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Methodology of "Shock and Kill" Therapy Approach for HIV-1 Treatment Using a Novel Zinc Finger Protein

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Abstract

Human immunodeficiency virus(HIV) causes Acquired Immune Deficiency Syndrome(AIDS) in human bodies. As this disease annually causes 690,000 death, how to treat and cure AIDS has been an important topic for the modern society. Recent researches of HIV-1 treatments have mentioned the "Shock and Kill" approach to eliminate the viral reservoir. One of the researches indicates a special C2H2-Zinc finger motif could be used to apply this approach. In this work, a C2H2-Zinc finger motif is modeled to target the NF-κB doublet sequence which has been proved able to highly regulate the viral transcription. By calculating with softwares, the affinity of this Zinc Finger motif is qualified theoretically. Also, several suggested experiments are raised to determine the possibility of clinical application. The similar ZF motifs could potentially be applied in "Shock and Kill" approach to substitute the traditional antiretroviral therapies, achieving great breakthrough in HIV-1 therapy.

Keywords

HIV, Zinc Finger Protein, Shock and Kill Approach.

1. Introduction

Treating the HIV infection has been known as a challenging problem. Over last few decades, AIDS is incurable due to its special infection mechanism.

Different from other viruses, HIV-1 integrates its gene into cells via mechanism of reverse transcription. Once attacking CD4+ T cells the reverse transcriptase uses viral RNA template to form a viral DNA, rather than directly translate into viral proteins. The viral DNA integrates into cell genome, forming lentivirus. Meanwhile, make CD4+ T cells unable to induce humoral immunity and cell-mediated immunity. As a lentivirus, it can successfully escaped from the recognition of immune system. Furthermore, this mechanism enables HIV to transcribe and translate more HIV particles without causing cell death.

Currently, the HIV therapies focus on attacking infected cells through ART[1] using several drugs. Drugs in ART specifically inhibits the reverse transcriptase, which blocks the HIV-1 replication and prevents AIDS attack. However, the viral DNA cannot be eradicated in the body and the interruption of ART leads to the continued replication of HIV-1. In order to eliminate the lentivirus in the cells, the infected cells need to be induced to death to destruct the viral genome so that more virus can no longer replicate.

Recently, a conceptual strategy known as "shock and kill" approach[2] has been established, which proposes to target and destructing the dormant infected cells. By signal intervention or regulator

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import, HIV-1 expression frequency could be altered. This alteration triggers a fiercer replication of HIV-1, which exceeds the infected cell tolerance, leading to passive programmed death.

The expression alteration process requires latency reversing agents (LRAs)[2]. LRAs can either interfere the histone or act on viral DNA sequences to increase the level of viral gene expression. In the majority of projects about LRAs proposed to act as histone deacetylase inhibitors (HDACis) in tumor treatment but also show LRA properties such as vorinostat. The lagger includes dCas9-VPR which directly associates to the DNA which is mainly discussed here.

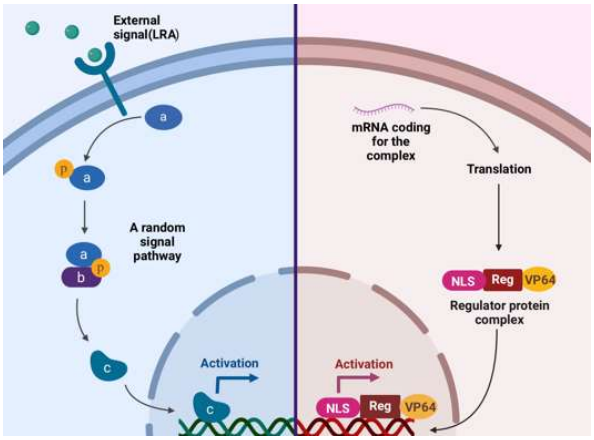

Figure 1. The mechanism of LRAs

Figure 1: The mechanism of LRAs. On the left side(blue), LRA more frequently triggers a signal pathway than the normal time. In the pathway, the final messenger, protein C, activates the transcription of viral gene. On the right side(pink), an mRNA which translates to regulatory protein is induced into the infected cell. The complex including Nuclear Localization Signal and VP64[5] enters the nucleus, associating with viral gene and start transcription. This complex is discussed in the subsequent context.

Some LRAs are able to directly or indirectly intervene the Long terminal repeating sequences (LTRs) [3] in the viral DNA. This region is located at both ends of HIV-1 viral genes and lacks of the viral gene but multiple enhancers. The activation of this sequence could potentially increase the overall rate of transcription leading to cell death and so on diminish the scale of lentiviruses. Because different sites have different efficiency of transcription activity. Therefore, choice of LRAs for association needs to be significantly concerned.

On the LTR, there is a unique Nuclear Factor-κB (NF-κB) doublet [4] which is only found in HIV-1 but nowhere on other human genomes. The NF-κB doublet has the following sequence:

5'—GGGACTTTCCGCTGGGGACTTTCC—3'

Each NF-κB section has identical conserved sequence:

5'—GGGACTTTCC—3'

As this sequence is unique to HIV-1, it can be a transparent target to the transcription regulatory proteins. It specifically recognizes and associates to the sequence rather than influences other uninfected cells. Meanwhile, the contiguous activation of NF-κB[6] doublet could give rise to a higher transcription level than other "shock and kill" [2]strategy using different sites.

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The Zinc Finger Protein (ZFP) [7] is a class of regulatory proteins which have been implicated in multiple cellular processes. ZFPs have a common structure which contains a conserved motif including one or more zinc ions. The zinc ions are normally coordinated by surrounding hydrophilic amino acids to stabilize the protein folding. One major type of zinc finger protein is Cys2-His2 (C2H2-ZFP)[8] type in which the zinc ion is coordinated by two cysteines and two histidines.

The specific amino acids in Zinc Finger Proteins could form non-covalent bonds with the base pairs in DNA or the carbon-phosphate backbone. Different combinations of amino acids enable Zinc Finger to target the specific base triplets on the DNA sequences. Normally, Zinc Finger Proteins have higher affinities to the triplet containing GNN[9] or ANN [10]sequences, followed by CNN sequences. However, CNN[11] can only occur one time and TNN[12] sequence is rarely associated to Zinc Finger proteins.

Although some sections of NF-κB doublet contains GNN and ANN sequences, the only problem is the part of the NF-κB doublet enriched by TNN sequence (TCC or TTC triplet occurs). So the upstream and downstream sequences which have less TNN triplets of NF-κB need to be considered parts of binding sites to allow four-finger ZFPs to associate with DNA. Also, our preliminary experiment is designed to examine whether the activation is enough to lead to the death of infected cells.

2. Result

2.1 Structure Analysis of Zfps

Following the concept in the "Shock and kill" approach, a novel Zinc Finger Proteins is designed to substitute the original transcription regulators in this work. Firstly, consider the upstream and downstream sequence of NF-κB doublet:

5'—CTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GAG —3'
NF-κB I NF-κB II

We examined this sequence on the Zinc Finger Tool website server[13] to find out the zinc finger binding region. Three target sequences have been identified as Zinc finger binding regions in the Zinc Finger Tool ver. 3.0.

Table 1. The alternative target sequences and their top scores

Position	Lengths(bp)	Number of Subsites	Top score	Target sequence
1	12	1	54.60	CTA CAA GGG ACT
15	12	1	54.97	CCG CTG GGG ACT
16	12	1	52.99	CGC TGG GGA CTT

Table 1: The alternative target sequences and their top scores. The subsites is the contiguous targets sites specifies the minimum target size of the target site to be found[13]. For instance, if the minimum target size required is 12 bp, and a target site found that is 16 bp, there will be two subsites of 12 bp (one from position 1-12 and another is from 4-16). The top score[13] is used to measure the quality of zinc finger on the website. In general, the higher top score indicates the better quality of zinc finger.

In order to bind both NF-κB region, two sequence needed to be selected out. The target for NF-κB II is unique, only sequence 1 could be selected out. For NF-κB I, we selected out the sequence 2 whose top score ranks the first. Then the tool is used to design the optimal Zinc Finger proteins. These two proteins do not have overlapped sequence and all of them are four-finger proteins. Each finger sequence composes six amino acids.

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Table 2. The selected target sequences and their amino acid sequences of fingers

Position	Top score	Target sequence	Amino acid sequence of Fingers
1	54.60	CTA CAA GGG ACT	THLDLIR RSDKLVR QSGNLTE QNSTLTE
15	54.97	CCG CTG GGG ACT	THLDLIR RSDKLVR RNDALTE RNDTLTE

Table 2: The selected target sequences and their amino acid sequences of fingers. Both Zinc finger proteins are four-finger type fitting with each target sequence. We omit other amino acid sequences like Cys2-His2 zinc holding sequences and the terminal sequences.

Then we examine these sequences on Swiss modelling website[14] to further analyse the quality of each zinc fingers, and to predict overall structure of each zinc fingers.

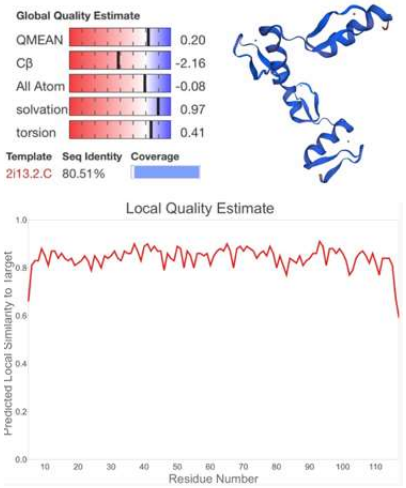

Figure 2. The estimate quality analysis for ZFP-NF I

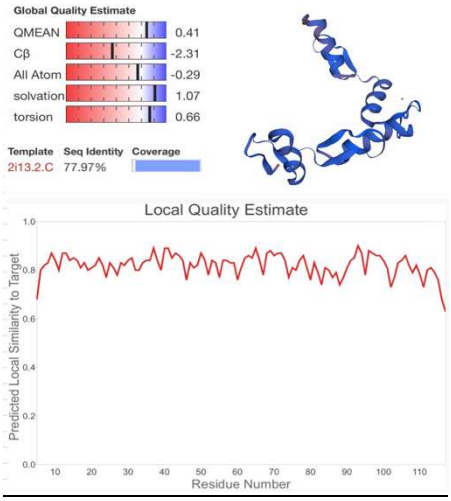

Figure 3. The quality estimate analysis of ZFP-NF II

Figure 2: The estimate quality analysis for ZFP-NF I; Figure 3: The quality estimate analysis of ZFP-NF II. For each figure: The overall quality estimation is measured by QMEAN score[15]. This term is a composite estimator based on different geometrical properties and provides both global (i.e. for the entire structure) and local (i.e. per residue) absolute quality estimates on the basis of one single model.

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QMEAN score measures the quality of zinc finger according to a model, higher score indicates the model has better better quality and higher confidence. Positive values indicate that the model scores higher than experimental structures on average. Negative numbers indicate that the model scores lower than experimental structures on average.

"Global Quality Estimate" measured by the term QMEAN Z-score[15] (The first row) estimates of the "degree of nativeness" of the structural features observed in the model on a global scale. Other terms, Cβ atoms, all atoms, the solvation potential and the torsion angle potential are the individual terms for QMEAN-Z. These terms use to give a total mark for QMEAN Z-score.

"Template" modifies the most similar linking pattern to the zinc finger proteins. The "Seq Identity" measures the similarities to this model template and this is shown geologically by "Coverage". The three-dimensional structure of zinc finger proteins are also shown.

The "Local Quality Estimate" measured by QMEAN local scores, or QMEANDisCo[16] which has higher accuracy. This measurement assesses the consistency of observed interatomic distances in the model with ensemble information extracted from experimentally determined protein structures that are homologues to the target sequence.

The core data is sorted in the chart:

Table 3. The core parameters of quality estimation

Zinc Finger Sequence	GMQE[14]	QMEAN Global	QMEAN Local	Sequence Identity (SMTL ID: 2i 13.2C)[17]
Standard	0.00-1.00	>-4.00	>6.00	100%
ZFP-NF I	0.81	0.20	Average 0.85	80.51%
ZFP-NF II	0.79	0.41	Average 0.87	77.97%

Table 3: The core parameters of quality estimation. The first row "standard" for GMQE is the range of GMQE. The higher value of GMQE, the reliability is higher. For QMEAN global and QMEAN Local, the standard shows the range of high quality zinc finger proteins.

All the parameters of the ZFP-NFs show the high quality of both zinc finger proteins. This implies that the feasibility of these zinc finger proteins is high.

Then we posted the two sequences into the Swiss Modelling website to predict the structure of protein-DNA complex according to the pattern SMTL ID: 2i 13.2C. The model is shown below:

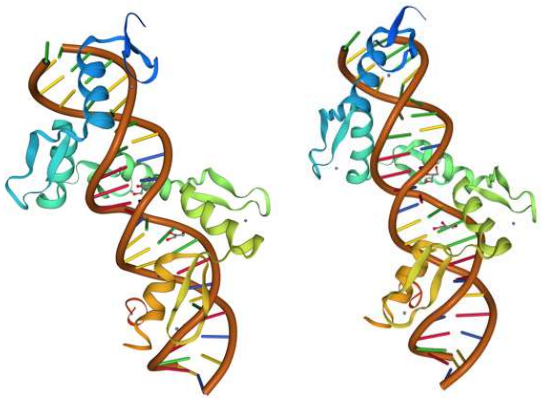

Figure 4. The DNA-protein binding models of both zinc fingers which are based on the protein type SMTL ID: 2i 13.2C.

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Figure 4: The DNA-protein binding models of both zinc fingers which are based on the protein type SMTL ID: 2i 13.2C. On the left is ZFP-NF I, on the right is ZFP-NF II. Different colours illustrate different model chains. The amino acid at the blue end is the "head" (position 1), which is isoleucine. The amino acid at the red end is the "tail" (position 190), which is serine. Both amino acid sequences of two zinc finger proteins have longer amino acids than the model. So the actual length of both proteins are longer than protein type SMTL ID: 2i 13.2C(length: 178). The two ZFP-NFs have almost the identical models.

Here, to ensure the successful binding process, we designed two zinc finger complexes called ZFP-NFI and ZFP-NFII. These two sequences will bind to two target sequences respectively, and have the following scheme consisted of different units:

Figure 5. The schematic representation of zinc finger complexes designed.

Figure 5: The schematic representation of zinc finger complexes designed. ZFP is the zinc finger protein domain, which is the main functional unit NLS is the nuclear localisation signal derived from simian virus (SV40). Tat is the transactivation domain of HIV-1. VP64 indicates the tetrameric repeat of the herpes simplex virus VP16 transactivation domain[2].

This design of zinc finger complex ensures the zinc finger proteins to penetrate the nuclear envelop, locating at proper target sites, and sustain the stable transcription process. As shown in the figure 1 in the right panel, the sequence is encoded by an mRNA which is induced into the cell and is transcribed into the protein sequence.

3. Proposed Methodology

3.1 Proposed Experiment to Test the Nuclear Envelop Penetration Efficiency

The nuclear envelop penetration of ZFP is crucial to the working efficiency. We design to use HEK293 mammalian cell line transfection to study and test whether the ZFP could be delivered to the desired location in nucleus.

In the cloning step, the mRNA encoding ZFP complex is added by a fluorescence tag such as GFP to either C-terminus or N-terminus as shown in figure 5. For example, adding GFP, this mRNA encoding for GFP-ZFP complex is transfected into the mammalian HEK293 cell line with the aid of transfection reagent. Then, once the protein is expressed, we will image and localize the GFP-tagged ZFP by the microscope under fluorescence channel.

Here, real-time fluorescence channel is used to observe the nuclear penetration process. As GFP tag shows green in the fluorescence, whether ZFP complex successfully enter the nuclear envelop and associated to viral genome could be justified.

3.2 Proposed Experiment to Test the Optimum Concentration of ZFP-Nfs

The usage of ZFP-NFs requires a concentration that could properly lead to cell death. This concentration is named as Optimum Concentration. The subsequent experiments' concentration of ZFP-NFs is based on this optimum concentration.

The optimum concentration is the concentration of ZFP-NFs that could trigger the most frequent transcription, but the count of white blood cell maintains above minimum of the theoretical value.

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The experiment carries out in vivo on HIV-1 infected humanized mice which highly restore the human cellular environment. Each mouse is injected by Zinc Finger Protein solution with different concentration ranging from 0.1g Zinc Finger+17mL sterile water (ddH2O) for injection to 2.0g Zinc Finger+17mL ddH2O for injection. The precision of each Zinc Finger Proteins' dosages is to 0.1g. Each group use a particular concentration of Zinc Finger Proteins and contain 10 mice as samples. This process using intravenous injection.

In the first period, all the samples intravenously inject the blocking agents (ARTs and so on) to prevent further infection. The count of CD4 T cells and the count of viral particles are examined at each 1 hour. The total duration is 6 hours. Then injecting ZFP solutions with different dosage. Each sample accepts blood test at 1 hours each time and total duration is 36 hours. At each time, the counts record and plot in the graph to illustrate the efficiency of each dosage.

The count of CD4 T cell plus white blood cell and the life condition are monitored for the tests of both ZFP-NFI and ZFP-NFII. At the optimum concentration, the CD4 T cell and white blood cell count need to be nearest to the lower boundary of count. But the physiological function of mice is stable.

3.3 Proposed Experiment to Test ZFP-Nfs Combination

The combination of ZFP-NFs may have accumulative impact. The experiment also contains a control group including no Zinc Finger Protein. The dosages of combination of ZFP-NFI+ZFP-NFII use different proportions but keeping the total volume the identical to the previous test for either ZFP-NFI and ZFP-NFII.

The first step is to deploy the ZFP-NFI and ZFP-NFII solutions with optimum concentration. Then blending two solutions with proper proportion with interval of 5%, ranging from 0% to 100%. The following test procedure is identical to the previous experiment to test the efficiency of the combined solution and select out the most effective composition.

4. Discussion

4.1 Tso Impact and Analysis

Specially, when the tool analyzing the target sequence, it shows the both sequences would have sequences would have the Target Site Overlap (TSO)[18]. This impact originates from the sequence GXG. This impact will make Zinc Finger less modular and reduce the targeting efficiency.

In order to confirm the impact of TSO condition, we designed an experiment to examine the influence of TSO impact. Firstly, we recall the NF-κB sequence:

5'—GGGACTTTCC—3'

In this sequence, the first triplet GGG exists TSO phenomenon. We substitute this triplet into GGC to prevent the TSO impact existing. So this NF-κB sequence changes into:

5'—GGCACTTTCC—3'

By testing this sequence on Zinc Finger Web Server, these two sequences have identical top scores. This means they have similar theoretical affinity to the DNA but ignore the TSO impact.

According to the two sequence, we designed two zinc fingers:

These two zinc fingers with same amount are divided into two DNA pools with same amount of DNA in vitro. DNA in each pool contains particular sequence that we mentioned before. Then the complementary zinc finger protein adds into each pool. The pools initially contain 250μL of 30% DNA solution and added 150μL of 40% Zinc Finger Proteins.

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Table 4. The Zinc Finger amino acid sequences to test the TSO impact

Sequence	Top score	Target sequence	Amino acid sequence of Fingers
1	61.69	GGG ACT	RSDKLVR THLDLIR
2	61.69	GGC ACT	DPGHLVR THLDLIR

The pool temperature maintains at body temperature of human (37 degrees Celsius) and leave for 3 hours for zinc finger targeting. Then, in order to prevent the zinc finger drops out from the nucleic acid sequence, we use formaldehyde to cross-link[19] and lock them covalently. This process uses formaldehyde covalently linking with nucleic acid and amino acid, making the whole structure tolerant to the environment change during subsequent analysis process.

At each hour, 10μL of the solution are taken to do the electrophoretic mobility shift assay (EMSA)[20]. On each band of concentration, the distances that travels at each time are recorded into a graph to measure the rate of binding. This is calibrated with the control group. Meanwhile, the increasing of width of each band at the same time are measured. This is calibrated with the control group and recorded in order to measure the amount binding at each time.

The difference between the experiments are compared. If the difference is within 5%, TSO impact is less likely to effect the previous experiments.

4.2 Comparison with Other Lras

As mentioned before, different LRAs have various mechanism to trigger the transcription process including signal pathway interference and gene expression regulation.

One type of LRAs is histone deacetylase inhibitors(HDACis) including suberoylanilide hydroxamic acid (SAHA) and panobinostat[21]. These HDACis have been widely used in tumour treatment and are proved that have LRA properties. However, their LRA mechanism is unrelated to the function of HDACis, instead, it triggers several signal pathways.

For example, the SAHA's operating mechanism, this LRA triggers the activation of NKG2D pathway, delivering a potent signal to all natural killer cells(NK cells) and CD8+ T cells. The activation of NKG2D would lead NK cells to act with MHC-class-related sequence A (MICA) and B (MICB) and six different cytomegalovirus UL16-binding proteins(ULBP-16)[21] which specially up-regulated in HIV-1 infected cells. Then, the NK cells and co-stimulated CD8+ T cells release cytokines, activate the cell-mediated elimination of infected CD4+ T cells.

This method could prevent uncontrolled expression of viral genome and could be safer. Also, this method could more effectively specifies the infected cells. However, the HIV-1 activation rate of this therapy is theoretically lower than the ZFP reactivation and could increase the burden of immune system. The cell-mediated immunity takes long time and it should ensure that the rate of cell-mediated immunity needs to surpass the rate of virus production to prevent leaving out infection. Also because of the multiple ART and blocking therapy, it could prevent further infection.

In the term of transcription regulator, dCas9-VPR is also widely researched. dCas9 is the dead enzyme used in CRISPR-Cas9 gene editing strategy. By using guide RNA, dCas9 has lower missing rate because of complementary base pairing which specifically binds to target sequence. Also, a VPR domain linking to dCas9 could maintain the fidelity during transcription However, dCas9-VPR has more complicated structure, resulting in the difficulty in nuclear envelop penetration process.

However, the comparison experiments could be established to test the efficiency of different strategies. Also, several strategies could be blended together to reply different situations of infection.

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4.3 Suggestions for Clinical Application

When clinically inject ZFP complex, it needs a method to penetrate cell membrane. A modified ZFP-coding gene carrier. Here, DNA fusion expression is used.

As mentioned before, HIV-1 infected cells specifically up-regulated MHC-class-related sequence A (MICA) and B (MICB) and six different cytomegalovirus UL16-binding proteins(ULBP-16). In DNA fusion expression method, DNA coding these genes, together with DNA coding mRNA of ZFP complex are integrated into adenovirus which is specially disposed to ensure safety.

The edited adenovirus transfects mammalian HEK293 cells to cultivate first generation of functional viruses existing antigen complementary to MICA, MICB and ULBP-16 and particularly associate to infected cells. By extraction, these transformed adenovirus is used to do the clinic experiments on humanised mice, apes and finally applied on patients.

Moreover, the protection of uninfected cells is also important. So several ART or blocking strategy need to be compared and combined. In clinical tests, the protection by ART and blocking strategy should be as much as the classic blocking method.

5. Conclusion

Although Zinc Finger Protein using in shock-and -kill approach is considered to be high-cost and not as precise as CRISPR, its high effectiveness and positive morality make it still possible for the HIV-1 treatment in near future. If the clinical examination could successfully prove the efficiency and availability of ZFP-NFs, the AIDS is considered to be curable and reduce the economic burden from the ART strategies. However, several issues need to be discussed further in the future, such as the detailed protecting schemes for uninfected cells and the interfering factors.

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