were tested to find their immunological suitability. Antigenicity, allergenicity and toxicity were determined in the designed vaccines to determine their potential in eliciting a safe and effective immune response. The prediction of antigenicity was done with the help of VaxiJen server and the prediction of allergenicity was done with the AllergenOnline database. Also, the analysis of the toxicity of the vaccine constructs was done using the T3DB server. These tests were conducted to ensure that the vaccine candidates designed have immunogenic properties and they do not cause allergenicity or toxicity.

Table 8: Evaluation of the designed vaccine constructs for antigenicity, allergenicity, and toxicity profiles

Vaccine No.	Antigenicity	Presence of Allergens	Presence Of Toxins
Vaccine-1	0.6740	Number of sequence with hits:0	Your search returned no results
Vaccine-2	0.6271	Number of sequence with hits:0	Your search returned no results
Vaccine-3	0.4799	Number of sequence with hits:0	Your search returned no results
Vaccine-4	0.5505	Number of sequence with hits:0	Your search returned no results
Vaccine-5	0.6636	Number of sequence with hits:0	Submitted sequence 1(1 Match)

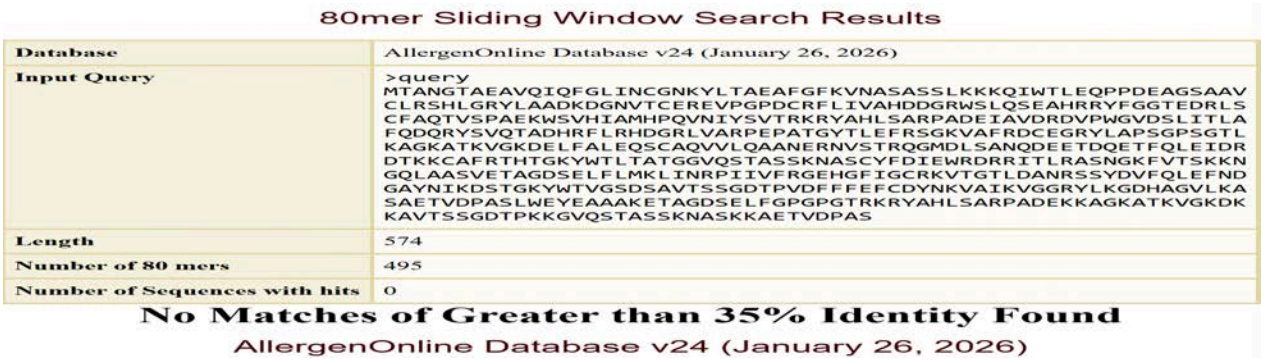


Figure 9: Allergenicity evaluation of the constructed vaccine candidate (i)

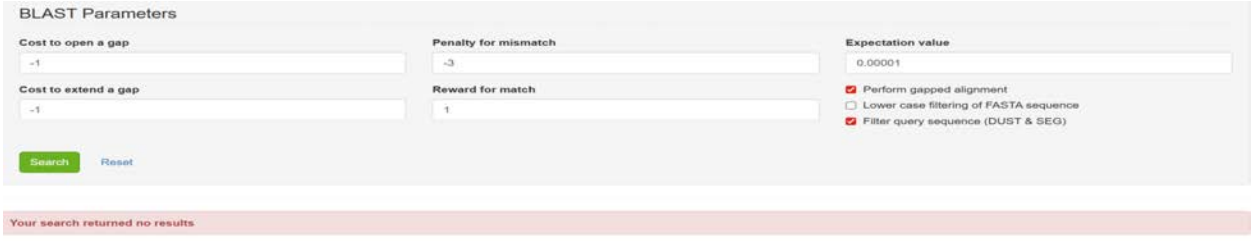


Figure 10: Toxicity evaluation of the constructed vaccine candidate (i)

3.6 Evaluation of Key Biochemical Parameters of the Prepared Vaccine Construct

ExPASy ProtParam online server was used to analyze the physicochemical properties of designed vaccine constructs. The tool is used to give comprehensive data about the biochemical properties of the vaccine candidates, through the analysis of parameters like molecular weight, amino acid composition, molecular formula, theoretical isoelectric point (pI), instability index, and the grand average of hydropathicity (GRAVY). Such physicochemical indicators are beneficial to identify the stability, solubility, and overall suitability of the vaccine constructs to proceed with immunological and structural analyses.

Table 9: Assessment of Physicochemical Characteristics of Designed Vaccine Constructs via ExPASy ProtParam

Parameters	Vaccine 1	Vaccine 2	Vaccine 3	Vaccine 4	Vaccine 5
Molecular Weight	62755.14	72366.25	58197.63	73813.24	65778.83
Theoretical PI	8.57	8.60	6.63	7.37	9.07
Instability Index	31.79	30.74	35.34	22.08	57.34
Aliphatic Index	65.85	80.09	79.07	79.66	82.23
GRAVY (Grand Average of Hydropathicity)	-0.539	-0.342	-0.392	-0.258	-0.461

Based on the summarized physicochemical analysis, all vaccine constructs exhibited negative GRAVY values, indicating their hydrophilic nature. GRAVY scores reflect the overall hydrophobicity or hydrophilicity of a protein, where negative values are generally associated with improved solubility and enhanced bioavailability. Such hydrophilic characteristics are considered favorable for vaccine development as they may reduce potential toxicity and support effective immune interactions. Furthermore, most vaccine candidates demonstrated instability index values below 40, suggesting satisfactory structural stability; however, Vaccine-5 showed a comparatively higher instability index, indicating lower stability relative to the other constructs. In addition, the theoretical isoelectric point (pI) values of the selected vaccine candidates remained within an acceptable range, supporting their potential suitability for therapeutic applications.

Number of amino acids: 574
Theoretical pI: 8.57
Molecular weight: 62755.14

Atomic composition:

Carbon	C	2748
Hydrogen	H	4307
Nitrogen	N	795
Oxygen	O	862
Sulfur	S	15

Formula: $C_{2748}H_{4307}N_{795}O_{862}S_{15}$
Total number of atoms: 8727

Extinction coefficients:
Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.
Ext. coefficient 69955
Abs 0.1% (=1 g/l) 1.115, assuming all pairs of Cys residues form cystines

Ext. coefficient 69330
Abs 0.1% (=1 g/l) 1.105, assuming all Cys residues are reduced

Estimated half-life:
The N-terminal of the sequence considered is M (Met).
The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:
The instability index (II) is computed to be 31.79
This classifies the protein as stable.

Aliphatic index: 65.85
Grand average of hydropathicity (GRAVY):-0.539

Figure 11: Physicochemical Analysis of Vaccine Candidate (i) Using ProtParam

3.7 Comparative (Homology) Modeling of the Prepared Vaccine Candidates

Phyre2 web-based protein modeling server was used to create three-dimensional structural models of the vaccine candidates constructed. The server predicts tertiary protein structures using homology-based fold recognition approaches and also offers clues to possible functional and ligand-binding domains. The sequences of the five vaccine constructs were uploaded to the server separately to predict their structures. In every submission, the server produced models and confidence values and coverage of the sequences that showed the trustworthiness of the modeled structures. The modeling process required approximately 30 minutes to 2 hours per construct and the summarized results are shown in the table below.

Table 10: Model Coverage and Confidence of Vaccine Constructs Assessed using Phyre2

Parameters	Vaccine 1	Vaccine 2	Vaccine 3	Vaccine 4	Vaccine 5
Confidence	100%	100%	100%	100%	100%
Coverage	84%	74%	81%	71%	66%

The vaccines had the highest sequence coverage as indicated in the table i.e. Vaccine candidate (i) had the highest coverage of about 84 and Vaccine (iii) had a coverage of about 81. The other vaccine constructs on the other hand portrayed lower coverage values of between 80 and 60 percent as compared to each other and were not deemed very favourable to the objectives of this research. Vaccine (i) was chosen based on these observations to carry out additional structural and functional analyses. The confidence score, as well as the detailed coverage information of Vaccine (i) and (iii) are as follows:

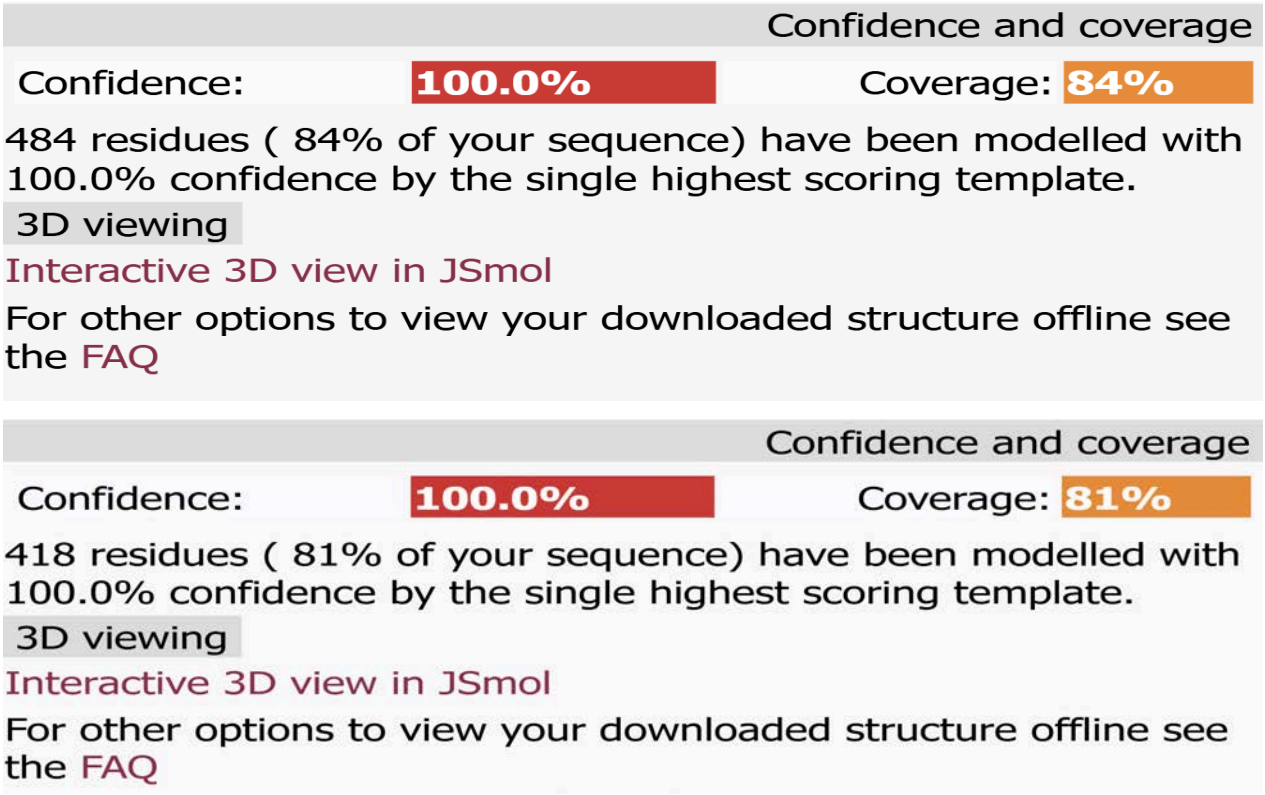


Figure 12: Phyre-2 results for vaccine (i) & (iii)

3.8 Z-Score Based Quality Evaluation of the Predicted 3D Vaccine Structure

The quality of the predicted vaccine model in the form of the structures was also assessed by the use of the ProSA-web server which scores the reliability of the protein structures in general using Z-score. This is an interactive web-based tool which gives graphical illustrations of quality scores and energy profiles to determine structural accuracy. Besides assessing modeled structures, the server makes comparisons with experimentally measured protein structures by X-ray crystallography and NMR spectroscopy.

In this analysis, the PDB file that was produced on the Phyre2 modeling server was entered into the ProSA-web interface. The server generated a Z-score indicating the quality of the overall structure of the vaccine model, and a local plot of quality indicating the energy values based on knowledge across the sequence positions. The energy profile showed variations between the residues, with some negative energy regions (below the baseline) and positive energy regions (above the baseline) which show variations in the local structural stability of the predicted model.

Table 11: ProSA-Web-Based Analysis of Vaccine Constructs by Z-Score Analysis

Z Scores	Vaccine 1	Vaccine 2	Vaccine 3	Vaccine 4	Vaccine 5
	-7.66	-9.48	-7.09	-10.71	-11.08

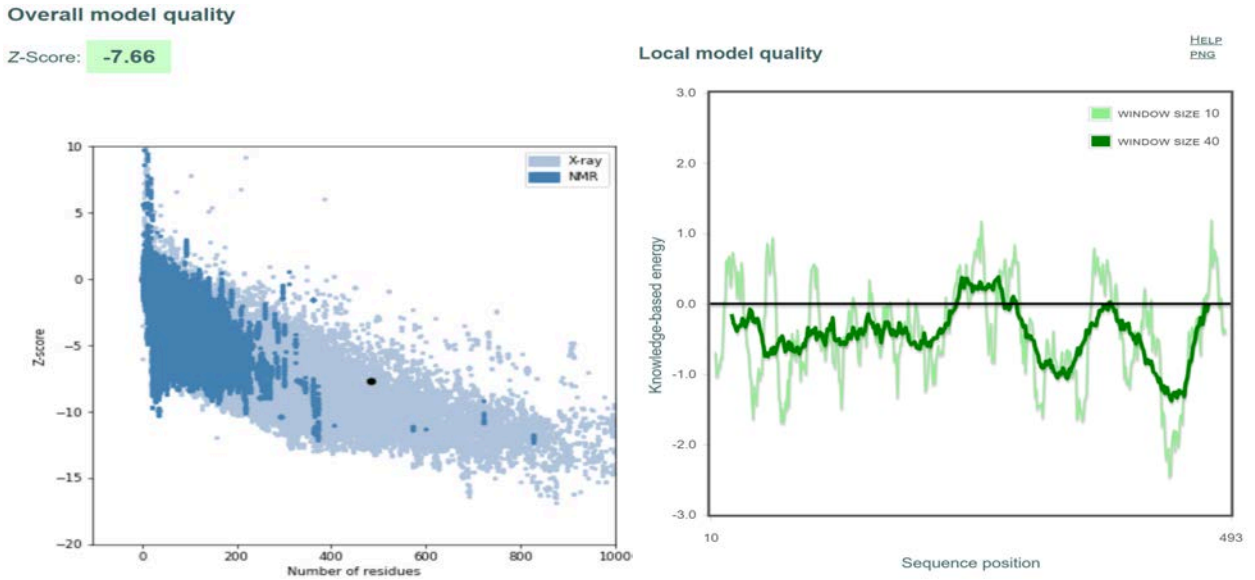


Figure 13: Evaluation of Overall model quality and local model quality of Vaccine (i)

3.9 Structural Reliability Checking Using Ramachandran Plots

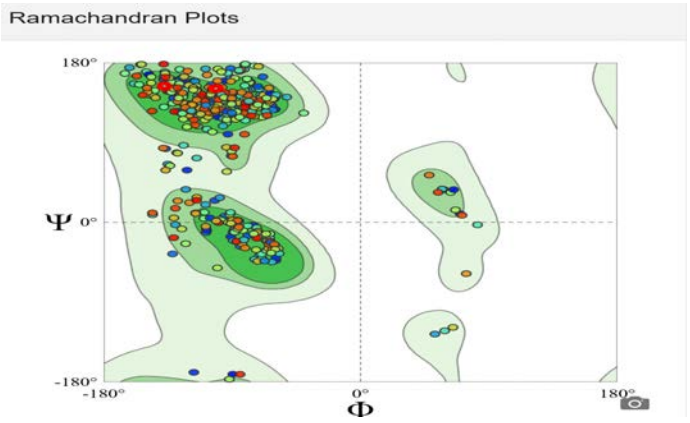
Analysis of the predicted vaccine structure in the form of Ramachandran plots was done with the SWISS-MODEL (ExPASy) structure validation program by uploading PDB files obtained after the Phyre2 server. This discussion gives a view of the stereochemical quality of the models based on the distribution of dihedral angles of the backbone. The percentages of residues that are contained within the preferred regions and the percentages of outliers of the five vaccine candidates are summarized as given in the table below.

Table 12 : Vaccine Model Structural validation using Ramachandran plot analysis

Parameters	Vaccine 1	Vaccine 2	Vaccine 3	Vaccine 4	Vaccine 5
Ramachandran Favored	96.27%	97.35%	81.97%	98.34%	94.36%
Ramachandran Outlier	0.00%	0.00%	3.85%	0.21%	0.77%
Rotamer outliers	0.00%	0.00%	0.00%	0.00%	0.00%
MolProbity	2.27	2.04	2.86	2.03	2.77

Re-examination of the Ramachandran plot showed that most of the residues in the predicted vaccine models were found in the preferred regions which suggested that the structures were of acceptable stereochemical quality. A smaller percentage of residues were found in the extra allowed regions with only a small percentage found as outliers. The abundance of residues in preferred regions indicates that the model vaccine is expected to have a stable and reliable backbone conformation that is likely to be further structurally analyzed.

Based on the table we can observe that, the most favored region of the vaccine is the no 1 which has Ramachandran Favored region and Ramachandran Outlier region of 96.27 and 0.00 respectively. Consequently, the outcome is satisfactory since Ramachandran Favored region is more than 90 percent and Ramachandran outlier is less than 2.



Ramachandran Favoured	96.27%
Ramachandran Outliers	0.00%
Rotamer Outliers	0.00%
C-Beta Deviations	0

Figure 14: Ramachandran Plot Analysis of Vaccine (i) Using the SWISS-MODEL Tool

Table 13: Comparative Overview of Assessed Parameters Across Vaccine Candidates

Parameters	Server	Vaccine 1	Vaccine 2	Vaccine 3	Vaccine 4	Vaccine 5
Antigenicity of vaccine	Vaxijen V.2.0	0.6740	0.6271	0.4799	0.5505	0.6636
Allergenicity	Allergen Online	None	None	None	None	None
Toxicity	T3DB	None	None	None	None	Present
Theoretical Isoelectric Point (pI)	Protparam	8.57	8.60	6.63	7.37	9.07
Instability Index		31.79	30.74	35.34	22.08	57.34
GRAVY		-0.539	-0.342	-0.392	-0.258	-0.461
Confidence	PHYRE-2	100%	100%	100%	100%	100%
Coverage		84%	74%	81%	71%	66%
Ramachandran Favored	Ramachandran SWISS MODEL	96.27%	97.35%	81.97%	98.34%	94.36%
Ramachandran Outlier		0.00%	0.00%	3.85%	0.21%	0.77%

According to the summary table, Vaccine (i) meets the prerequisites. Moreover, the physicochemical parameters values were not out of the acceptable range: the theoretical pI value was between 8 and 10 (8.57), the instability index (31.79) was less than 40, and the GRAVY value was negative (-0.539). These findings indicate that the vaccine is not volatile and has hydrophilic properties. The vaccine also showed good antigenicity with a score of 0.6740 which means that the vaccine is likely to trigger an immune response. Moreover, it did not show any allergenicity or toxicity.The model reliability was also supported by the structural validation parameters. The coverage was 84% and the confidence score was 100%. Besides that, Ramachandran favored region was found to be more than 90 (96.27) and no Ramachandran outliers were found (0.00) indicating strong stereochemical quality of the predicted structure.Comparatively, Vaccine (v) also had a good antigenicity (0.6636) and a 100 percent confidence value, but with lower coverage (66%). Although its physicochemical characteristics were satisfactory, forecasted toxicity and a relatively low structural coverage indicated that it was not as preferable as Vaccine (i).Vaccine (ii) and Vaccine (iv) also had good properties but Vaccine (i) had relatively better antigenicity and structural stability than the other candidates.

Overall, Vaccine (i) showed higher antigenicity and a satisfactory percentage of residues within the favored regions of the Ramachandran plot, indicating that it may serve as a promising candidate for further investigation. Therefore, Vaccine (i) was selected for subsequent analyses to evaluate its immune response potential and binding affinity. To validate this, molecular docking and immune simulation studies were performed to further assess its suitability for vaccine development.

The primary aim of this experiment was to assess the individual vaccine constructs in terms of antigenicity, physicochemical characteristics, and structural stability and then compare the two to find the most appropriate vaccine candidate.

Comprehensively, Vaccine (i) was more antigenic and had a good percentage of residues in the preferred locations of the Ramachandran plot and this means that it could be a viable candidate to be experimented upon. Thus, Vaccine (i) was chosen as a further subject of analyses to check the potential of its immune response and binding affinity. To confirm this, molecular docking and immune simulation were also conducted to further evaluate its applicability in the development of vaccines.

3.10 Molecular Docking Analysis of the Vaccine Construct with Toll-Like Receptors (TLRs)

Analysis of molecular docking was done on the ClusPro server to assess the interaction between the designed vaccine construct and Toll-like receptor 3 (TLR-3). TLR-3 PDB structure was the receptor and the modeled vaccine structure obtained in the Phyre2 server was provided as the ligand. Ten possible docked complexes that depicted various binding orientations of the receptor and ligand were produced in the docking simulation.

Among these complexes whose docking was predicted, the first docked model showed the most stable interaction with a docking score of -714.9. A further analysis was conducted using cluster analysis in order to determine the most preferable binding geometry. Cluster 0 was chosen as the best docked complex as it showed the highest cluster population (94 members) and lowest energy score (−714.9) of the interaction between the vaccine candidate (i) and the TLR-3 receptor.

Cluster	Members	Representative	Weighted Score
0	94	Center	-692.9
		Lowest Energy	-714.9
1	81	Center	-641.4
		Lowest Energy	-780.8
2	70	Center	-750.4
		Lowest Energy	-751.1
3	67	Center	-626.6
		Lowest Energy	-777.9
4	54	Center	-598.6
		Lowest Energy	-793.6
5	54	Center	-636.7
		Lowest Energy	-735.0

Figure 15: Analysis and Selection of the Most Energetically Favorable Docked Complex Using ClusPro

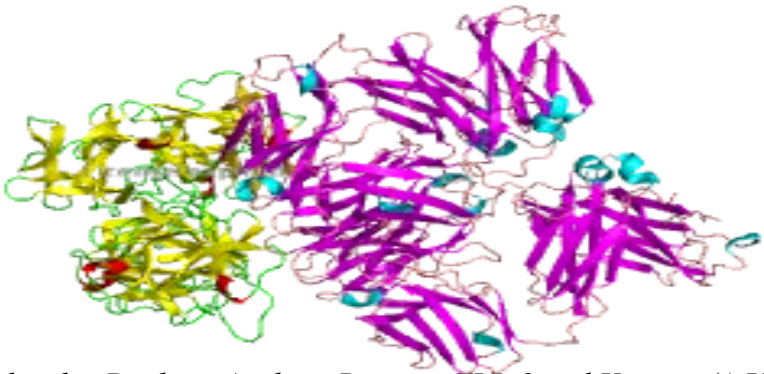


Figure 16: Molecular Docking Analysis Between TLR-3 and Vaccine (i) Using ClusPro

3.11 Immune Simulations

Immune response simulation of the selected vaccine construct was performed using the C-ImmSim server to evaluate its potential immunogenic behavior. After submitting the vaccine sequence, the simulation completed successfully and generated graphical outputs illustrating the predicted immune responses, as shown in Figure 18. The results indicated an initial increase in antigen concentration following vaccination, as observed in graph (a).

Subsequently, the simulation demonstrated notable peaks in IgM and IgG antibody levels around day 28, with a substantial increase in antibody production after approximately 60 days. These patterns suggest that the vaccine construct is capable of eliciting a strong immune response. In addition, the activation and proliferation of lymphocytes were observed, leading to the generation of both antibodies and immunological memory cells. The formation of these memory cells is essential for maintaining long-term immune protection and enabling a rapid immune response upon future exposure to the target antigen.

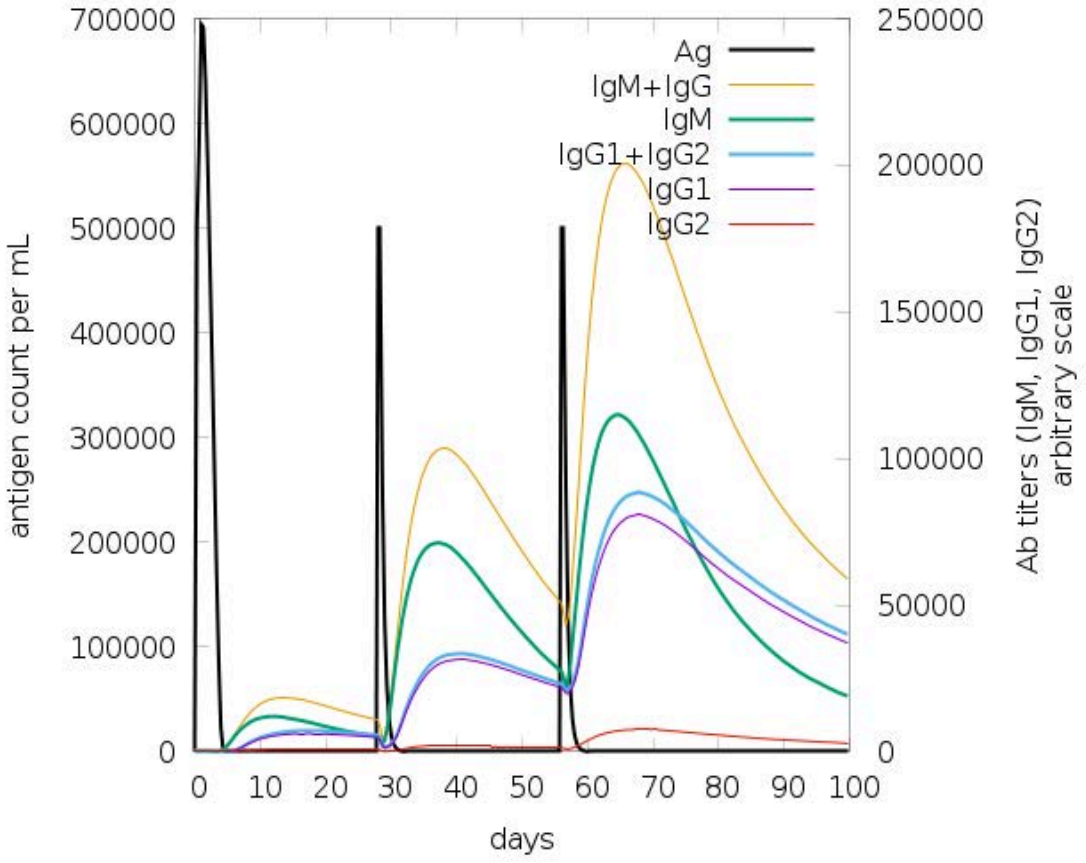


Figure 17: Immune simulation of vaccine (i) illustrated graphically via C-ImmSim

Chapter 4

Discussion

One of the most severe and resistant brain tumors which cannot be treated is glioblastoma, which is characterized by rapid development, high recurrence rate, and few treatment options. Nevertheless, surgery, radiotherapy, and chemotherapy have improved but the prognosis of the patients is poor. As a result, the design of efficient immunotherapeutic methods, in particular, peptide-based vaccines, has become a topic of significant interest. In this regard, the use of in-silico immunoinformatics methods offers a quick and methodical framework of screening promising vaccine candidates as well as saving a lot of time and expense of traditional experimental vaccine development.

The five glioblastoma-associated proteins were identified as the possible targets of vaccine creation in the current study. To determine immunogenic epitopes that can stimulate both cellular and humoral immune response, these proteins were analyzed systematically with several bioinformatics tools. First, antigenicity, allergenicity, and toxicity were used to predict and screen CTL, HTL, and B-cell epitopes to achieve immunological safety and efficacy. Multiplexing of selected epitopes into multi-epitope vaccine constructs was then employed with the use of suitable peptide linkers to preserve the structural integrity as well as enhance the immunogenic activity. Out of the five vaccine constructs created, only Vaccine (i) remained capable of meeting all the defined immunological, physicochemical, and structural validation objectives, with the rest of the constructs being eliminated because of constraints like inadequate antigenicity, predicted toxicity, or decreased structural stability, which further supports the strength of the selection strategy.

After constructing the vaccines, every candidate was tested using a series of computer-based tests, such as antigenicity tests, allergenicity tests, toxicity tests, and physicochemical tests. All these analyses were done to ascertain the stability, solubility, and immunological capabilities of the vaccine constructs. Tertiary structures were also predicted with structural modeling with the Phyre2 server and structural validation was done with the use of Z-score analysis and Ramachandran plot analysis to determine the quality of the stereochemical structure of the predicted models. The comparative analysis of the vaccine constructs made it possible to identify the candidate with the most desirable structural and physicochemical properties.

In addition, molecular docking with immune receptors including the Toll-like receptors (TLRs) was done to determine possible interactions between the vaccine construct and host immunological recognition molecules. The docking outcomes were able to give information about the binding affinity and potential immunostimulatory property of the designed vaccine candidate. Moreover, the C-ImmSim server was used to analyze the immune simulation to forecast the immune response after immunizing the body with the vaccine. According to the simulation, the production of immunological responses such as the production of antibodies and activation of immune cells occurred, which may indicate that the designed construct has the potential to trigger a humoral and cellular immune response.

Lastly, the Protein (i)-based vaccine construct was further tested to induce immune responses by using the C-ImmSim server. This immune simulation tool forecasts how a vaccine candidate will cause humoral and cellular immune response upon administration. The analysis produced graphical illustrations to show how antibodies including IgG and IgM are produced, as well as how CD4+ helper T cells and CD8+ cytotoxic T cells are activated. These modeled immune response patterns allowed the ability of the developed vaccine (i) to elicit a robust antibody response and cellular immunity.

Chapter 5

Conclusion

Conclusively, this paper explored the possibility of coming up with a multi-epitope vaccine candidate through the analysis of various glioblastoma-associated proteins. Predicted and evaluated epitope sequences of the chosen target proteins were done in-silico through an in-silico immunoinformatics framework. All the proteins were then evaluated on their suitability as a vaccine in terms of antigenicity, allergenicity, toxicity, physicochemical traits as well as structural stability. This was then compared to arrive at the most promising construct among the predicted constructs. An example of the vaccine designs evaluated showed relatively better antigenic potential, physicochemical properties and structural stability and this demonstrates its potential to be explored further. These results underscore the usefulness of computational immunology methods in speeding up the process of identifying possible therapeutic vaccine targets.However, even though the computational results are promising, further experimental validation is needed. To verify the safety, stability and immunogenic effectiveness of the proposed vaccine candidate, laboratory-based research and clinical testing will be vital before it can be potentially used in therapeutic approaches.

5.1 Limitations of the Study

Although the current study has brought positive results, it has a number of limitations inherent in it. First of all, the whole process of designing a vaccine and its testing was based on in-silico techniques, which are not based on experimental testing, but on computational prediction. Though these approaches offer a speedy and economical platform of finding possible vaccine candidates, it is unable to reproduce the complexity of biological systems entirely. Thus, in-vitro and in-vivo experiments are necessary to confirm the antigenicity, immunogenicity, safety and binding interaction of the proposed vaccine construct.

The other weakness of this study is the choice of candidate proteins. Though 5 glioblastoma-associated protein sequences were first identified to be used in designing the vaccine, one construct finally met all the established immunological, physicochemical and structural requirements. The fact that the remaining four candidates were sifted out is indicative of how rigorous the selection process was, but also suggests there was a narrow range of viable vaccine constructs, which could limit the diversity and strength of the final candidate.

Moreover, the outcomes of immune simulation obtained using a computational platform might not be representative of the dynamic and complex immune responses that happen in a living organism. Host genetic variability, tumour heterogeneity and microenvironmental effects are factors of great importance to the vaccine performance but cannot be fully modeled in the in-silico models.

Therefore, although the results of this research give a solid initial background to develop the glioblastoma vaccine, extensive experimental verification and clinical research is needed to identify the feasibility of the given vaccine candidate and its potential therapeutic effect.

5.2 Future Prospects

To take this work beyond computational predictions, one research should be aimed in future to experimentally validate the proposed vaccine candidate. To ensure that it can induce a robust immune response and be safe under actual biological conditions, in-vitro and in-vivo experiments will be required. Moreover, more sophisticated delivery technology, including nanoparticle-based methods, might be of benefit to the stability of the vaccine and provide it with a better targeted delivery inside the body.

Additional enhancement can also be done by extending the research to cover more proteins associated with glioblastoma or use multitarget vaccine designs to enhance efficiency. The combination of artificial intelligence and machine learning tools may be used to optimize epitope prediction and speed up the process of vaccine creation in general. In case of their successful implementation, they can help create a more personalized and effective immunotherapy in treating glioblastomas.

References

S. Akira and K. Takeda, “Toll-like receptor signalling,” Nature Reviews Immunology, 2004.

D. Bhattacharya, J. Nowotny, R. Cao and J. Cheng, “3Drefine: An interactive web server for efficient protein structure refinement,” Bioinformatics, 2016.

F. Castiglione, F. Celada and M. Bernaschi, “The immune system as a computational system,” Immunological Reviews, 2012.

S. R. Comeau, D. W. Gatchell, S. Vajda and C. J. Camacho, “ClusPro: An automated docking and discrimination method for the prediction of protein complexes,” Bioinformatics, 2004.

I. A. Doytchinova and D. R. Flower, “VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines,” BMC Bioinformatics, 2007.

O. Finn, “A believer’s overview of cancer immunosurveillance and immunotherapy,” Journal of Immunology, 2018.

E. Gasteiger, C. Hoogland, A. Gattiker et al., “Protein identification and analysis tools on the ExPASy server,” in The Proteomics Protocols Handbook, Humana Press, 2005.

L. Heo, H. Park and C. Seok, “GalaxyRefine: Protein structure refinement driven by side-chain repacking,” Nucleic Acids Research, 2013.

M. C. Jespersen, B. Peters, M. Nielsen and P. Marcatili, “BepiPred-2.0: Improving sequence-based B-cell epitope prediction,” Nucleic Acids Research, 2017.

A. Kiel et al., “Immunoinformatics approaches in vaccine design,” 2025.

L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass and M. J. E. Sternberg, “The Phyre2 web portal for protein modeling, prediction and analysis,” Nature Protocols, 2015.

Y. Mineharu, N. Kamran and P. R. Lowenstein, “Dendritic cell-based immunotherapy for glioblastoma: Current status and future directions,” Frontiers in Immunology, 2011.

M. R. Neagu and D. A. Reardon, “An update on the role of immunotherapy and vaccine strategies in glioblastoma,” Current Treatment Options in Oncology, 2015.

M. R. Olin, M. Ampudia-Mesias, L. L. Pennell and I. F. Parney, “Treatment combining CD200 immune checkpoint inhibition and tumor-lysate vaccination after surgery for glioblastoma,” Journal for ImmunoTherapy of Cancer, 2014.

L. Potocnakova, M. Bhide and I. B. Pulzova, “An introduction to B-cell epitope mapping and in silico epitope prediction,” Journal of Immunology Research, 2016.

J. Ponomarenko and P. E. Bourne, “Antibody–protein interactions: Benchmark datasets and prediction tools evaluation,” BMC Structural Biology, 2007.

M. Prajapat et al., “Prediction and analysis of conserved B-cell epitopes,” Journal of Biomolecular Structure and Dynamics, 2021.

N. Rapin, O. Lund, M. Bernaschi and F. Castiglione, “Computational immunology meets bioinformatics,” PLoS ONE, 2010.

B. Reynisson, B. Alvarez, S. Paul, B. Peters and M. Nielsen, “NetMHCpan-4.1 and NetMHCIIpan-4.0,” Nucleic Acids Research, 2020.

B. Sahay, R. K. Choudhury and S. K. Gupta, “In silico prediction and validation of vaccine candidates,” Infectious Disorders - Drug Targets, 2020.

T. N. Schumacher and R. D. Schreiber, “Neoantigens in cancer immunotherapy,” Science, 2015.

A. Singh, M. Thakur and A. Sharma, “Immunoinformatics-based vaccine design and immune simulation analysis,” Computational Biology and Chemistry, 2021.

G. Studer et al., “SWISS-MODEL: Homology modelling of protein structures and complexes,” Nucleic Acids Research, 2020.

O. Trott and A. J. Olson, “AutoDock Vina: Improving the speed and accuracy of docking,” Journal of Computational Chemistry, 2010.

M. Weller et al., “Glioblastoma,” Nature Reviews Disease Primers, 2015.

M. R. Wilkins et al., “Protein identification and analysis tools in the ExPASy server,” Methods in Molecular Biology, 1999.

M. Andreatta and M. Nielsen, “Gapped sequence alignment using artificial neural networks: Application to the MHC class I system,” *Bioinformatics*, 2016.

J. A. Greenbaum, P. H. Andersen, M. Blythe et al., “Towards a consensus on datasets and evaluation metrics for developing B-cell epitope prediction tools,” *Journal of Molecular Recognition*, 2007.

M. Nielsen and M. Andreatta, “NetMHCpan-3.0: Improved prediction of binding to MHC class I molecules integrating information from multiple receptor and peptide length datasets,” *Genome Medicine*, 2016.

P. A. Reche and E. L. Reinherz, “Prediction of peptide-MHC binding using profiles,” *Methods in Molecular Biology*, 2007.

J. Yang, R. Yan, A. Roy, D. Xu, J. Poisson and Y. Zhang, “The I-TASSER Suite: Protein structure and function prediction,” *Nature Methods*, 2015.

R. Vita, S. Mahajan, J. A. Overton et al., “The Immune Epitope Database (IEDB): 2018 update,” *Nucleic Acids Research*, 2019.

B. Peters and A. Sette, “Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method,” *BMC Bioinformatics*, 2005.

A. Sette and J. Fikes, “Epitope-based vaccines: An update on epitope identification, vaccine design and delivery,” *Current Opinion in Immunology*, 2003.

R. Solanki and M. Tiwari, “Cancer immunotherapy and peptide vaccine strategies: Current perspectives,” *Molecular Biology Reports*, 2021.

D. Hanahan and R. A. Weinberg, “Hallmarks of cancer: The next generation,” *Cell*, 2011.

C. R. Iacobuzio-Donahue, “Epigenetic changes in cancer,” *Annual Review of Pathology*, 2009.

D. N. Louis, H. Ohgaki, O. D. Wiestler et al., “The 2016 World Health Organization classification of tumors of the central nervous system,” *Acta Neuropathologica*, 2016.

T. Cloughesy, W. Cavenee and P. Mischel, “Glioblastoma: From molecular pathology to targeted treatment,” *Annual Review of Pathology*, 2014.

A. B. Heimberger and D. A. Reardon, “Immunotherapy advances for glioblastoma,” *Neuro-Oncology*, 2015.

D. A. Reardon, H. Okada and M. Weller, “Vaccines and cellular immunotherapy in glioblastoma,” *Neuro-Oncology*, 2013.

H. Okada, J. Attanucci, C. M. L. Hambardzumyan et al., “Immunotherapeutic approaches for glioma,” *Critical Reviews in Immunology*, 2009.