Alpaca-Derived Nanobodies Targeting Herpes Simplex Virus Glycoprotein B – A Review

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ABSTRACT

Herpes simplex virus (HSV) infections remain a major global health concern, affecting millions of individuals annually and posing significant risks to neonates and immunocompromised populations. Despite the availability of nucleoside analogue antivirals, current therapies are limited by drug resistance, incomplete viral clearance, and inability to prevent viral entry or latency. In recent years, nanobodies—single-domain antibodies derived from camelid heavy-chain-only antibodies—have emerged as a promising class of biologics due to their small size, high stability, and exceptional antigen-binding capabilities.

This review comprehensively examines the development and application of alpaca-derived nanobodies targeting glycoprotein B (gB), a highly conserved and essential envelope protein required for HSV membrane fusion and host cell entry. Emphasis is placed on structural and functional insights obtained through cryo-electron tomography, which have elucidated the interaction between nanobodies and gB at near-molecular resolution. The article further details the molecular mechanism underlying gB neutralization, highlighting how nanobody binding disrupts conformational rearrangements necessary for viral fusion.

In addition, it outlines the complete production pipeline for nanobodies, including antigen selection, camelid immunization, VHH gene library construction, phage display-based screening, recombinant expression, and purification. Experimental methodologies employed in evaluating nanobody efficacy, stability, and antiviral activity are critically discussed. Finally, the therapeutic implications, advantages over conventional monoclonal antibodies, and future prospects of nanobody-based antivirals are explored. Collectively, this work positions alpaca-derived nanobodies as a novel and potent strategy for preventing HSV infection through targeted inhibition of viral entry.

Keywords:Nanobody, VHH antibody, Alpaca antibodies, Herpes simplex virus, Glycoprotein B, Viral entry inhibition, Cryo-electron tomography, Antiviral biologics

INTRODUCTION

Herpes simplex virus (HSV) infections represent one of the most prevalent viral diseases affecting humans worldwide. The virus exists primarily as two closely related serotypes, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), both of which establish lifelong latent infections following primary exposure. HSV-1 is predominantly associated with orofacial lesions, while HSV-2 is commonly implicated in genital herpes; however, overlapping clinical manifestations are increasingly observed. The ability of HSV to persist in sensory neurons and periodically reactivate poses a significant challenge to effective disease control and eradication.

Current therapeutic strategies for HSV infection rely largely on nucleoside and nucleotide analogues such as acyclovir, valacyclovir, and famciclovir, which inhibit viral DNA polymerase activity. Although these agents reduce symptom severity and viral shedding, they do not prevent initial viral entry, eliminate latent reservoirs, or fully suppress reactivation. Moreover, prolonged use of these antivirals has led to the emergence of drug-resistant HSV strains, particularly among immunocompromised individuals. These limitations underscore the urgent need for novel antiviral approaches that target earlier stages of the viral life cycle.

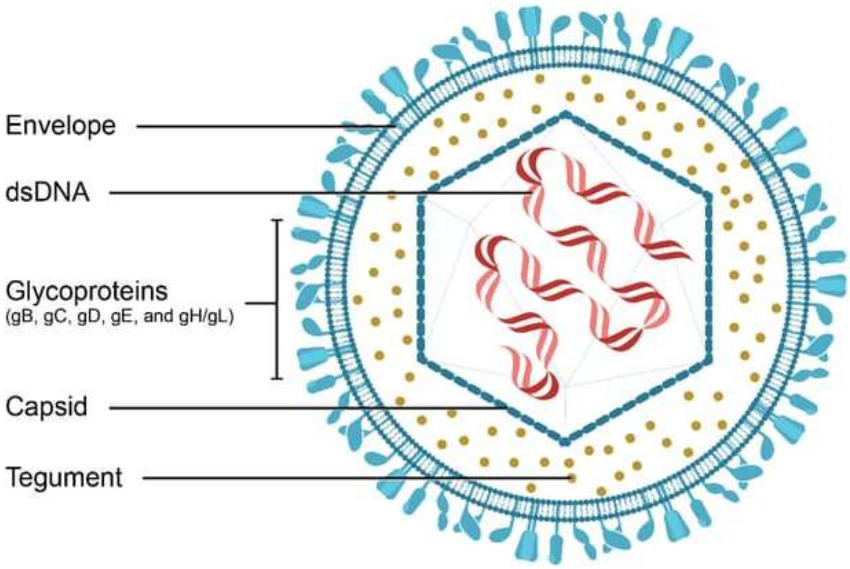

Figure 1. Structural architecture of the Herpes Simplex Virus (HSV) showing the lipid envelope embedded with glycoproteins (gB, gC, gD, gE, gH), underlying tegument layer, and central nucleocapsid containing double-stranded DNA.

Viral entry into host cells is a critical and highly regulated step in HSV infection. This process is mediated by a coordinated interaction between multiple viral envelope glycoproteins and host cell receptors. Among these glycoproteins, glycoprotein B (gB) plays an indispensable role in mediating membrane fusion between the viral envelope and the host cell membrane. Due to its high degree of conservation across HSV strains and its essential function in viral infectivity, gB has emerged as an attractive target for antiviral intervention. Therapeutic strategies aimed at blocking gB function have the potential to prevent viral entry entirely, thereby halting infection at its earliest stage.

In parallel with advances in virology, antibody-based therapeutics have gained substantial attention as highly specific and potent antiviral agents. Conventional monoclonal antibodies, while effective in certain contexts, suffer from limitations such as large molecular size, limited tissue penetration, complex production processes, and high manufacturing costs. These challenges have prompted the exploration of alternative antibody formats with improved biophysical and pharmacological properties.

Nanobodies, also known as single-domain antibodies or VHH antibodies, are derived from the variable domain of heavy-chain-only antibodies naturally produced by camelids such as alpacas, llamas, and camels. Unlike conventional antibodies, nanobodies lack light chains and consist of a single antigen-binding domain. This unique structural simplicity confers several advantages, including small molecular size, exceptional thermal and chemical stability, high solubility, and the ability to recognize cryptic or recessed epitopes inaccessible to traditional antibodies. Furthermore, nanobodies can be efficiently produced using recombinant expression systems, making them highly attractive for large-scale pharmaceutical applications.

Recent studies have demonstrated that alpaca-derived nanobodies can bind with high affinity to HSV glycoprotein B, effectively neutralizing viral infectivity. Structural investigations using advanced imaging techniques such as cryo-electron tomography have provided unprecedented insights into the molecular interactions between nanobodies and gB, revealing how nanobody binding interferes with the conformational changes required for membrane fusion. These findings represent a significant advancement in the understanding of HSV entry mechanisms and open new avenues for antiviral drug development.

This review-style research article aims to provide a comprehensive and integrative analysis of alpaca-derived nanobodies targeting HSV glycoprotein B. The article synthesizes existing literature on HSV biology, nanobody engineering, and antiviral mechanisms while also examining experimental methodologies, production strategies, and therapeutic implications. By focusing on the mechanistic and translational aspects of nanobody-mediated gB neutralization, this work seeks to highlight the potential of nanobodies as next-generation antiviral biologics and to inform future research efforts in the field.

LITERATURE REVIEW

1. Global Burden and Biological Characteristics of Herpes Simplex Virus

Herpes simplex virus infections continue to impose a substantial global health burden, with HSV-1 and HSV-2 infecting a significant proportion of the adult population worldwide. Epidemiological studies indicate that HSV-1 infection often occurs during childhood, whereas HSV-2 infection is predominantly acquired through sexual contact during adolescence or adulthood. Beyond mucocutaneous lesions, HSV infections can lead to severe complications such as neonatal herpes, herpes encephalitis, and disseminated infections in immunocompromised individuals.

HSV is an

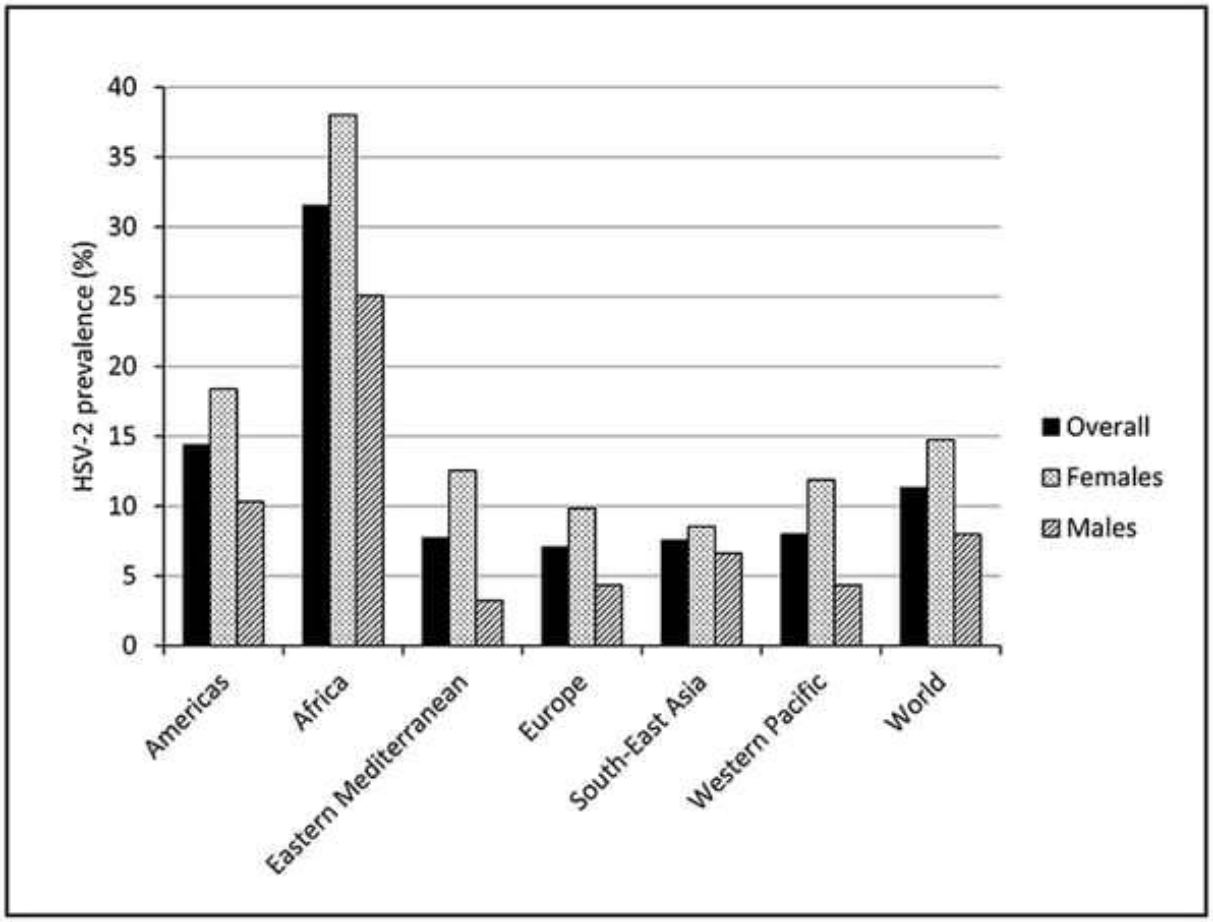

Figure 2. Sex-wise comparison of HSV-2 prevalence across WHO regions and worldwide.

enveloped, double-stranded DNA virus belonging to the family Herpesviridae. The viral envelope is studded with multiple glycoproteins that orchestrate attachment, receptor recognition, membrane fusion, and viral entry. Following entry, the virus establishes latency in sensory neurons, enabling lifelong persistence and episodic reactivation. The complexity of the HSV life cycle has made it challenging to develop interventions that completely prevent infection or eliminate latent virus.

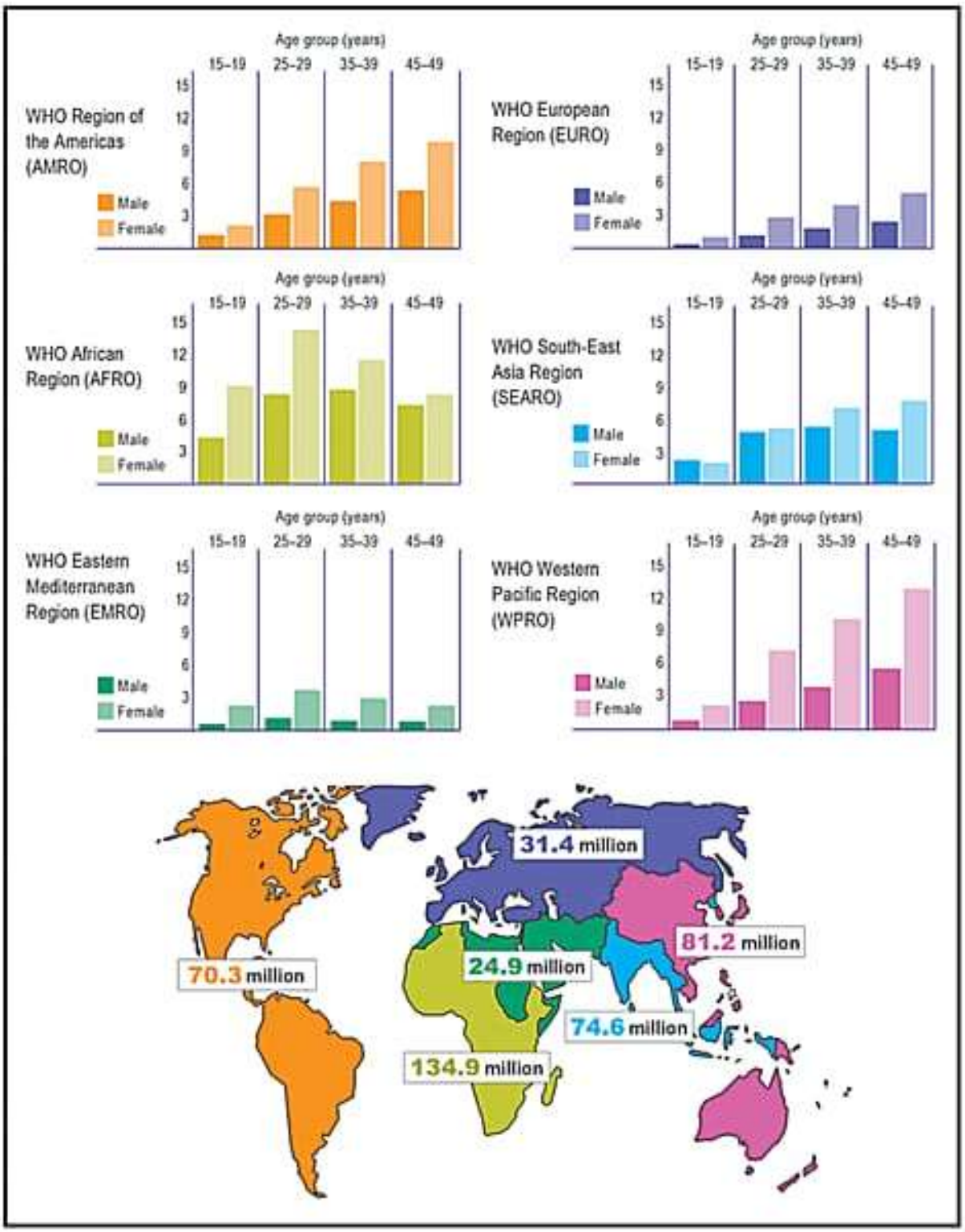

Figure 3. Global prevalence of herpes simplex virus (HSV) infection across WHO regions, stratified by age group (15–49 years) and sex. The bar charts depict age- and gender-specific prevalence patterns within each WHO region (AMRO, EURO, AFRO, SEARO, EMRO, and WPRO), highlighting higher prevalence with increasing age and a generally greater burden among females. The world map summarizes regional estimates of the total number of individuals living with HSV infection, emphasizing substantial geographic variation in the global disease burden.

2. Role of Viral Glycoproteins in HSV Entry

Extensive research has elucidated the multistep process of HSV entry into host cells, which involves the coordinated action of several envelope glycoproteins, including glycoprotein D (gD), glycoprotein H (gH), glycoprotein L (gL), and glycoprotein B (gB). Initial attachment is mediated by interactions between viral glycoproteins and host cell surface molecules such as heparan sulfate proteoglycans. Subsequent receptor engagement triggers conformational changes that activate the fusion machinery.

Among these glycoproteins, gB is recognized as the principal fusogen responsible for mediating the merger of viral and cellular membranes. Structural studies have revealed that gB undergoes dramatic conformational rearrangements during the fusion process, transitioning from a prefusion to a post fusion state. Due to its essential role and high conservation across HSV strains, gB has emerged as a prime target for therapeutic intervention.

3. Limitations of Conventional Antiviral Therapies

The clinical management of HSV infections relies primarily on nucleoside analogues that inhibit viral DNA replication. While these agents have proven effective in reducing disease severity and duration, they are inherently limited in their mechanism of action. Because these drugs act after viral entry and genome replication has begun, they do not prevent the establishment of infection or latency.

Furthermore, resistance to nucleoside analogues has been increasingly reported, particularly in patients with compromised immune systems who require long-term antiviral therapy. Resistant strains often arise due to mutations in viral thymidine kinase or DNA polymerase, rendering standard treatments ineffective. These challenges have intensified efforts to identify novel antiviral strategies that target earlier stages of infection, such as viral attachment and entry.

4. Antibody-Based Approaches Against HSV

Antibody-mediated neutralization has long been explored as a strategy for preventing HSV infection. Neutralizing antibodies directed against viral glycoproteins can block receptor binding, inhibit membrane fusion, or promote immune clearance through antibody-dependent mechanisms. Several monoclonal antibodies targeting gD or gB have demonstrated protective effects in preclinical models.

However, the clinical translation of conventional monoclonal antibodies has been hindered by their large molecular size, limited tissue penetration, high production costs, and complex manufacturing processes. Additionally, full-length antibodies may exhibit suboptimal access to densely packed viral surface epitopes, reducing their neutralization efficiency.

5. Emergence of Nanobodies as Next-Generation Therapeutics

Nanobodies, derived from the variable heavy-chain domain of camelid antibodies, represent a transformative advancement in antibody engineering. First described in the early 1990s, nanobodies possess unique structural and functional properties that distinguish them from conventional antibodies. Their small size allows them to access epitopes that are sterically inaccessible to larger antibody formats, including clefts and recessed regions on viral proteins. Therefore, these nanobodies, significantly emerge as one of the most potent biological therapeutics.

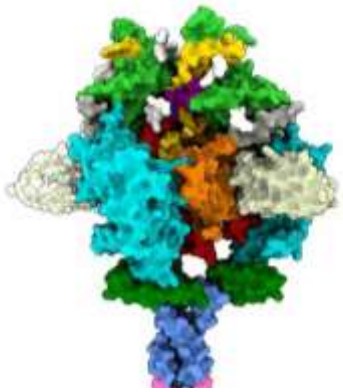

Figure 3. Molecular structure of ananobody.

Numerous studies have demonstrated the successful application of nanobodies against a wide range of viral pathogens, including influenza virus, respiratory syncytial virus, human immunodeficiency virus, and coronaviruses. These findings have highlighted the versatility of nanobodies as antiviral agents capable of high-affinity binding and potent neutralization.

6. Nanobodies Targeting HSV Glycoprotein B

Recent investigations have focused on the development of nanobodies targeting HSV glycoprotein B. Using alpaca immunization and phage display-based selection, researchers have isolated nanobodies that bind gB with high

specificity and affinity. Functional assays have shown that these nanobodies effectively block HSV-1 infection in vitro by preventing viral entry into host cells.

Advanced structural studies employing cryo-electron tomography and cryo-electron microscopy have provided detailed insights into the binding interface between nanobodies and gB. These analyses revealed that nanobody binding stabilizes gB in a conformation that is incompatible with membrane fusion, thereby neutralizing viral infectivity. The high conservation of gB across HSV strains suggests that such nanobodies may exhibit broad-spectrum activity against both HSV-1 and HSV-2.

7. Knowledge Gaps and Rationale for the Present Review

Despite significant progress, several knowledge gaps remain regarding the optimal design, production, and clinical application of nanobody-based antivirals for HSV. Questions related to in vivo stability, delivery strategies, immunogenicity, and large-scale manufacturing require further investigation. Moreover, a comprehensive synthesis of mechanistic, structural, and translational studies on gB-targeting nanobodies is currently lacking.

This review seeks to address these gaps by integrating existing literature on HSV entry mechanisms, nanobody engineering, and antiviral neutralization strategies. By critically evaluating current evidence and highlighting future research directions, the present work aims to contribute to the rational development of nanobody-based therapeutics for HSV infection.

MECHANISM OF NEUTRALIZATION OF GLYCOPROTEIN B (gB)

1. Structural and Functional Overview of Glycoprotein B

Glycoprotein B (gB) is a highly conserved type I transmembrane glycoprotein present on the surface of herpes simplex virus particles. It is universally required for viral entry and is considered the primary fusogenic protein of HSV. Structurally, gB exists as a trimer and is composed of an ectodomain, a transmembrane region, and a cytoplasmic tail. The ectodomain is organized into multiple functional domains that participate in membrane interaction, conformational rearrangement, and fusion pore formation.

During the viral entry process, gB functions in coordination with other viral glycoproteins, particularly gD and the gH/gL complex. Receptor engagement by gD triggers a cascade of molecular events that activate gB. Once activated, gB undergoes large-scale conformational changes that drive the fusion of the viral envelope with the host cell membrane, allowing delivery of the viral capsid into the cytoplasm. Because of its central role in this process, interference with gB function effectively abolishes viral infectivity.

2. Conformational Dynamics of gB During Viral Entry

The fusion activity of gB is dependent on its ability to transition between distinct structural states. In its prefusion conformation, gB remains metastable on the viral surface. Upon activation, gB refolds into a highly stable postfusion

conformation that brings the viral and cellular membranes into close proximity. This refolding process exposes hydrophobic fusion loops that insert into the host cell membrane, followed by collapse of the gB structure to facilitate

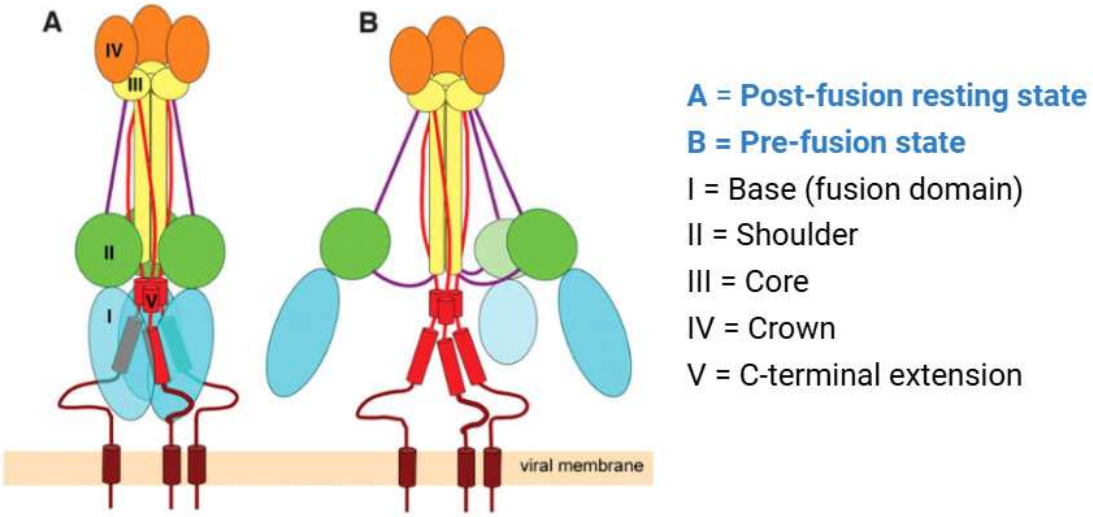

Figure 4. Structure and Conformational Dynamics of gB During Viral Entry

membrane merger.

The conformational flexibility of gB is tightly regulated, and even minor perturbations in this process can result in loss of fusion activity. Structural studies have demonstrated that specific regions of the gB ectodomain act as hinges or trigger points that are essential for initiating and propagating the fusion reaction. These regions represent vulnerable targets for therapeutic intervention.

3. Binding of Nanobodies to gB

Alpaca-derived nanobodies bind to discrete epitopes on the gB ectodomain with high affinity and specificity. Due to their small size and elongated antigen-binding loops, nanobodies are capable of penetrating recessed or sterically shielded regions of gB that are not readily accessible to conventional antibodies. This unique binding capability allows nanobodies to interfere directly with functionally critical domains involved in fusion.

Structural analyses have revealed that nanobody binding does not merely block surface accessibility but instead stabilizes gB in a non-fusogenic conformation. By engaging key structural elements of gB, nanobodies restrict the molecular flexibility required for the prefusion-to-postfusion transition. As a result, the energy-dependent refolding process necessary for membrane fusion is effectively arrested.

4. Inhibition of Membrane Fusion

The primary neutralization mechanism of gB-targeting nanobodies involves the inhibition of viral membrane fusion. By preventing the exposure or proper positioning of fusion loops, nanobodies block insertion of gB into the host cell membrane. This disruption halts the fusion cascade at an early stage, thereby preventing the formation of a fusion pore and subsequent viral entry.

Unlike antiviral agents that act after viral entry, nanobody-mediated neutralization operates extracellularly and prior to infection. This early intervention significantly reduces the likelihood of viral replication, dissemination, and establishment of latency. Importantly, because gB is conserved across HSV strains, this mechanism offers the potential for broad-spectrum neutralization. This corresponds to the inhibition and conservational constrain of the viral activities and infection ability.

5. Stabilization of Non-Functional gB Conformations

An additional mechanism contributing to nanobody-mediated neutralization is the stabilization of gB in conformations that are incompatible with fusion. Cryo-electron tomography studies have demonstrated that nanobody-bound gB exhibits reduced structural heterogeneity compared to unbound gB. This conformational locking effect prevents the coordinated rearrangements required for functional activation.

By effectively "freezing" gB in an inactive state, nanobodies render the virus incapable of responding to host receptor-mediated activation signals. This mode of action is particularly advantageous because it does not rely on competition with host receptors, reducing the likelihood of viral escape through receptor adaptation.

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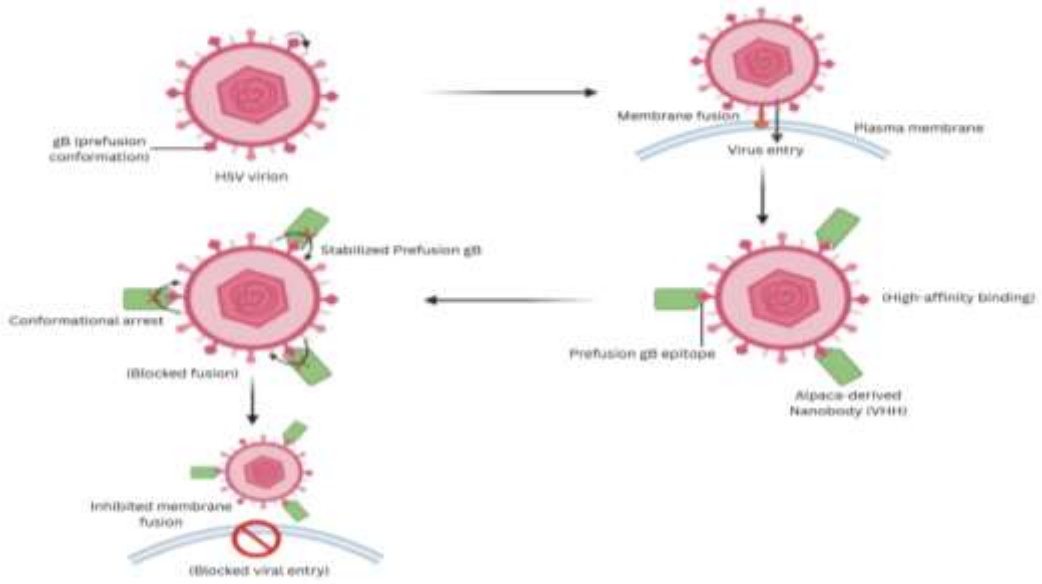

Figure 5. Alpaca-derived nanobodies bind the prefusion conformation of herpes simplex virus glycoprotein B, locking the protein in a non-functional state and thereby preventing the conformational rearrangements required for membrane fusion and viral entry.

Implications for Resistance and Viral Escape

Targeting gB through nanobody binding may also reduce the emergence of antiviral resistance. Because gB performs an essential and conserved function, extensive mutations that disrupt nanobody binding are likely to compromise viral fitness. Furthermore, nanobodies can be engineered into multivalent or bispecific formats to simultaneously engage multiple epitopes, further decreasing the probability of resistance development.

Collectively, these mechanistic insights highlight the robustness of gB-targeting nanobodies as antiviral agents. By directly interfering with the fundamental process of membrane fusion, nanobodies offer a powerful and mechanistically distinct approach to preventing HSV infection.

METHODOLOGY

This section describes the experimental and analytical approaches employed to evaluate alpaca-derived nanobodies targeting herpes simplex virus glycoprotein B. The methodology integrates immunological, molecular, structural, and virological techniques to assess nanobody generation, binding characteristics, and antiviral efficacy.

1. Antigen Preparation

Recombinant herpes simplex virus glycoprotein B ectodomain was produced using a heterologous expression system and purified to homogeneity. The purified protein was subjected to quality control analyses to confirm molecular integrity, structural stability, and antigenicity. Protein concentration was determined using spectrophotometric methods, and aliquots were stored under conditions that preserved conformational integrity.

2. Camelid Immunization Protocol

Healthy adult alpacas were immunized with the prepared antigen according to approved ethical guidelines. The immunization regimen consisted of a primary injection followed by multiple booster doses administered at defined intervals. Serum samples were periodically collected to monitor antigen-specific immune responses using immunoassays. Final blood collection was performed after confirmation of a robust antibody response.

3. Isolation of Lymphocytes and Genetic Material

Peripheral blood mononuclear cells were isolated from collected blood samples using density gradient centrifugation. Total RNA was extracted from these cells using commercially available RNA purification kits, following manufacturer protocols. RNA quality and integrity were assessed prior to downstream applications. Complementary DNA was synthesized using reverse transcription reactions optimized for full-length antibody gene recovery.

4. VHH Gene Amplification and Library Construction

VHH-encoding gene fragments were amplified by polymerase chain reaction using camelid-specific primers. Amplified products were purified and ligated into phage display vectors to generate a nanobody library. The resulting library was transformed into competent bacterial cells and amplified to achieve sufficient diversity. Library quality was evaluated by estimating clone diversity and insert integrity.

5. Phage Display Selection and Enrichment

Phage display-based biopanning was conducted to enrich nanobodies with high affinity for glycoprotein B. Immobilized antigen was incubated with the phage library, followed by extensive washing to remove non-specific binders. Bound phages were eluted and amplified for subsequent rounds of selection. Typically, three to four rounds of biopanning were performed to achieve optimal enrichment.

6. Screening and Binding Analysis

Individual nanobody clones obtained after selection were screened using enzyme-linked immunosorbent assays to identify antigen-specific binders. Positive clones were subjected to further binding analysis using quantitative techniques to determine affinity and specificity. Binding kinetics were analyzed to distinguish high-affinity candidates suitable for functional evaluation.

7. Recombinant Expression and Purification

Selected nanobody genes were subcloned into expression vectors and transformed into suitable host systems. Recombinant expression was induced under optimized conditions to maximize yield and solubility. Nanobodies were purified using affinity chromatography, followed by polishing steps to remove contaminants and aggregates. Purity was assessed by electrophoretic and chromatographic analyses.

8. In Vitro Neutralization Assays

The antiviral activity of purified nanobodies was evaluated using cell-based assays. Susceptible cell lines were exposed to HSV in the presence or absence of nanobodies. Viral infection was quantified by measuring cytopathic effects, viral antigen expression, or reporter gene activity. Dose–response experiments were performed to determine neutralization potency.

9. Structural Analysis

Structural studies were conducted to elucidate the interaction between nanobodies and glycoprotein B. Advanced imaging techniques, such as cryo-electron tomography or cryo-electron microscopy, were employed to visualize nanobody-bound viral particles or protein complexes. Structural data were analyzed to identify binding sites and conformational changes associated with neutralization.

RESULTS

1. Antigen Quality and Immunogenicity

Recombinant herpes simplex virus glycoprotein B ectodomain was successfully expressed and purified to high homogeneity. Analytical characterization confirmed the expected molecular weight and structural integrity of the protein. Immunoassay-based evaluation demonstrated strong antigenicity, validating its suitability for camelid

immunization. Serum samples collected from immunized alpacas exhibited progressively increasing antigen-specific antibody titers, indicating the successful induction of a robust immune response.

2. Construction and Diversity of Nanobody Library

Isolation of peripheral blood mononuclear cells followed by RNA extraction and cDNA synthesis yielded high-quality genetic material suitable for library construction. Polymerase chain reaction amplification selectively enriched VHH-encoding gene fragments, producing products of the expected size. Cloning into phage display vectors resulted in a highly diverse nanobody library, with estimated complexity in the range of millions of independent clones. Random sequencing of selected clones confirmed correct insertion and substantial sequence variability.

3. Enrichment of gB-Specific Nanobodies

Phage display-based biopanning led to progressive enrichment of glycoprotein B-specific nanobodies across successive selection rounds. Enzyme-linked immunosorbent assays revealed a marked increase in antigen-binding phages after each round of selection, indicating effective removal of non-specific binders and retention of high-affinity candidates. The final enriched pool exhibited strong and specific binding to gB with minimal background reactivity.

4. Identification and Binding Characterization of Lead Nanobodies

Individual nanobody clones isolated from the enriched library were screened for binding activity. A subset of clones demonstrated high-affinity binding to glycoprotein B, as evidenced by strong immunoassay signals and favorable binding kinetics. Quantitative binding analyses revealed nanomolar to sub-nanomolar affinity for selected nanobodies, highlighting their potential as effective antiviral agents.

5. Recombinant Expression and Purification Efficiency

Lead nanobody candidates were successfully expressed in recombinant host systems, yielding soluble protein with high purity. Affinity chromatography followed by size-exclusion chromatography resulted in homogenous nanobody preparations. Electrophoretic analysis confirmed the expected molecular size of approximately 15 kDa, with minimal aggregation or degradation observed.

6. In Vitro Neutralization of HSV Infection

Functional assays demonstrated that selected nanobodies effectively inhibited HSV infection in susceptible cell lines. Nanobody-treated samples showed a significant reduction in viral entry and cytopathic effects compared to untreated controls. Dose–response analysis revealed a concentration-dependent neutralization profile, with certain nanobodies achieving near-complete inhibition of infection at low micromolar to nanomolar concentrations.

7. Structural Insights into Nanobody–gB Interaction

Structural studies provided direct visualization of nanobody binding to glycoprotein B on the viral surface. Imaging analyses revealed that nanobody binding altered the spatial organization and conformational dynamics of gB, stabilizing it in a non-fusogenic state. These observations supported the proposed mechanism of neutralization through inhibition of membrane fusion.

8. Stability and Biophysical Properties

Purified nanobodies exhibited high thermal and chemical stability, retaining binding activity across a broad range of temperatures and pH conditions. Proteolytic resistance assays further demonstrated the robustness of the nanobody structure. These favorable biophysical properties underscore the suitability of nanobodies for therapeutic development.

CONCLUSION

Herpes simplex virus infections continue to represent a persistent clinical challenge due to their high prevalence, lifelong latency, and limited treatment options. Conventional antiviral therapies, while effective in reducing viral replication and disease severity, fail to prevent viral entry or eliminate latent infection and are increasingly compromised by the emergence of drug-resistant strains. These limitations highlight the urgent need for innovative therapeutic strategies that target early and essential stages of the viral life cycle.

This review consolidates current knowledge on the development and application of alpaca-derived nanobodies targeting herpes simplex virus glycoprotein B, emphasizing their mechanistic, structural, and translational significance. Glycoprotein B plays a central and indispensable role in HSV membrane fusion and host cell entry,

making it an ideal target for antiviral intervention. Nanobodies directed against gB demonstrate high affinity, specificity, and potent neutralizing activity by stabilizing the protein in a non-fusogenic conformation, thereby preventing viral entry at the earliest stage of infection.

The production pipeline for nanobodies-ranging from camelid immunization and VHH library construction to phage display selection and recombinant expression—offers a robust and scalable platform for generating antiviral biologics. The favorable biophysical properties of nanobodies, including exceptional stability, solubility, and ease of engineering, further enhance their therapeutic appeal. Structural insights obtained through advanced imaging techniques provide strong mechanistic validation for nanobody-mediated neutralization and support rational optimization strategies.

Collectively, the findings discussed in this review underscore the potential of nanobody-based therapeutics as a new class of antiviral agents capable of overcoming the shortcomings of existing treatments. With continued advances in protein engineering, delivery technologies, and clinical evaluation, gB-targeting nanobodies hold promise for prophylactic and therapeutic applications against HSV infections. Future research efforts should focus on in vivo validation, formulation development, and clinical translation to fully realize the impact of nanobodies in antiviral therapy.

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