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CRISPR-Cas13 for HIV-1 RNA Targeting: Advances, Challenges, and NGS-Driven Insights

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Abstract

Background: The main obstacle to eliminating HIV-1 from the body exists because HIV-1 latent reservoirs continue to survive despite antiretroviral treatments which successfully manage HIV-1 infection. The DNA-editing method CRISPR-Cas9 faces hazards of unintended DNA changes but scientists developed CRISPR-Cas13 as a synthetic RNA-targeting nuclease that destroys viral RNA without affecting host DNA. The review presents recent scientific developments in Cas13-based HIV-1 control while showing how directing treatment at viral RNA transcripts produces better treatment results than direct treatment of proviral DNA. We study how Cas13 orthologs including Cas13a, Cas13b, Cas13d, and the compact Cas13X operate at the molecular level while we analyze their RNase activities which create potential safety risks for medical treatment. The existence of several major obstacles still needs resolution because the system experiences problems with delivering treatments to tissue reservoirs and the virus develops escape mutations and the system deals with unintentionally destroying RNA and the body responds to Cas proteins which come from bacteria. Next-generation sequencing has become an essential method which researchers use to determine target effectiveness while they track how mutations develop and study the impacts of various RNA sequences on gene expression. We proposed that the implementation of NGS-enabled personalized Cas13 treatment, which uses patient proviral sequencing to create crRNA designs and monitor post-treatment results, provides a practical solution to achieve successful clinical implementation.

Introduction

HIV-1 infection has become a chronic condition which can be treated because combination antiretroviral therapy transformed it from a fatal progressive disease. Patients who maintain treatment at undetectable viral levels will see their immune systems protected from infections while they stop disease spread through horizontal transmission. The ART treatment method does not result in complete disease elimination [1,2]. Most people will experience viral rebound within three weeks after they stop their treatment because their body retains CD4+ T cells which carry dormant HIV-1 provirus integrated into their genetic material [3].

The latent reservoir reaches its complete establishment within two days following the first infection while maintaining its stability for an extended period. The studies which assessed intact proviral DNA levels in patients who had ART treatment for multiple decades found that their decay rate extended over many years instead of the typical months thus showing that ART would need more than seventy years of ongoing treatment to fully eliminate the virus [4,5]. The reservoir exists as the primary location in resting memory CD4+ T cells which also contains other cellular compartments including myeloid cells and tissue-resident macrophages and cells that exist in anatomical sanctuaries which include the gut-associated lymphoid tissue and central nervous system. The extensive

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genetic variation found in HIV-1 quasispecies creates challenges for eradication because all effective cure methods need to overcome this genetically diverse group of target organisms [6,7].

Scientists have tested different methods to remove latent reservoirs but all methods encountered specific restrictions. The "shock and kill" method uses latency-reversing agents to restart viral transcription which makes infected cells vulnerable to both immune system destruction and virus-induced cellular damage [8]. Researchers have studied all available latency-reversing agents through clinical trials but results showed no agents could effectively decrease reservoir size because the agents could not fully reactivate all cells while immune effectors failed to control specific reservoir cells that naturally resist destruction [9].

The method called "block and lock" works to create permanent silencing of proviral sequences which stops reactivation while keeping infected cells intact. The system looks perfect in theory because it needs permanent upkeep to maintain its operation while failing to remove the hidden virus reserve [10]. The CCR5Δ32 homozygous donor allogeneic hematopoietic stem cell transplant has successfully cured two patients the "Berlin patient" and the "London patient" by replacing their HIV-1 vulnerable immune systems with cells that resist the virus. The procedure has high death risk and requires concurrent hematologic cancer treatment for its safe application in all patients [11,12].

We have studied CRISPR-Cas9 as a DNA editing tool to investigate its potential for directly interrupting integrated proviral DNA. The proof-of-concept studies show that Cas9 can remove or disable provirus yet researchers still worry about unintended genetic changes and double-strand breaks at repetitive HIV-1 sequences which result in chromosomal rearrangements and the permanent effects of DNA editing. Cas9 does not have the capability to remove any existing viral RNA or viral transcripts that come from intact provirus before the editing process begins [13].

Theoretical benefits of these methods arise from their RNA-targeting mechanism which allows for temporary genetic changes and enables fast destruction of both genomic and spliced viral RNA while using latency reversal methods to reach newly made RNA from reactivated provirus [14]. CRISPR-Cas13 has become the best RNA-targeting platform because it allows users to program its functions while maintaining exactness and delivering strong RNase activity [15].

Molecular Mechanisms of CRISPR-Cas13

- **Discovery and Classification of Cas13 Orthologs**

The CRISPR-Cas13 system belongs to Class 2, Type VI of the CRISPR effector family because it uses a single-protein structure to target RNA molecules. Cas13 identifies and destroys single-stranded RNA through its two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains, which operate differently from Cas9 and Cas12 that cut DNA [16]. Scientists discovered the Cas13 family through their analysis of microbial metagenomic databases which they compiled through computational mining. Cas13a (initially designated C2c2) was discovered in 2015, representing the first characterized RNA-guided RNA-targeting CRISPR system. Researchers discovered Cas13b (Type VI-B) in 2017 and Cas13d in 2018, which contains 930 amino acids that enable its delivery through adeno-associated virus vectors [17]. The Cas13X system represents the smallest known RNA editing tool because it contains only 775 amino acids, whereas its high-fidelity variants (hfCas13X) have reduced off-target activity yet preserve their ability to target specific locations [18].

Every ortholog displays specific characteristics which are important for drug development. Cas13a needs a protospacer flanking site (PFS) to prefer A or U bases which should be next to the target area while the other two types of Cas13b and Cas13d demonstrate more flexible PFS needs. The research community employs Cas13d as its primary ortholog for mammalian studies because the protein's compact structure and high functionality and low detrimental effects allow easy use in research on mammalian cells [19].

- **Mechanism of RNA Recognition and Cleavage**

The Cas13 nuclease is guided to its RNA target by a CRISPR RNA (crRNA) molecule of approximately 20-30 nucleotides in length. The Cas13 protein develops from its pre-crRNA through its own RNase mechanisms while Cas9 requires its development through a separate trans-activating crRNA (tracrRNA) process. The HEPN-2 domain processes pre-crRNA into mature crRNA which remains attached to the Cas13 protein [20]. Target recognition occurs through Watson-Crick base pairing between the crRNA spacer sequence and complementary sequences in the target RNA. Binding of the Cas13 protein causes a structural change that brings the two HEPN domains closer together, which activates their catalytic sites [21]. The activated HEPN domains cut the target RNA at sites that are far from where crRNA binding occurs, leading to complete RNA degradation. The HEPN domains become active through their activation process, which initiates the breakdown of nearby single-stranded RNAs this unique feature distinguishes Cas13 from other CRISPR systems and enables its use in diagnostics and treatments [22].

- **Collateral Activity: Mechanism and Consequences**

The collateral RNase activity of Cas13 the indiscriminate degradation of non-target RNAs following target engagement functions as a double-edged sword. Diagnostic tests use collateral activity because it improves nucleic acid detection through signal amplification which occurs when reporter molecules get cleaved [23]. The use of collateral activity in therapeutic applications creates major safety problems. The activated state of Cas13 leads to the degradation of cellular mRNAs and non-coding RNAs which results in cell damage and the activation of innate immune systems that recognize RNA fragments. The different orthologs show varying degrees of collateral activity in mammalian systems which results in

Cas13a showing maximum activity while Cas13d shows least and Cas13b showing intermediate activity [19,24]. The presence of low-level collateral activity during chronic expression has the potential to cause cellular dysfunction. Scientists use three strategies to reduce collateral effects which involve developing high-fidelity variants, creating crRNA designs that prevent off-target activation, and implementing transient delivery systems that restrict Cas13 exposure time [19,25,26].

❖ Distinguishing Cas13 from RNAi and Antisense Technologies

The CRISPR-Cas13 system expands the available RNA-targeting techniques which already include RNA interference (RNAi) and antisense oligonucleotides (ASOs). The different features of each platform determine which one will work best for HIV-1 research purposes [27,28]. RNAi uses small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) to activate the RISC complex which then destroys matching RNA molecules. RNAi can achieve effective results but it leads to RISC component saturation problems while producing unwanted effects because of its seed-region binding ability and it faces competition from natural microRNAs. ASOs function as synthetic oligonucleotides which attach to specific RNA targets and bring RNase H to destroy the RNA [29,30]. The treatment provides precise targeting abilities through its chemical modifications which improve stability but patients must receive treatment at high doses with multiple sessions [31].

The Cas13 system provides multiple beneficial features to users. The system can be quickly modified to detect new viral strains because its guide RNA can be changed without needing protein engineering work. The system enables easier delivery operations because its single-effector design requires less effort than multi-component systems [32]. The target recognition mechanism shows better accuracy because it has fewer seed-region off-target effects than RNAi technology. Cas13 provides two delivery options through mRNA and ribonucleoprotein formats which enable temporary use while decreasing the chance of causing lasting damage. The bacterial origin of Cas13 proteins creates immune response problems which synthetic ASOs and natural RNAi triggers do not have [27].

Preclinical Advances in Cas13-Mediated HIV-1 Suppression

❖ In Vitro Proof-of-Concept Studies

The initial assessment of Cas13 effectiveness against HIV-1 was performed using immortalized cell lines that included HEK293T and Jurkat T cells. The transfection of Cas13a or Cas13d with crRNAs that target conserved regions of the HIV-1 genome resulted in major decreases of viral RNA and p24 antigen and reverse transcriptase activity [33]. The most effective target sites include the 5' untranslated region, the gag-pol overlap, and the tat/rev regulatory region—sequences that are relatively conserved across HIV-1 subtypes and essential for viral replication. Researchers have found that using multiple guide strategies outperforms single guide methods for preventing viral escape [34]. The expression of three to four crRNAs that target different regions showed a decrease in viral RNA of up to 95% and stopped the emergence of resistant variants during extended culturing. The time-course experiments demonstrated that Cas13-mediated degradation happens instantly because RNA levels decrease significantly during the first 6-12 hours after induction which differs from the slow RNAi and latency reversal methods [35].

❖ Ex Vivo Studies in Primary Human Cells

Scientists have successfully developed multiple delivery systems which enable them to convert primary human CD4+ T cells into HIV-1 target cells. The team achieved successful CRISPR editing through electroporation which delivered Cas13 mRNA together with synthetic crRNAs while maintaining low toxicity levels [33,36]. The researchers observed significant decreases in both viral RNA and p24 protein production after Cas13 began expressing in cells which had undergone latency reversal from J-Lat and ACH-2 latently infected cell lines. The ex vivo research conducted with primary CD4+ T cells which medical professionals had obtained from ART-suppressed patients demonstrated that Cas13 could decrease the number of cells which developed the capacity to produce viruses after they received stimulation [37]. The research team treated cells with Cas13 ribonucleoprotein complexes which targeted specific HIV-1 sequences before exposing them to conditions which would activate the virus. The treated group of wells showed a 50-80% reduction in detectable viral outgrowth compared to the untreated control group which indicates that Cas13 effectively removes or disables most replication-competent reservoir viruses [38].

Myeloid reservoirs, including monocyte-derived macrophages and microglia, have also been investigated. While these cells support lower levels of HIV-1 replication than CD4+ T cells, they represent important sanctuary sites that may contribute to rebound. Cas13 delivered via lentiviral vectors achieved efficient transduction of macrophages and reduced viral production from infected cells, supporting the feasibility of targeting these reservoirs [39].

❖ In Vivo Studies in Animal Models

The study conducted preclinical testing of Cas13 against HIV-1 by using humanized mouse models which involved reconstituting NSG or NOG mice with human CD34+ hematopoietic stem cells [40]. The researchers employed mice which had received human immune system transplants to study their response to HIV-1 infection. The team used AAV vectors and lipid nanoparticles and lentiviral vectors to deliver Cas13 after the establishment of infection [41]. The published studies show that AAV-delivered Cas13d reduces plasma viremia by 1-2 logs while decreasing proviral DNA levels in

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spleen bone marrow and gut-associated lymphoid tissue through the use of CD4+ T cell-targeting AAV9 and AAV-DJ serotypes. Multi-guide Cas13 constructions demonstrate better effectiveness than single guide designs [42]. The control group experienced viral rebound after Cas13 treatment but this process took longer than it did in the treated group which shows that some reservoir cells remained alive after treatment [43].

The EcoHIV model a chimeric HIV-1 capable of infecting mice without humanization—has also been employed, offering advantages of immunocompetent hosts. The use of lipid nanoparticles to deliver Cas13 achieved outstanding brain viral RNA reduction results which indicate its potential function in treating central nervous system viral reservoirs [44]. The ongoing non-human primate studies which have not yet been published will provide critical delivery efficiency and immunogenicity and efficacy data for testing in an outbred immunocompetent model before human trials begin [45].

Delivery Strategies for Cas13 Therapeutics

- **Viral Vector Delivery Systems**

The most developed method for delivering genes inside living organisms uses adeno-associated virus (AAV) vectors as their most advanced delivery system. More than 12 AAV-based gene therapies have received approval from regulatory authorities, while clinical trials have confirmed their safety through hundreds of studies [46]. The AAV system delivers Cas13 through its packaging capacity, which limits the selection of orthologs to genes under 4.7 kilobases in size. The Cas13d protein, which has 930 amino acids and a 2.8 kb coding sequence, meets the capacity limit when combined with a pol III or pol II promoter and a crRNA expression cassette. Both Cas13a (3.2 kb) and Cas13b (2.9 kb) require the implementation of dual-AAV delivery methods that use split-intein or overlapping vector designs, which lead to lower delivery efficiency [47].

The process of selecting serotypes establishes which cells a virus can infect and which body tissues it will spread throughout. AAV9 and AAV-DJ have demonstrated efficient transduction of human CD4+ T cells in humanized mouse models [48]. The AAV-BBB system which includes brain-broad barrier variants enables payload delivery through the blood-brain barrier to reach microglial reservoir sites. Resting CD4+ T cells which serve as the main viral reservoir show poor AAV transduction because they do not possess the essential co-receptors and intracellular components needed for effective AAV entry and second-strand synthesis [48].

Lentiviral vectors enable sustained Cas13 expression through their ability to package larger genetic material and permanently integrate into host DNA. The technology requires assessment because its biosafety profile includes two main hazards: insertional mutagenesis and possible germline transmission [49]. The use of lentiviral vectors becomes acceptable for ex vivo applications which involve harvesting patient cells and performing genetic editing before reinfusion. The safety risks associated with integrating vectors become more severe when they are used for in vivo applications [50].

- **Non-Viral Delivery Platforms**

Lipid nanoparticles (LNPs) have become the top non-viral delivery system because mRNA vaccines for SARS-CoV-2 have proven their effectiveness in clinical trials. The use of LNP-formulated Cas13 mRNA together with synthetic crRNAs enables temporary Cas13 expression, which decreases the chances of enduring unintentional effects and immune system reactions [51]. The four-component LNP formulation (ionizable lipid, cholesterol, PEG-lipid, and helper lipid) can be optimized for CD4+ T cell targeting through incorporation of antibodies or ligands recognizing surface markers such as CD4 or CD3[52].

The use of LNPs provides multiple benefits for research into curing HIV-1. Transient expression accomplishes its purpose by destroying all viral RNA and reactivated transcripts which need to be eliminated without the need for continuous enzyme activity [37]. The company uses a manufacturing process which creates products through cell-free operations that eliminate the need for expensive and complicated viral vector production operations. The treatment plan includes repeat dosing to counteract the possibility that initial therapy will not reach all hidden infection sites [53]. The current LNP formulations show a tendency to accumulate in the liver after intravenous administration, while researchers still face difficulties in delivering LNPs to lymphoid tissues and the central nervous system [54].

The Ribonucleoprotein RNP complexes which consist of pre-assembled Cas13 protein together with crRNA represent the delivery method that produces the least amount of time which can operate between hours and days. RNPs can be delivered through electroporation for ex vivo applications and through nanoparticle formulations for in vivo applications [55]. The system achieves maximum safety through its design which prevents Cas13 nucleic acid encoding from entering the organism since this mechanism eliminates integration and prolonged expression time [49].

- **Tissue-Specific Targeting Challenges**

The anatomical distribution of the HIV-1 reservoir exists across multiple body compartments which include peripheral blood, lymph nodes, spleen, gut-associated lymphoid tissue, bone marrow, and central nervous system. The delivery process encounters specific challenges because each compartment has its own set of barriers which must be overcome [56]. Systemic vector administration fails to reach lymph nodes and gut-associated lymphoid tissue because their tight endothelial barriers block high endothelial venules. Scientists improve lymphatic targeting through three methods which include creating ligands that bind to lymphatic endothelial receptors and using lipids which enable chylomicron binding and performing direct intranodal injections during research studies [57].

The blood-brain barrier safeguards the central nervous system reservoir which exists in perivascular macrophages and microglia. AAV9 and AAV-BBB variants show some ability to cross this barrier, but efficiency remains low. Intrathecal administration which involves injecting medication into the cerebrospinal fluid allows doctors to deliver CNS-targeted therapy but this invasive method presents safety risks [58].

❖ Immunogenicity of Cas13 Proteins

The immunogenicity of bacterial-derived Cas proteins presents a major challenge which remains largely unrecognized by most people. Human populations have documented pre-existing immunity to Cas9 with 30-60% of individuals showing antibody and T cell responses which scientists believe resulted from previous encounters with Staphylococcus aureus and Streptococcus pyogenes [59]. Scientists have not yet conducted a complete assessment of pre-existing immunity to Cas13 orthologs even though it seems likely because people constantly encounter environmental bacteria that possess Type VI CRISPR systems [60].

The immune system can develop new responses to Cas13 which doctors deliver through three methods of protein administration and mRNA treatment and viral vector delivery for patients who do not have pre-existing immunity. The effectiveness of treatment decreases after multiple doses because neutralizing antibodies prevent its action while cytotoxic T cells destroy Cas13-expressing cells together with off-target edited cells which should have been eliminated [61]. Scientists use less immunogenic orthologs together with transient delivery systems which prevent antigen presentation and immunosuppressive preconditioning techniques that resemble AAV gene therapy trial methods to develop their immunogenicity reduction techniques [62].

NGS-Driven Insights: Efficacy, Escape, and Off-Target Assessment

❖ The Essential Role of Next-Generation Sequencing

The standard techniques used to evaluate Cas13 performance which include quantitative PCR for viral RNA and p24 ELISA plus viral outgrowth assays generate average results from diverse cell groups and different viral quasispecies [63]. The techniques cannot identify uncommon escape mutants while they fail to describe clonal behavior and they do not provide complete evaluations of off-target impacts. The limitations of existing methods are resolved by next-generation sequencing which serves as an essential method to deliver single-nucleotide details and complete viral population analysis and transcriptome-wide evaluation of off-target effects [64]. NGS provides three methods for validating Cas13 which include measuring on-target efficacy through viral RNA depletion and detecting viral escape mutations and evaluating off-target RNA degradation across the transcriptome [19].

❖ Quantifying On-Target Efficacy with High Resolution

The RNA-seq analysis of Cas13-treated cells enables researchers to measure HIV-1 transcript levels which remain undetectable through targeted PCR methods. The researchers used viral read counts to determine how much each viral transcript class unspliced genomic RNA, singly spliced transcripts, and multiply spliced transcripts encoding tat and rev was reduced compared to host and spike-in controls [65]. Cas13 targeting of the 5' UTR results in equal degradation of all viral transcripts, while splice junction targeting leads to different effects on spliced isoforms. Single-cell RNA-seq (scRNA-seq) research shows that Cas13 activity differs greatly among infected cells. The majority of cells show >90% reduction in viral RNA, but some cells with lower Cas13 expression or higher initial proviral loads achieve partial clearance [66]. The resistant cells will develop into the cells that initiate the upcoming viral resurgence. In order to develop strategies that successfully eradicate refractory cells researchers need to discover the transcriptional and surface markers which enable cells to resist Cas13 treatment [67].

Long-read sequencing using Oxford Nanopore or PacBio platforms enables full-length HIV-1 transcript isoform analysis. This is particularly important for assessing whether Cas13 induces aberrant splicing or generates truncated viral transcripts that might retain biological activity despite lacking target sites [68].

❖ Detecting and Characterizing Viral Escape Mutations

HIV-1 can develop resistance against selective pressure because its high mutation rate together with its extensive population size create multiple chances for the virus to escape detection. The deep sequencing of viral populations during Cas13 treatment demonstrates how escape mutations develop at specific targeted sites through longitudinal monitoring [66]. In vitro passaging experiments show that escape mutations develop 3-6 weeks after researchers select Cas13 treatment candidates. The crRNA seed region (positions 1-10 of the guide) contains single nucleotide mismatches which lead to most severe target recognition problems [67].

The escape mutational landscape shows different patterns of evolution which depend on the selected target areas. The TAR element and the gag-pol frameshift site function as highly conserved regions which provide essential support for viral replication [69]. The variable regions of the virus allow escape mutations to develop without causing significant fitness penalties. The data shows that simultaneous targeting of multiple conserved sites decreases the chances that organisms will develop escape mutations [70].

Bioinformatic pipelines for escape detection from NGS data must distinguish true escape mutations from pre-existing polymorphisms and sequencing errors. The CRISPResso2 tool

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together with custom workflows enables scientists to measure mismatch occurrences at the target site and determine whether treatment mutations developed or already existed in the original inoculum [71]. The presence of escape mutations in humanized mouse studies demonstrates why researchers need to use multiple guide systems while monitoring viral outbreaks throughout clinical testing [41].

❖ **Transcriptome-Wide Off-Target Assessment**

The RNase activity that Cas13 exhibits as collateral activity creates a risk that it will destroy all RNA molecules present in cells. The complete off-target evaluation needs RNA-seq analysis of the entire transcriptome which compares samples treated with Cas13 to their respective control groups [19]. Researchers need to use statistical methods to identify differentially expressed genes while they evaluate total RNA integrity in their study of RNA-seq data from Cas13-treated samples. Off-target activity occurs when multiple transcripts experience substantial decreases in RNA levels while sequencing shows a 3' coverage bias which suggests that RNA molecules have been broken into smaller pieces and there is an accumulation of reads that connect to repetitive elements which may serve as unintentional targets [72]. The Cas13 variants hfCas13X and other high-fidelity Cas13 variants show lower global transcriptome disruption when tested than their wild-type orthologs yet they still achieve their intended on-target effects [19].

Ribosome profiling (Ribo-seq) gives scientists extra information about how off-target RNA degradation affects translation results. Ribo-seq detects protein synthesis impairment from small mRNA abundance decreases because it shows these effects better than RNA-seq alone. The regulatory submission process necessitates a complete off-target testing package which includes RNA-seq and Ribo-seq and targeted tests of predicted off-target sites for clinical development [73].

❖ **NGS for Reservoir Characterization**

The NGS system, which assesses Cas13 activity, serves its primary function by identifying HIV-1 reservoirs in patients who have undergone treatment. The Intact Proviral DNA Assay (IPDA) uses double digital droplet PCR technology to measure intact and defective proviruses through targeted measurement of two genomic areas (gag and env/pol). The technology of IPDA enables users to identify all shown amplicon sequences except for the specific sequence variations existing within those amplicons [74]. Scientists obtain genetic details about the complete reservoir existence through full-length proviral sequencing technology which requires more labor but delivers complete sequence information about the entire reservoir and all existing mismatches to crRNA candidates [75].

The study used NGS-based methods (LEGO-PCR, integration site amplification) to examine integration sites which showed that Cas13 treatment changed the pattern of proviral integration sites in infected cells. The remaining viral reservoir will contain more proviral integrations in heterochromatic areas which scientists find challenging to activate when Cas13 treatment selectively targets cells with proviruses that exist in specific genomic regions like active transcription sites. Understanding these dynamics could inform the design of combination strategies [76].

Key Challenges and Limitations

❖ **The Specificity-Toxicity Trade-Off**

The main obstacle which scientists face in developing Cas13 therapies involves creating effective treatments which do not cause off-target effects or unwanted toxic reactions. High-activity Cas13 variants reach their maximum viral suppression potential yet they produce excessive cellular RNA destruction which scientists consider intolerable [19]. The low-activity variants provide better safety profiles yet they risk losing their power to eliminate the viral reservoir. The engineered variants which achieve high-fidelity performance deliver an intermediate result; however, their effectiveness needs to be confirmed through additional studies in actual living systems. The assessment of collateral activity becomes difficult because even small amounts of RNA degradation that occur during ongoing Cas13 expression result in cumulative effects [27]. The two delivery methods (mRNA-LNP, RNP) establish transient exposure limits which decrease risk through their time-restricted emissions. The reservoir can only be removed through continuous activities which need to address viral RNA that emerges from reactivation events which happen throughout time [77].

❖ **Viral Escape and the Need for Multi-Guide Strategies**

The genetic diversity and adaptive capacity of HIV-1 guarantee that all single-target treatments will lead to the development of escape mutants. The use of three to four guide strategies which focus on conserved regions reduces the risk but does not completely eliminate it. The ideal guide set would target essential regions which possess high fitness costs for escape mutations and which remain constant across all circulating subtypes [34].

The alternative method involves targeting host factors which cells need to replicate viruses instead of attacking the viral genome. The use of Cas13 to target CD_4 and CCR_5 mRNA has been proposed as a method to protect cells from infection while avoiding the development of viral escape mechanisms. The method would fail to remove existing infected cells from the body and it would lead to immunosuppression problems [78].

❖ **Immunogenicity and Pre-Existing Immunity**

As noted above, pre-existing immunity to Cas13 orthologs has not been systematically characterized but may limit therapeutic application in a substantial fraction of the population. Even in seronegative individuals, administration of Cas13 may induce immune responses that preclude repeat dosing. Strategies to address immunogenicity include using humanized or de-immunized Cas13 variants, transient delivery

formats that minimize antigen presentation, and immunosuppressive preconditioning regimens [42].

❖ **Regulatory and Manufacturing Hurdles**

The regulatory pathway for Cas13-based HIV-1 therapy is uncertain. If delivered as a gene therapy (e.g., via AAV or lentiviral vector), the product would be regulated by the Office of Tissues and Advanced Therapies (OTAT) at the FDA. If delivered as an mRNA-LNP formulation, regulation would fall under the Center for Biologics Evaluation and Research (CBER) with potential involvement of the Office of Vaccines. The absence of approved Cas13 therapeutics creates uncertainty regarding the required non-clinical studies, potency assays, and clinical endpoints [49].

Manufacturing at scale presents additional challenges. GMP production of Cas13 mRNA requires rigorous purification to remove double-stranded RNA contaminants that trigger innate immunity. Lipid nanoparticle formulation must achieve consistent size, encapsulation efficiency, and sterility. For viral vector approaches, establishing stable producer cell lines and achieving high-titer, high-purity vector preparations is resource-intensive [79].

Future Directions and Clinical Roadmap

❖ **Engineering Next-Generation Cas13 Variants**

Ongoing protein engineering work will create Cas13 variants which have enhanced therapeutic effects. Presently available high-fidelity variants (hfCas13X) demonstrate on-target performance while reducing off-target results yet they need additional testing through in vivo studies [19,26,80]. Chemical or light-mediated dimerization-based split-Cas13 systems enable scientists to achieve both spatial and temporal control over their functions. The fusion of dCas13 which lacks catalytic activity with RNA-modifying enzymes (ADAR for base editing and methyltransferases for epitranscriptomic control) enables researchers to therapeutically modify viral RNA without causing harmful effects through RNA degradation [27].

❖ **NGS-Guided Personalized Cas13 Therapy**

The highly diverse genetic makeup of HIV-1 which exists between different people and changes throughout the life of an infected person requires custom crRNA design methods [81]. The complete sequencing of the patient's proviral reservoir is performed through long-read techniques to locate regions that maintain their structure across different sequences and can be used as treatment targets [82]. The design process creates a set of three to five crRNAs which match the patient's viral sequences while excluding targets that already have mismatches which would lead to resistance. The NGS monitoring system tracks treatment progress through time to identify new escape mutations which prompt the need for crRNA redesign [34].

The current diagnostic system already supports drug resistance testing while this method provides personalized treatment for HIV-1 patients. The path for regulatory approval of guide RNAs which are customized for individual patients remains untested while manufacturing times need to be synchronized with the speed of viral genetic change [83].

❖ **Combinatorial Approaches for Reservoir Elimination**

- Cas13 is unlikely to cure HIV-1 as a monotherapy. Rational combination strategies include:
- Cas13 + latency reversal: Reactivate latent provirus with LRAs, then degrade nascent viral RNA with Cas13 before virions assemble and spread. This "shock and clear" approach avoids the immune toxicity concerns of "shock and kill [84]."
- Cas13 + broadly neutralizing antibodies (bNAbs): bNAbs can clear Cas13-exposed infected cells through antibody-dependent cellular cytotoxicity and may also neutralize residual virions [85,86].
- Cas13 + immune checkpoint inhibitors: Blockade of PD-1 or other exhaustion markers may enhance endogenous T cell responses against infected cells [42].

Sequential Cas13 + Cas9: Cas13 degrades viral RNA and reduces viral protein production, followed by Cas9-mediated proviral disruption in the remaining infected cells [43].

❖ **Path to Clinical Trials**

A clinical development pathway for Cas13-based HIV-1 therapy might proceed as follows:

Phase 0/I: Ex vivo editing of autologous CD4+ T cells from ART-suppressed individuals. Cells are harvested, edited with Cas13 RNP or lentiviral vectors, and reinfused. Safety endpoints include infusion reactions, off-target editing, and emergence of escape mutants [87].

Phase I/II: In vivo delivery of Cas13 mRNA-LNP or AAV vectors to ART-suppressed individuals with measurable reservoir. Key endpoints include reduction in intact proviral copies by IPDA, safety (adverse events, immunogenicity), and time to viral rebound upon analytical treatment interruption (ATI) [88].

Phase II/III: Randomized controlled trials comparing Cas13 plus ART to ART alone, with ATI as the primary endpoint. Long-term follow-up for delayed adverse events including autoimmunity and malignancy [89].

The first-in-human trials will require careful patient selection (suppressed on ART for >2 years, CD4 count >500, no evidence of pre-existing immunity to Cas13), extensive informed consent regarding risks of ATI, and long-term monitoring protocols [90].

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Conclusion

CRISPR-Cas13 has emerged as a programmable RNA-targeting platform with distinct advantages for HIV-1 therapy: it degrades viral RNA without altering host DNA, can be delivered transiently to limit off-target risks, and is readily reprogrammable to address viral escape. Preclinical studies demonstrate potent suppression of HIV-1 replication in vitro, ex vivo, and in humanized mouse models, with multi-guide strategies reducing viral outgrowth from latently infected cells.

However, substantial challenges remain before clinical translation. Delivery to all reservoir compartments, particularly lymph nodes and the central nervous system, is inefficient with current vectors. Viral escape mutations inevitably emerge under selective pressure, necessitating multi-guide approaches and ongoing monitoring. The collateral RNase activity of Cas13 raises off-target concerns that require comprehensive NGS-based assessment. Immunogenicity of bacterial-derived Cas proteins may limit efficacy and preclude repeat dosing.

Next-generation sequencing has proven indispensable for addressing these challenges. RNA-seq quantifies on-target efficacy at single-base resolution. Deep sequencing of viral populations reveals escape mutation landscapes and informs guide design. Transcriptome-wide off-target assessment provides safety data essential for regulatory approval. As high-fidelity Cas13 variants and improved delivery platforms reach maturity, NGS-guided personalized Cas13 therapy may enter clinical testing as a component of combination HIV-1 cure strategies.

The path forward requires continued collaboration among virologists, gene therapists, bioinformaticians, and regulatory scientists. The tools are advancing rapidly. The need for an HIV-1 cure remains urgent. CRISPR-Cas13, while not yet ready for the clinic, represents a promising addition to the armamentarium of approaches targeting the persistent reservoir.

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Declarations:

Authors' Contribution:

- All Authors Conceptualization, data collection, interpretation, drafting of the manuscript and intellectual revisions
- The authors agree to take responsibility for every facet of the work, making sure that any concerns about its integrity or veracity are thoroughly examined and addressed

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