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Sepideh Saeb

Tarbiat Modares University

Mehrdad Ravanshad

Tarbiat Modares University

Mahmoud Reza Pourkarim

Katholieke Universiteit Leuven Universitair Psychiatrisch Centrum KU Leuven

Fadoua Daouad

Universite de Strasbourg

Kazem Baesi

Pasteur Institute of Iran

Olivier Rohr

Universite de Strasbourg

Clémentine Wallet

Universite de Strasbourg

Christian Schwartz (✉ schwartz.christian@unistra.fr)

Universite de Strasbourg

Short report

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Brain HIV-1 latently-infected reservoirs targeted by the suicide gene strategy

Sepideh Saeb^{1,2}, Mehrdad Ravanshad^{1,*}, Mahmoud Reza Pourkarim³, Fadoua Daouad², Kazem Baesi⁴, Olivier Rohr², Clémentine Wallet² & Christian Schwartz^{2,*}

¹ Department of virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

² University of Strasbourg, Research Unit7292, DHPI, IUT Louis Pasteur, Schiltigheim, France

³ KU Leuven, Department of Microbiology, Immunology and Transplantation, Division of Clinical and Epidemiological Virology, BE3000 Leuven, Belgium.

⁴ Hepatitis and AIDS department, Pasteur Institute of Iran, Tehran, Iran.

& The authors contributed equally to this work

* Corresponding authors:

schwartz.christian@unistra.fr

Ravanshad@modares.ac.ir;

Abstract

Several strategies are currently investigated to reduce the pool of all HIV-1 reservoirs in infected patients in order to achieve functional cure. The most prominent HIV-1 cell reservoirs in the brain are microglial cells. Virus infection may be lifelong. Infected microglial cells are believed to be the source of peripheral tissues reseeding and responsible for the emergence of drug resistance. Clearing infected cells from the brain is therefore crucial. However, many characteristics of microglial cells and the central nervous system prevent the eradication of brain reservoirs. Current trials, such as “shock and kill”, the “deep and lock” and the gene editing strategies do not respond to these difficulties. Therefore, new strategies have to be designed when considering brain reservoirs such as microglial cells. We set up an original gene suicide strategy using a latently infected microglial model. In this paper we provide proof of concept of this strategy. Our results demonstrate that this strategy enables the eradication of latently-infected microglial cells.

Keywords: HIV-1; microglial; latent reservoirs; suicide gene

Introduction

Since the introduction of the combination antiretroviral therapy (cART) in 1996 the lethal HIV-1 infection had evolved to a chronic disease. However, despite continual efforts definitive cure has not been achieved. This is principally due to the existence of latently-infected HIV-1 reservoirs. The principal reservoir is the resting CD4⁺ T cells but we have now abundant proofs that many other cell reservoirs such as hematopoietic stem cells, dendritic cells, microglial cells and cells from the monocyte-macrophage lineage (reviewed in [1–3]) exist. Furthermore, some of these reservoirs are found in sanctuaries such as the genital tract, the adipose tissue, the bone marrow and the brain.

A prerequisite to successfully purge reservoirs is to understand first the molecular mechanisms, which support the establishment and maintenance of HIV-1 latency. Better understandings of these mechanisms is essential in order to identify new targets and/or when targeting the viral cycle at other stages than cART is currently directed to [4]. These molecular mechanisms, however are partially understood. We have notably shown that the cellular cofactor CTIP2 (Bcl11b) supports both the establishment and the maintenance of HIV-1 post-integration latency in microglial cells [5–7]. CTIP2 works as a scaffold protein by recruiting at least two different complexes in microglial cells. As part of a chromatin remodelling complex CTIP2 is associated with the lysine demethylase LSD1, the histone deacetylases HDAC1 and HDAC2, and the histone methyltransferase SUV39H1[5,8–10]. Moreover, CTIP2 also participates to control the elongation process of gene transcription by inhibiting P-TEFb functions. [11,12].

To date, three strategies are considered, which have the perspectives to achieve functional cure: these are pharmacological approaches including the “Shock and Kill” strategy and the alternative “Block and Lock” strategy and the gene therapy including the gene editing tool based strategy. The “Shock and Kill” strategy is based on the reactivation of the latent virus followed by an intensification of cART. This strategy seeks the clearance of reservoirs either by the cytopathic effect of the reactivated virus or by the immune system via the actions of cytotoxic T cells (CTLs) [13]. The feasibility of shock and kill strategy is today only conceivable with circulating reservoirs such as the resting T CD4⁺ cells. Indeed, targeting the brain reservoirs with the “Shock and Kill” strategy is at present not feasible due to several characteristics of the CNS:

- i. The CNS is a sanctuary with barriers (Brain Blood Barrier and choroid plexus) which lower the penetration of drugs into the brain [14].

- ii. The main cellular targets are CNS resident macrophages including perivascular macrophages and microglial cells. However, few drugs are able to target the monocyte-macrophages lineage and microglial cells [15].
- iii. Reactivation of the virus with Latency Reversing Agents (LRAs) will lead to the synthesis of neurotoxic viral proteins such as Tat, Vpr and the gp120 as there are no drugs currently available targeting HIV transcription. Furthermore, reactivation of the virus activates macrophage/microglial cells and it is often correlated with CNS inflammation [16,17].

An alternative approach to achieve long term control over HIV-1 infection without the need of cART is to induce long lasting inhibition of HIV-1 gene expression [18]. Molecules inhibiting HIV-1 expression, called latency-promoting agents (LPAs), induce deep latency state (the Block) and prevent HIV-1 gene transcription (the Lock) [19]. Among LPAs, didehydrocorticostatin (dCA), an inhibitor of the transactivator Tat, had promising effect. However, a recent *in vitro* study described virus resistance to dCA [20]. In addition, gene therapy has recently been developed and shows promising results. This includes bone marrow transplantation to replace blood cells with CCR5-mutated stem cells or gene editing such as the CRIPR/cas9 technology to disrupt the HIV genome. However, a major off-target of the gene editing strategy is the rise of unwanted gene mutations and chromosomal translocations [21]. Moreover, HIV-1 can elude CRISPR/cas9 mediated repression. Indeed, subsequently to an initial trial to ascertain proof of concept of gene editing, which target specifically HIV-1 [22], it quickly appeared that the virus was able to subvert the DNA repair machinery to evolve rapidly into CRISPR/Cas9 resistant strains [23–26]. A more promising approach to explore is a variant of the CRISPR/Cas9 technology, which predisposes latently infected cells to reactivate or to induce deep latency [27]. The method makes use of a defective Cas9 (dCas9) protein fused to virus activators or to repressors in order to reactivate latently-infected cells or to suppress HIV-1 expression, respectively [27] [28]. These experiments showed reactivation of HIV expression in CD4+ T cells and in microglial cell lines [29]. Remarkably, CRISPR/dCas9 when associated with HDAC inhibitors and PKC activators reactivates HIV in a synergistic manner [30].

New strategies have to be designed when considering brain reservoirs such as microglial cells, due to the difficulties to target them. An original gene therapy has been recently considered, which relies on the so called suicide gene therapy already well studied in the field of cancer [31,32]. Indeed, this approach has already been used in *in vitro* experiments to inhibit HIV-1 replication and proved to be safe and effective [33,34]. Briefly, this strategy is based on the expression of herpes simplex virus thymidine kinase, which subsequently converts the prodrug Ganciclovir into a toxic drug inducing apoptosis. We redesigned this strategy in order to target

specifically latent brain reservoirs. As evoked above and in [7,35] brain reservoirs of HIV-1 are in sanctuaries in which cART does not work efficiently and new techniques are needed to overcome these limitations. A recombinant AAV vector including a Long Terminal Repeat-Thymidine kinase (LTR-TK) gene has been developed. Our idea relies on the fact that latently-infected reservoirs reactivated produce the trans-activator Tat which can bind onto the HIV-1 promoter LTR which drives the expression of thymidine kinase. In the presence of the prodrug Ganciclovir, we thus expected to induce apoptosis preferentially in reactivated cells of the latently-infected reservoirs. For this purpose we used an AAV vector which is nonpathogenic for human and has the ability to infect both dividing and non-dividing cells [36]. Moreover, the vector exhibits stable transgene expression with low risk of insertional mutagenesis [36]. Last but not least, some AAV serotypes have been shown to infect cells in the brain following intravascular administration in preclinical trials [37,38]. We show in this paper that a model of latently-infected microglial cell when reactivated by Romidepsin and transduced by a recombinant AAV becomes apoptotic.

Materials and methods

1. AAV production

1.1 Design of the recombinant AAV viral vector

We designed a Recombinant AAV based vector (rAAV) (figure 1) which contains Herpes Simplex Virus-1 (HSV-1) Thymidine Kinase (TK) gene under the control of the HIV-1 promoter LTR (rAAV-LTR-TK), as follows:

AAV-DJ Helper Free Promoterless Expression System was purchased from CELL BIOLABS (Catalog number: VPK-411-DJ).

The whole sequence of wild type LTR which contains the TAR structure (ClaI and EcoRI enzyme sites at the 5' and 3' end, respectively) was synthesized. The sequence was obtained from a 2nd generation lentivector; pLEX - jRED – TurboGFP ; (Catalog number: OHS4736). Then the sequence cloned in PUC57 cloning vector, was subcloned in the above mentioned restriction enzyme sites of pAAV-MCS promoterless expression vector.

Concerning the TK gene cloning, HSV-1 TK (Accession number: AB009254.2) was amplified by PCR from a PCDH-CMV-MCS-EF1-Puro (Catalog number: CD510B-1) plasmid carrying the coding region of TK. This was performed with primers containing EcoRI site at 5' and BamHI at 3' end. Primers sequences are as follows:

TK Forward primer: 5' CAT CGA TGG AAT TCC TCC GTG TTT CAG TTA GCC 3'

TK Reverse primer: 5' CG GGA TCC CAC CGA GCG ACC CTG 3'

The PCR product was inserted downstream of the HIV-1 LTR which was cloned before in pAAV-MCS promoterless expression vector, and the final vector was constructed (rAAV-LTR-TK).

1.2 Production of the Recombinant AAV LTR-TK virus

One day before transfection, HEK-293 cells were cultured to achieve 70 to 80% confluence. Cotransfection of HEK-293 cells with rAAV-LTR-TK vector, pAAV-DJ and pHelper (with the ratio of 1:1:1 respectively) was achieved by using Calcium Phosphate transfection. 48-72 hours after transfection rAAV-LTR-TK viruses were harvested by Freeze/thaw cycles based on the manufacturer instructions. rAAV LTR-TK crude lysate was used directly. rAAV LTR-TK aliquots were stored in -80°C. After viral DNA extraction, titer of viruses was determined by qPCR.

2. Viral Infection and Cell Treatments

2.1 rAAV-LTR-TK transduction

CHME5/HIV- and CHEM5/HIV+ cell lines cultured in DMEM, supplemented with 10% Fetal Calf serum and 1% penicillin/streptomycin, were plated on 24-well plates. 24h later, cells were infected with rAAVs with a titer of 10,000 genome copies / cell (GC/cell). After 24 h of infection, cells were washed with PBS twice and treated with Romidepsin (Concentration: 0.0175 µM) to reactivate the latent virus. Next day, Ganciclovir (GCV) was added to the cells and 24h later, apoptosis assay was done according to “ab219918 Annexin V-iFluor 594 Apoptosis Detection Kit” (Abcam). The Percentage of cell reactivation and apoptosis was determined by flow cytometry. For each analysis, at least 5000 events were counted.

2.2 The cell treatments

The treatment conditions (on CHME5/HIV+ and CHME5/HIV- cells) are as follows (Table 1)

CHME5 /HIV-	Romidepsin	rAAV-TK	GCV
Control III.	-	-	-
Control IV.	-	+	+
			-
Control V.	+	+	+
			-
Control VI.	+	-	-
CHME5/HIV+	Romidepsin	rAAV-TK	GCV
Test	+	+	+
			-
Control I.	-	+	+
			-
Control II.	+	-	-

Table 1. The treatment conditions (on CHME5/HIV+ and CHME5/HIV- cells)

Statistical analysis

All experiments were done in triplicate. Means and SDs were calculated using Independent (Unpaired) t test. P-value < 0.05 was considered statistically relevant (*p < 0.05, **p < 0.01, *** p < 0.001, ****p < 0.0001).

Results

The suicide gene strategy

Suicide gene therapy is based on the transduction of infected cells including latently-infected cells with a recombinant AAV (rAAV). In the experiments we used CHME5/HIV+, a model of latently-microglial cell line. The rAAV contains a gene coding for the herpes simplex (HS) thymidine kinase and the transcription of the vector is driven by the 5' Long Terminal Repeat (LTR) HIV-1 promoter. Following transduction of latently-infected cells by rAAV (figure 2 step 1), cells are treated with the HDAC inhibitor Romidepsin in order to reactivate the virus

(figure 2 step 2), which consequently produces Tat proteins. Tat in turn binds on the LTR-TK promoter activating the synthesis of the thymidine kinase (TK). In the presence of TK, the added prodrug Ganciclovir is phosphorylated leading to the formation of Ganciclovir triphosphate (figure 2 step 3), a toxic form of the drug inducing apoptosis (figure 2 step 4). This strategy aims to reactivate specifically latently-infected cells. We expected that only these reactivated cells will be apoptotic when treated with Ganciclovir.

Transduction of rAAV leads to the death of CHME5/HIV+ but not that of CHME5/HIV- cells.

Negative control experiments

In a first set of experiments, we used as control the non-latently-infected microglial cell line CHME5 (referred as CHME5/HIV-). The signal level of GFP reflects the percentage of reactivation of latently infected CHME5 cells (referred as CHME5/HIV+), while the signal level of RFP reflects the percentage of apoptotic cells. As expected, the non-treated and rAAV non-transduced CHME5/HIV- cells are negative for both GFP and RFP (figure 3A lane 1 and 2). We next checked that the various treatments (Romidepsin and Ganciclovir) did not affect CHME5/HIV- cells transduced or not with rAAV. We showed that cells transduced with rAAV but not treated with Romidepsin, the percentage of RFP + (figure D lane 3 and 4) cells in the presence or absence of Ganciclovir were comparable and very low, suggesting that the rAAV vector did not induce apoptosis of HIV- cells. In cells that were not transduced with rAAV but treated with Romidepsin, the percentage of GFP + and RFP + cells were also very low supporting that Romidepsin has no effect on its own (figure 3 B). The slight increase of GFP+ cells in the presence of romidepsin (around 10% of GFP+) versus in the absence of romidepsin (around 5% of GFP+) might reflect the fact that romidepsin induce autofluorescence (compare figure 3B lane 1 or 3C lane 2 with figure 3D lane 2). Finally, cells transduced with rAAV and treated with Romidepsin in the presence and absence of Ganciclovir (figure 3C), the signal level of GFP (figure 3C lane 1 and 2) and RFP (figure 3C lane 3 and 4) were also very low, suggesting that Ganciclovir alone did not affect cell viability. The low level of positive (GFP+) cells observed in CHME5/HIV- treated with combination of drugs (Romidepsin and Ganciclovir) and transduced with rAAV (figure 3D lane 1 and 2) reflects the non-specific background luminosity. To be note, we might expect a transcriptional activation of the TK gene driven by the LTR following a Romidepsin treatment which could lead to unexpected apoptosis of non-reservoirs (CHME5 without HIV referred as CHME5/HIV-). However we do not observed such

an off-target effect with Romidepsin. If we compare CHME5/HIV- transduced with rAAV with and without a Romidepsin treatment, we can notice that the level of positive cells (RFP+) observed is low which reflects a non-specific background luminosity (figure 3C lane 3 versus figure 3D lane 3)

Experiments using the latently-infected microglial cell line CHME5/HIV+

We next used a latently-infected microglial cell line, CHME5/HIV+, as a model and applied the same protocol as for the control CHME5/HIV- experiments (figure 4). On the dot plots representing cells gates for expression of GFP+ (Y axis) and RFP+ (X axis), we can monitor respectively the CHME5/HIV+ being reactivated or apoptotic (figure 4E). We visualize the GFP+ cells on the Y axis following the Romidepsin treatment (compare right and left representation of the dot plots figure E). Interestingly, we only observe RFP+ cells in CHME5/HIV+ cells transduced with rAAV-TK and treated with both Romidepsin and Ganciclovir (compare on figure 4E at the bottom the right and left representation of the dot plots). We observed more than 50% reactivation of CHME5/HIV+ cells following Romidepsin treatment in the presence or absence of Ganciclovir (figure 4A lane 1 and 2). On the other hand, in the absence of Romidepsin, reactivation of CHME5/HIV+ is low and comparable to the non-specific background observed with CHME5/HIV- cells (compare figure 4B lane 1 and 2 with figure 3 D lane 1 and 2). Around 37% of the cells are RFP + in the presence of Ganciclovir which represents the number of apoptotic cells (figure 4A lane 3 and 4). In CHME5/HIV+ cells transduced with rAAV-TK and treated with Ganciclovir, we still observe some GFP+ cells when the cells are treated with Romidepsin but many of them are becoming RFP+ which means apoptotic. In CHME5/HIV+ cells not transduced with rAAV, Romidepsin treatment reactivated 60 % of cells similarly as observed in cells transduced with rAAV (compare figure 4A lane 2 and figure 4 C lane 1). Interestingly, the level of RFP + cells was very low with a similar background level of fluorescence as observed in CHME5/HIV- cells (compare figure 4C lane 3 and 4 with respectively figure 3B lane 2 and figure 3A lane 2). Finally, CHME5/HIV+ cells transduced with rAAV and treated with Ganciclovir needed to be reactivated by Romidepsin to generate fluorescent cells (GFP + and RFP +) (figure 4 D and figure 4E).

A synthesis of our results is shown in figure 5. In the figure we can easily compare the effects of the various treatments on CHME5/HIV+ and CHME5/HIV- cells. The figure demonstrates well that exclusively CHME5/HIV+ cells were reactivated by Romidepsin (GFP+ lane 1 and 3) and only cells treated with Ganciclovir were apoptotic (RFP + lane 5).

Discussion

There is now numerous evidence supporting the findings that the major cellular reservoir of HIV-1 in the brain are microglial cells [7,35]. These cells are believed to be the source of peripheral tissues reseeding and responsible for the emergence of drug resistance. Moreover, these lifelong reservoirs are also responsible for the development of HIV-1 associated neurocognitive diseases (HAND). Microglial cells differentiate from the myeloid precursors of the embryonic yolk sac at a very early stage of embryogenesis [39,40]. They colonize the entire brain parenchyma before the formation of the Brain Blood Barrier and form a slowly dividing population of cells in the brain [39]. In a recent study, the average age of microglial cells was evaluated to 4.2 years and the great majority of them were shown to be capable of cell division all their life [41]. This means that infected microglial cells makes brain infection permanent contrary to other infected cells found in the brain.

Targeting these cells located in anatomic and pharmacologic sanctuaries is however very challenging [7]. In the classic “Shock and Kill” strategy latently-infected T CD4+ cells are first reactivated by the Shock strategy and the newly formed viruses are eliminated by cART. On the other hand reactivated HIV-1 positive cells are eliminated by the Kill strategy. A main backlash of the Kill strategy is that it is unable to eradicate all reservoirs despite latent cells becoming productive following reactivation. The failure is essentially due to the complexity of the Kill strategy, which does not allow the direct elimination of reactivated cells. An original way to circumvent this problem is to use suicide gene therapy. A major advantage of the strategy is that it eliminates reactivated cells directly and specifically by apoptosis. Furthermore, in case of microglial cells production and the release of neurotoxic viral proteins such as Tat and Vpr are also inhibited as a consequence of apoptosis. This is a major improvement compared to classical Kill strategies which are unable to inhibit viral transcription even when enhanced cART is applied. Although much efforts have been employed to discover new molecules inhibiting the transcriptional step no drugs has been put on the market to date [4].

Here we have set up a gene suicide strategy using a latently infected microglial model. We used the potent HDAC inhibitor Romidepsin to reactivate latently-infected microglial cells. HDAC contributes to the transcriptional silencing of HIV-1 by inducing the formation of heterochromatin. In the first step we showed that Romidepsin is able to specifically reverse

heterochromatin silencing of the virus. We also showed that HIV-1 negative cells are not affected, and rAAV transduction does not change the scale (amplitude, degree) of reactivation. In the next step we demonstrated that Romidepsin activated recombinant cells became apoptotic to Ganciclovir treatment. Control cells, which were not transduced with rAAV were unaffected by treatments. Remarkably, only reactivated cells became apoptotic. These results strongly support the concept that the suicide gene strategy allows the specific elimination of latently infected cells.

It is noteworthy that Romidepsin treatment does not allow complete reactivation of latently infected cells. As in other strategies aiming to reactivate latent reservoirs, a combination of LRAs chosen from an existing pool of 160 LRA drugs should improve the level of reactivation[42,43].

Approach still needs to be improved in order to achieve functional cure. Two main directions should be investigated: (i) designing efficient LRAs for the CNS and (ii) improving delivery of HIV drugs into the CNS and into the various CNS cells.

Several HDAC inhibitors (HDACi) have been tested in primary cells (astrocytes and macrophages) [44,45]. Preliminary data showed that some LRAs including panobinostat [46] and JQ1 [47] are relatively nontoxic and are able to trigger HIV reactivation at a therapeutic concentration [45]. Bryostatins-1, an LRA drug which activates Protein Kinase C and crosses the blood brain barrier could be exploited in the suicide gene strategy especially if designed for microglial cells [48,49].

Improvement of both bioavailability and bio-distribution of LRAs are also awaited with the development of carriers such as liposomes, dendrimers and micelles. Adeno-associated viruses are good candidates for delivering a transgene such as the LTR-TK transgene and/or drugs into the brain. Among the many serotypes tested, AAV9 and AAV8 were shown to cross the BBB which allow the intravascular administrations of the vectors [50]. In primary human brain microvascular endothelial cells used as a model of human BBB, AAV9 was shown to cross the BBB and transduce brain cells following systemic administration [51]. rAAV9 was further tested in a clinical trial to treat neurological disorders [52]. However gene transfer with rAAV9 was less effective in the adult than in the neonatal brain. A recent study found that systemic administration of an AAV8 mutant resulted in a more widespread brain gene transfer than that of rAAV9. Moreover, the study showed that BBB shuttle peptides improved AAV transduction in the brain [53].

In conclusion, we provided proof of concept of the gene suicide strategy in this paper. Our results demonstrate that this strategy enables the eradication of latently-infected microglial cells. We expect that suicide gene therapy could be successfully applied *in vivo*. This strategy could efficiently target latently-infected microglial cells in the brain, reactivating these cells by LRAs followed by their apoptosis. This approach thus prevents inflammation and the release of viral neurotoxic proteins which are secondary to reactivation. Indeed, the suicide gene approach aims to kill reactivated infected cells and simultaneously inhibit the release of new particles and viral products. The ultimate purge or at least considerable reduction of brain reservoirs, especially of microglial cells, is conceivable.

List of Abbreviations

AAV: adeno associated virus

BBB: blood brain barrier

cART: combination antiretroviral therapy

CNS: Central nervous system

CD4: cluster of differentiation 4

CTLs: cytotoxic T cells

CTIP2: COUP-Tf interacting protein 2

CCR5: chemokine receptor type 5

CHME5/HIV⁻: non-latently-infected microglial cell line

CHME5/HIV⁺: HIV-1 latently-infected microglial cell line

dCA: didehydro-corticostatin

GCV: Ganciclovir

GFP: green fluorescent protein

gp120: glycoprotein 120

HAND: HIV-1 associated neurocognitive diseases

HIV-1: Human Immunodeficiency Virus 1

HDAC: Histone deacetylase

HSV: herpes Simplex Virus

LRAs: Latency-Reversing Agents

LPAs: Latency-Promoting Agents

LSD1: Lysine-specific histone demethylase 1

LTR: Long Terminal Repeat

PKC: protein kinase C

P-TEFb: positive transcription elongation factor

RFP: red fluorescent protein

SUV39H1: Suppressor of Variegation 3-9 Homolog 1

TK: Thymidine kinase

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publication

Not applicable

Availability of data and material

Contact the corresponding authors christian schwartz or Mehrdad Ravanshad

Competing interests

The author(s) declare(s) that they have no competing interests

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Authors' contributions

SS performed all the experiments. SS, MR, MRP and KB designed the gene suicide TK vector and revised the manuscript. SS, CW and FD made the acquisition of the datas. CW, OR, MR and CS interpreted the datas. CW and SS designed the figures. CW and OR revised the manuscript. CW and CS designed the experiments. CS drafted the work.

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Figure 1. Characterization of rAAV-TK vector. HIV-1 LTR sequence was cloned in multiple cloning site of pAAV-MCS promoterless expression vector(1A), between ClaI and EcoRI restriction enzyme sites. The coding region of TK gene was also incorporated into this vector at the downstream of LTR; between EcoRI and BamHI sites; (1B). and rAAV-TK vector was constructed (1C).

Figure 2: schematic design of the experimental strategy

Figure 3. A-D CHME5 cells were treated with certain concentration of Romidepsin and rAAV-TK viruses in different conditions. GFP+ percentage represent the cells that are reactivated. RFP+ percentage represents the cells that are apoptotic and Bifluo+ fluorescent signal shows the percentage of the cells that are reactivated and apoptotic, Mock cells (Romidepsin-, rAAV-TK-, GCV-), (A); cells treated only with Romidepsin (B); Cells treated with Romidepsin and infected with rAAV-TK, with or without GCV (C); Cells infected with rAAV-TK with or without GCV in the absence of Romidepsin(D);

Figure 4. A-E CHME5/HIV cells were treated with certain concentration of Romidepsin and rAAV-TK viruses in different conditions and latent virus reactivation and cell apoptosis was measured by flow cytometry. GFP+ percentage represent the cells that are reactivated. RFP+ percentage represents the cells that are apoptotic. Cells treated with Romidepsin and infected with rAAV-TK, with or without GCV(A); Cells infected with rAAV-TK with or without GCV in the absence of Romidepsin (B); Cells with or without Romidepsin in the absence of rAAV-TK and GCV(C) ; A comparison between the cells that are treated with Romidepsin, rAAV-TK and GCV and those cells that infected with rAAV-TK and treated with GCV in the absence of Romidepsin (D). Cytometry analysis of GFP expression (green fluorescence), in CHME5/HIV+ cells in presence of a specific antibody for apoptotic cells coupled with annexin (red fluorescence). Assays were done with or without Romidepsin, rAAV-TK and GCV (E°).

Figure 5. A comparison between CHME5/HIV cells and CHME5 cells in different conditions, GFP+ percentage represent the cells that are reactivated. RFP+ percentage represents the cells that are apoptotic. Romidepsin(R), rAAV-TK (A) and GCV (G).

Figures

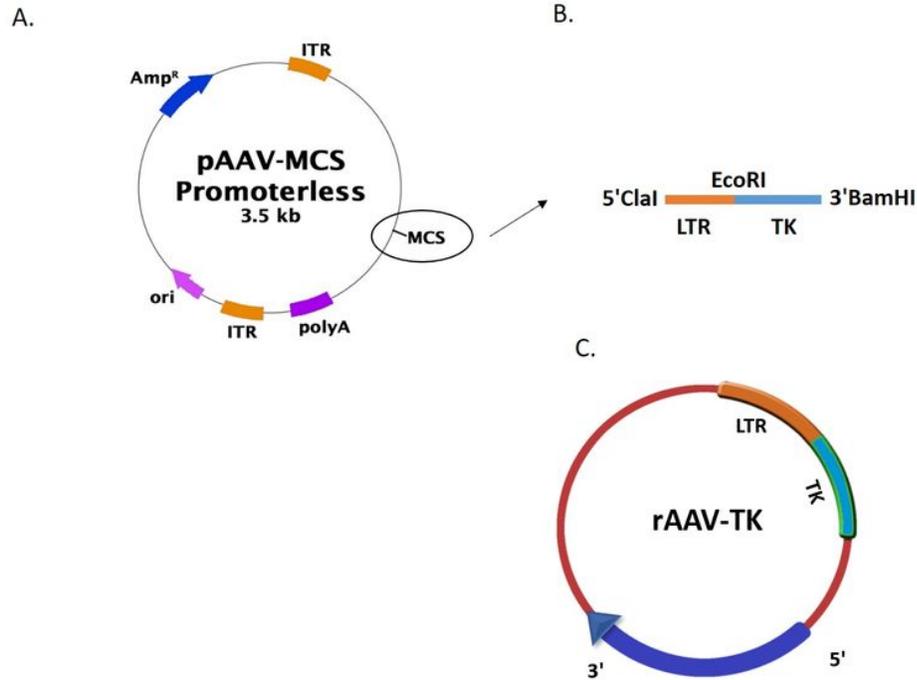
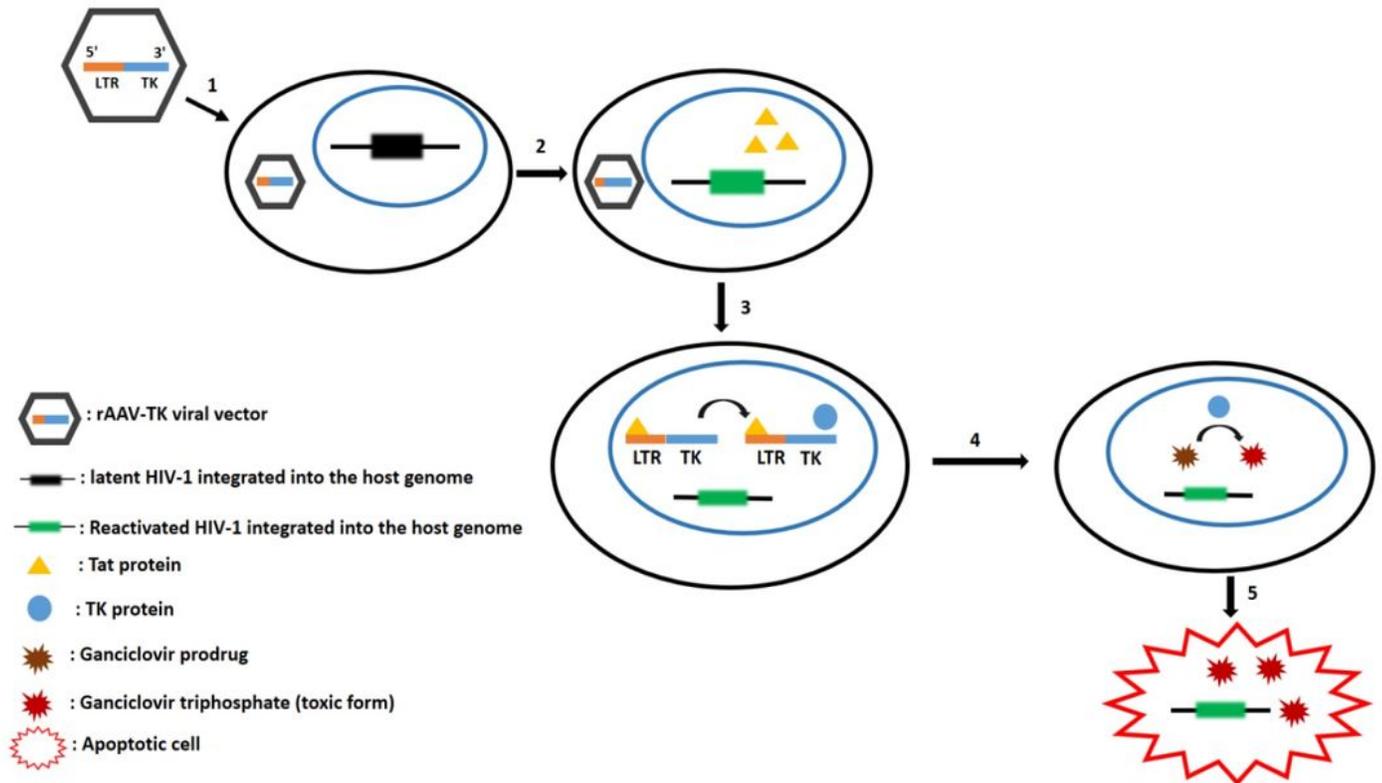


Figure 1

Characterization of rAAV-TK vector. HIV-1 LTR sequence was cloned in multiple cloning site of pAAV-MCS promoterless expression vector(1A), between **Clal** and **EcoRI** restriction enzyme sites. The coding region of **TK** gene was also incorporated into this vector at the downstream of **LTR**; between **EcoRI** and **BamHI** sites; (1B). and rAAV-TK vector was constructed (1C).



• This figure shows a schematic design of the experiment strategy:

1. **Recombinant AAV-TK (rAAV-TK) Transduction:** At first, Cells were transduced by rAAV-TK viral vector. This viral vector contains thymidine kinase gene (TK) and wildtype HIV-1 LTR at the upstream as a promoter structure.
2. **Romidepsin Treatment:** After 24 hours of rAAV-TK transduction, transduced cells were treated with Romidepsin in order to reactivate latent HIV-1 which is integrated into the host cell genome.
3. **Tat Protein Production:** Following latent HIV-1 reactivation by Romidepsin, Tat protein expression is induced. Binding of Tat to the LTR of rAAV-TK viral vector transactivates the LTR which leads to induce the expression of thymidine kinase enzyme protein.
4. **Ganciclovir Treatment:** Next day, Ganciclovir was added to the cells. TK and cellular kinase convert Ganciclovir prodrug to Ganciclovir triphosphate(the toxic form). Then, it is incorporated into the cellular DNA, terminates the replication of dsDNA and the apoptosis process is induced.
5. **Apoptosis Assay:** After 24 hours, Apoptosis assay was performed using "Annexin V-iFluor 594 apoptosis detection kit".

Figure 2

schematic design of the experimental strategy

CHME5/HIV-

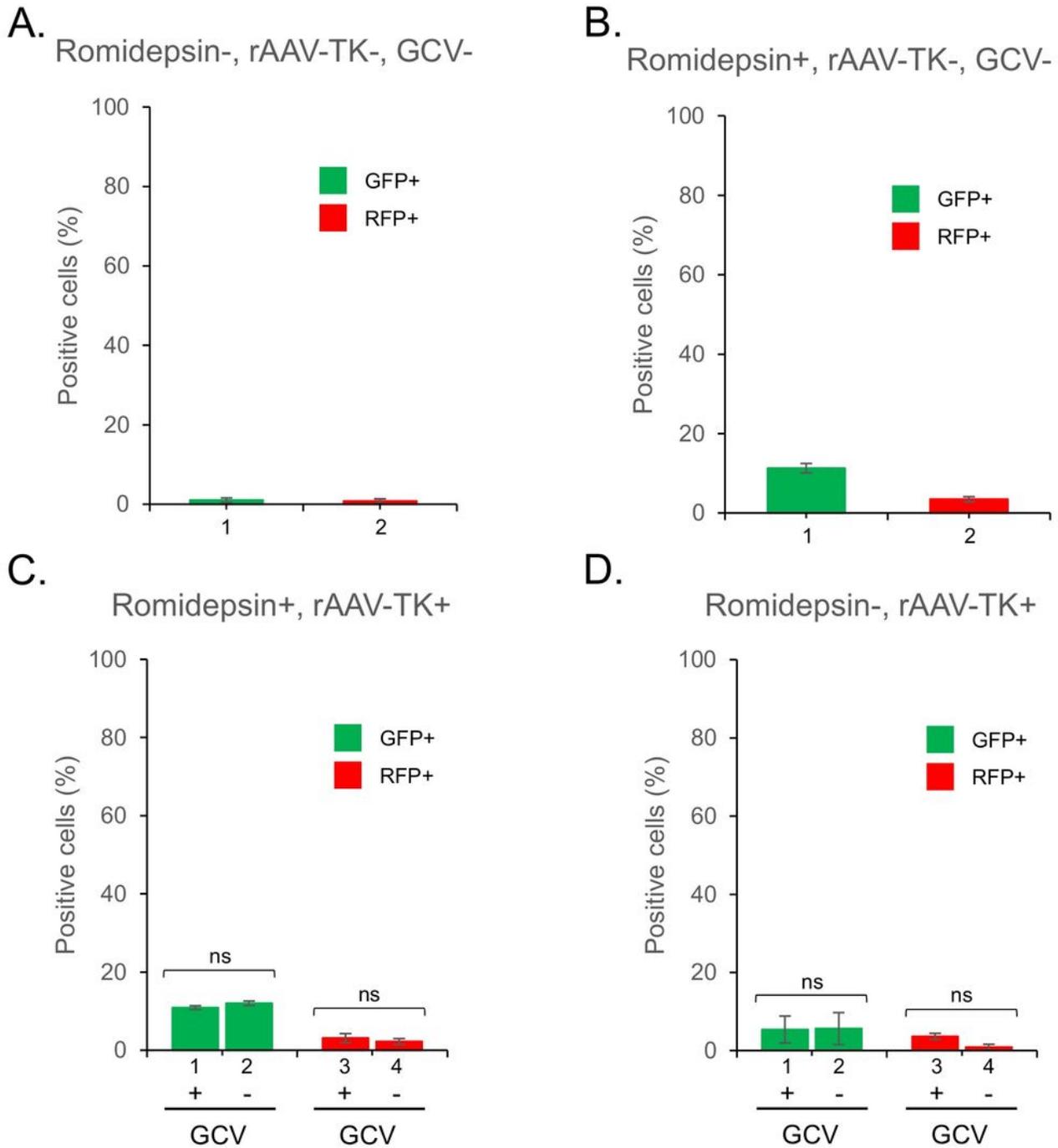


Figure 3

A-D CHME5 cells were treated with certain concentration of Romidepsin and rAAVTK viruses in different conditions. GFP+ percentage represent the cells that are reactivated. RFP+ percentage represents the cells that are apoptotic and Bifluo+ fluorescent signal shows the percentage of the cells that are reactivated and apoptotic, Mock cells (Romidepsin-, rAAVTK-,GCV-), (A); cells treated only with Romidepsin (B); Cells

treated with Romidepsin and infected with rAAV-TK, with or without GCV (C); Cells infected with rAAV-TK with or without GCV in the absence of Romidepsin(D);

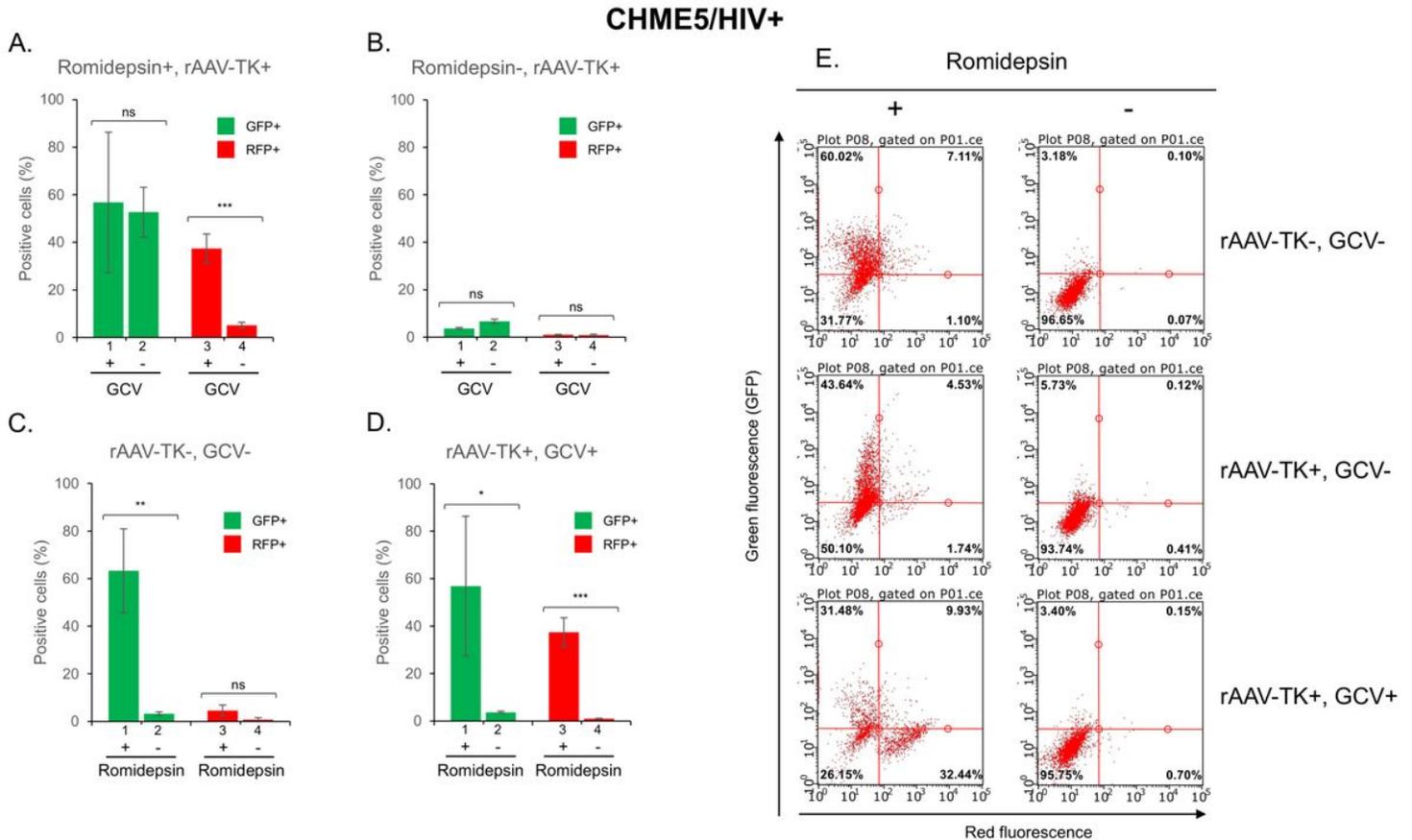


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A-E CHME5/HIV cells were treated with certain concentration of Romidepsin and rAAV-TK viruses in different conditions and latent virus reactivation and cell apoptosis was measured by flow cytometry. GFP+ percentage represent the cells that are reactivated. RFP+ percentage represents the cells that are apoptotic. Cells treated with Romidepsin and infected with rAAV-TK, with or without GCV(A); Cells infected with rAAV-TK with or without GCV in the absence of Romidepsin (B); Cells with or without Romidepsin in the absence of rAAVTK and GCV(C) ; A comparison between the cells that are treated with Romidepsin, rAAVTK and GCV and those cells that infected with rAAV-TK and treated with GCV in the absence of Romidepsin (D). Cytometry analysis of GFP expression (green fluorescence), in CHME5/HIV+ cells in presence of a specific antibody for apoptotic cells coupled with annexin (red fluorescence). Assays were done with or without Romidepsin, rAAV-TK and GCV (E°).

Romidepsin+, rAAV-TK+,

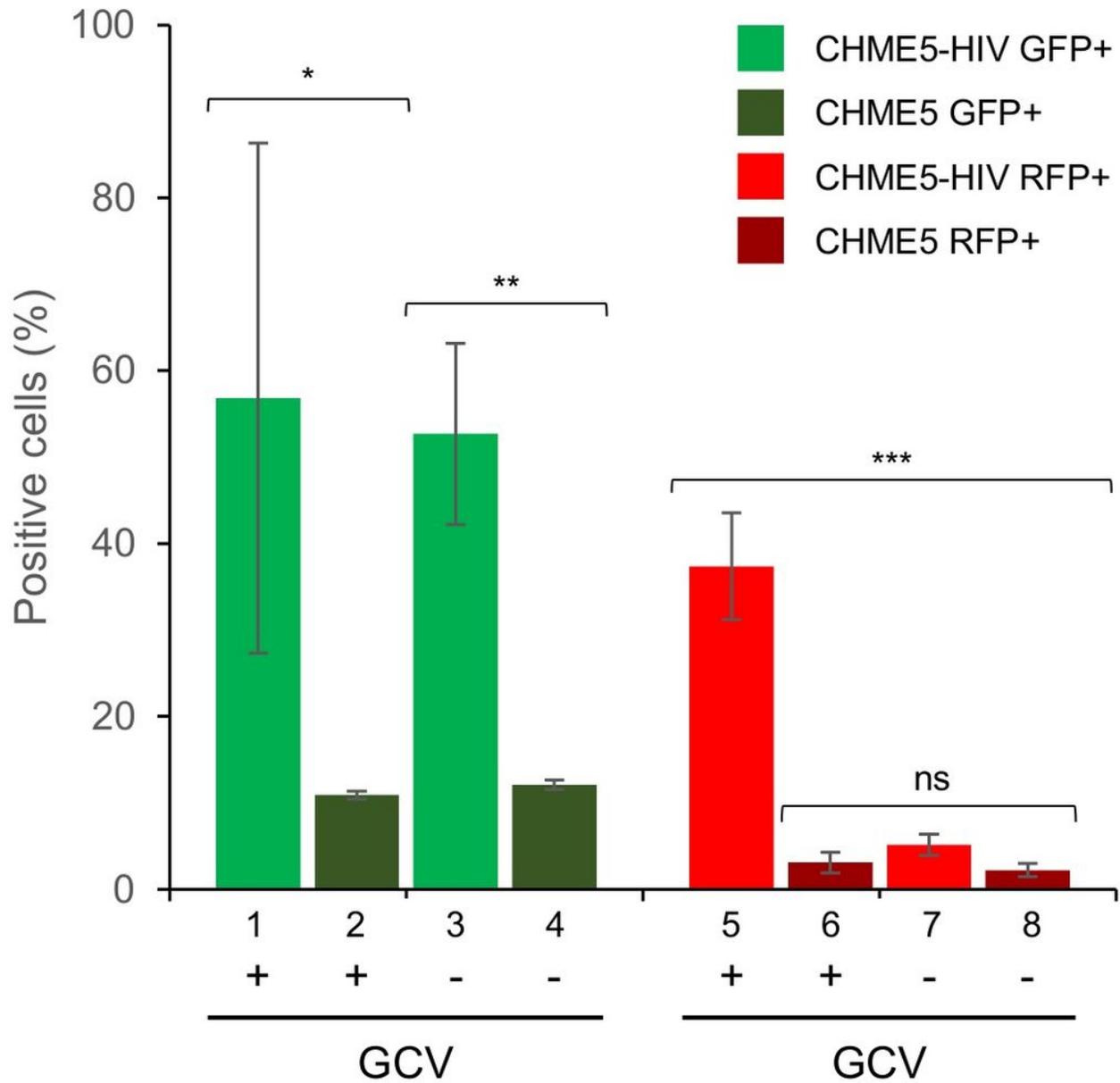


Figure 5

A comparison between CHME5/HIV cells and CHME5 cells in different conditions, GFP+ percentage represent the cells that are reactivated. RFP+ percentage represents the cells that are apoptotic. Romidepsin(R), rAAV-TK (A) and GCV (G).