EVALUATING THE ELIMINATION OF ICASP9-IL15 CAR-T CELLS USING PHOTOSENSITIZERS AP20187 *IN VITRO*

Can Van Mao[1,✉], Le Duy Cuong[2]
[1]Vietnam Military Medical University
[2]Military Central Hospital 108

This study was conducted to evaluate the ability to eliminate iCasp9-IL15 Chimeric Antigen Receptor-T (CAR-T) cells using the photosensitizers AP20187 in vitro. Peripheral blood mononuclear cells (PBMC) were activated with Dynabeads Human T-Activator CD3/CD28 and IL-2 to optimize the concentration of the antibiotic blasticidin for screening iCasp9-IL15 CAR-T cells. iCasp9-IL15 CAR-T cells after proliferation with 1D2 artificial antigen-presenting cells, were screened using blasticidin. Subsequently, the iCasp9 suicide gene of these cells was activated at various concentrations of AP20187 photosensitizer. The results showed that after 5 days of culture, a blasticidin concentration of 15.0 µg/mL was used to screen iCasp9-IL15 CAR-T cells (activated, non-transformed PBMC showed > 95% cell death). The proportion of iCasp9-IL15 cells after blasticidin screening reached approximately 75%. Using AP20187 at the concentration of 20 nM resulted in almost complete elimination of iCasp9-IL15 CAR-T cells (survival rate: 2.14%). So, iCasp9-IL15 CAR-T cells can almost be eliminated by AP20187 at a concentration of 20 nM in case of high toxicity during treatment.

Keywords: iCasp9-IL15 CAR-T cells, AP20187 photosensitizer, blasticidin.

I. INTRODUCTION

The currently clinically approved CAR designs do not allow control over CAR-T cells after infusion. As a result, the management of toxicity relies on immunosuppression using systemic corticosteroids and the IL-6 receptor antagonist antibody, tocilizumab. Unfortunately, the use of immunosuppressive drugs severely limits the duration of CAR-T cell activity.[1] Given the severity of toxicity and the cost of production, there is a clinical need to regulate the number and activity of CAR-T cells when deployed in patients. Existing approaches focus on regulating and controlling CAR-T cells.[2]

An important issue in using CAR-T cell therapy for cancer treatment is controlling CAR-T cells to avoid complications such as cytokine release syndrome or the emergence of malignant cells.[3] Various methods have been proposed by researchers, including passive control (transient transduction, affinity tuning) and inducible control (suicide genes, marker elimination, systemic CAR-T cell inhibition). Implementing the suicide gene strategy in CAR-T cell design aims to control the proliferation of CAR-T cells within the patient's body, thereby limiting the occurrence of the "on-target, off-tumor" effect or the emergence of malignant T cells. One of the most commonly utilized suicide genes is HSV-TK (herpes simplex virus thymidine kinase). Upon the administration of ganciclovir (GCV), HSV-TK, in conjunction with human kinases, catalyzes the phosphorylation of GCV, leading to the formation of GCV-triphosphate. This compound acts as a chain

Corresponding author: Can Van Mao
Vietnam Military Medical University
Email: canvanmao@vmmu.edu.vn
Received: 09/04/2025
Accepted: 23/04/2025

terminator during DNA synthesis, ultimately inducing programmed cell death.[4]

Recently, the inducible caspase-9 (iCasp9) system has demonstrated significant potential in clinical trials, particularly within the context of hematopoietic stem cell transplantation. The administration of the dimerizing agent AP20187, a chemical inducer of dimerization, facilitates the oligomerization of two caspase-9 subunits, resulting in the formation of an active caspase-9 complex that subsequently triggers apoptotic cell death. In a study conducted by Hoyos et al., the CD19 CAR/IL-15/iCasp9 construct was shown to enhance the eradication of tumor cells *in vivo* while also effectively eliminating CAR-T cells following exposure to AP20187.[5] The primary advantage of the iCasp9 system over the HSV-TK system is the remarkably rapid induction of apoptosis by AP20187, typically occurring within minutes. Due to this rapid response, recent studies have increasingly favored the utilization of iCasp9 over HSV-TK for CAR-T cell regulation. In this study, we aim to evaluate the elimination of iCasp9-IL15 CAR-T cells using photosensitizers AP20187 *in vitro*.

II. MATERIALS AND METHODS

1. Subjects

CAR-T cells were cultured in RPMI 1640 medium (Biowest), Fetal Bovine Serum (Hyclone), and CTS Optimizer T cell Expansion (Thermo Fisher). PBMCs were activated using Dynabeads Human T-Activator CD3/CD28 and recombinant human IL-2 (Peprotech), photosensitizer AP20187 (MedChemExpress), and Blasticidin S HCl (Thermo Fisher).

2. Methods

Optimization of blasticidin concentration for iCasp9-IL15 CAR-T cells screening

Following a 72-hour proliferation period, PBMCs were centrifuged at 200×g for 10 minutes to ensure the complete removal of magnetic beads in accordance with the manufacturer's instructions. The cells were subsequently washed twice with 1X PBS to eliminate residual beads. The resulting cell pellet was resuspended in a culture medium (RPMI supplemented with 10% FBS) at a density of 10^6 cells/mL. Fresh Dynabeads Human T-Activator CD3/CD28 were added at a 1:1 ratio, along with recombinant human IL-2 (50 U/mL), and the cell suspension was transferred to 6-well plates. Subsequently, blasticidin S HCl was added to each well at final concentrations of 0, 2.5, 5, 10, and 15 µg/mL. The cells were incubated for 5 days at 37°C in a humidified atmosphere containing 5% CO_2. Following the incubation period, cell viability was assessed to determine the optimal blasticidin concentration.

Collection and screening method for iCasp9-IL15 CAR-T cells

iCasp9-IL15 CAR-T cells, after expansion at a density of 10^6 cells/mL, were transferred to culture flasks and supplemented with blasticidin at an optimized final concentration. Dynabeads Human T-Activator CD3/CD28 and IL-2 (50 U/mL) were added to sustain cell proliferation. After 5 days, all cells were harvested, and viability was assessed using an automated cell counter following the manufacturer's protocol, including: Mix 10 µL of the cell suspension with 10 µL of 0.4% Trypan Blue solution in a 1:1 ratio. This mixture was gently pipetted up and down to ensure proper mixing and incubated at room temperature for 2–3 minutes. The stained cell mixture of 10 µL was loaded into a chamber of the Countess II FL slide. Subsequently, the slide was inserted into the Countess II FL to analyze and record results (total cell count, viable cell count and dead cell count).

Activation of the iCasp9 suicide gene by AP20187

Purified iCasp9-IL15 CAR-T cells were washed with 1X PBS, resuspended in fresh culture medium at a density of 10^6 cells/mL, and seeded into the wells of a 6-well culture plate. AP20187 was then added at final concentrations of 5nM, 10nM, and 20nM. After 12 hours, cells were harvested, and viability was assessed using an automated cell counter.

Statistical analysis

Excel and GraphPad Prism v9.0 software were used to graph and analyze data. Variables were expressed as quantity (n) and percentage (%).

3. Research ethics

This study was approved by the Ethics Committee of Military Hospital 103, Hanoi, Vietnam (Grant number: 180/2021/CNChT-HĐĐĐ, August 10th, 2021). Written consent to participate in this study was obtained through a direct survey and collected by researchers before the study procedure.

III. RESULTS

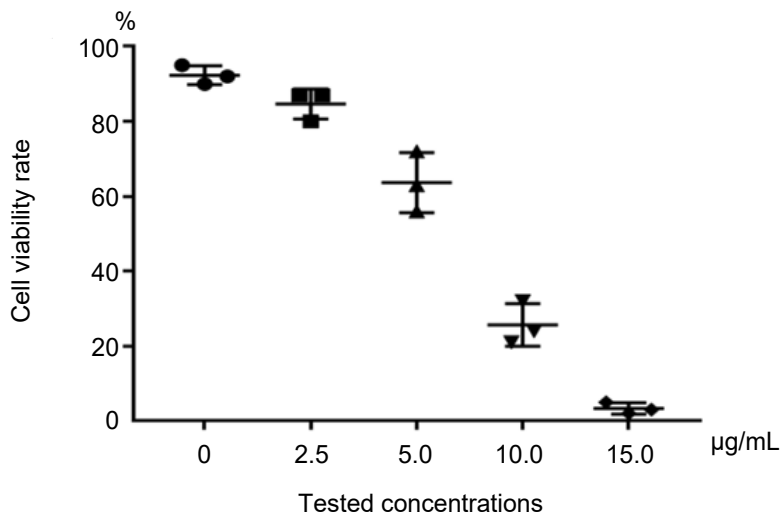

Chart 1. Optimization of blasticidin concentration for iCasp9-IL15 CAR-T cells screening

The selection of iCasp9-IL15 CAR-T cells was performed using Blasticidin S HCl. In this study, blasticidin was applied at concentrations of 2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 15 µg/mL. Following a 5-day incubation period, the results indicated that activated, non-transduced PBMCs exhibited a mortality rate exceeding 95% when exposed to blasticidin at a concentration of 15.0 µg/mL. Therefore, this concentration was selected for the subsequent screening of iCasp9-IL15 CAR-T cells (Chart 1).

Following a 5-day screening period with the selected blasticidin concentration, the iCasp9-IL15 cells demonstrated a viability rate of approximately 75% (Chart 2).

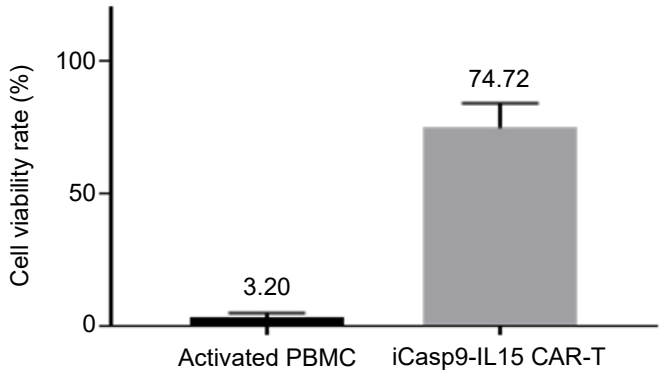

Chart 2. Screening of iCasp9-IL15 CAR-T cells using 15 µg/mL blasticidin

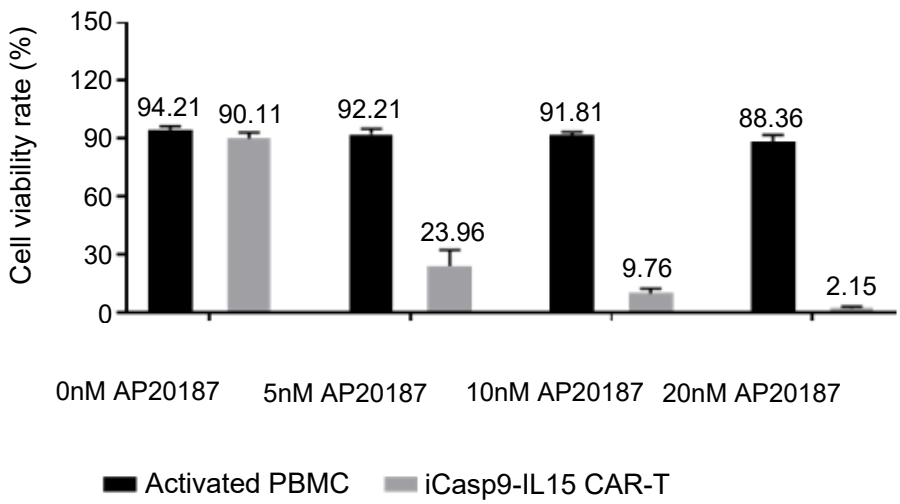

Chart 3. Activation of the iCasp9 suicide gene to eliminate iCasp9-IL15 CAR-T cells using AP20187

This study evaluated the inducible activation of the iCasp9 suicide gene using the chemical dimerization inducer AP20187 at concentrations of 5nM, 10nM, and 20nM. Results demonstrated that AP20187 concentrations of 10nM or higher effectively activated the iCasp9 suicide gene, resulting in the elimination of more than 90% of iCasp9-IL15 CAR-T cells. However, a concentration of 20nM AP20187 was required to achieve near-complete eradication, with residual cell viability of only 2.14% observed post-induction (*Chart* 3).

IV. DISCUSSION

Since the CAR-T iCasp9-IL15 construct contains a gene encoding Blasticidin S Deaminase, which confers resistance to the antibiotic blasticidin, this study utilized Blasticidin S HCl to selectively screen for iCasp9-IL15 CAR-T cells. Initially, the optimal antibiotic concentration for cell selection was determined using peripheral blood mononuclear cells (PBMCs) activated by Dynabeads Human T-Activator CD3/CD28. Based on previous studies, blasticidin concentrations of 2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 15 µg/mL were tested.[7,8] After a 5-day culture period, cell viability was evaluated using the Countess™ II Automated Cell Counter. The results showed that

activated, non-transduced PBMCs exhibited a mortality rate exceeding 95% after screening at a blasticidin concentration of 15.0 µg/mL (Chart 1). Therefore, this concentration was selected for screening iCasp9-IL15 CAR-T cells. These results align with prior studies indicating that mammalian cells lacking blasticidin resistance genes typically exhibit cell death at antibiotic concentrations between 2.5 and 10 µg/mL within a 10-day culture period.[6,7] Nevertheless, certain cell lines require higher blasticidin concentrations of up to 15 µg/mL.[9] Screening of iCasp9-IL15 CAR-T cells at the selected concentration resulted in approximately 75% cell viability (Chart 2).

After achieving a relatively homogeneous population of iCasp9-IL15 CAR-T cells through antibiotic selection, the present study evaluated the inducible activation of the iCasp9 suicide gene utilizing the chemical inducer of dimerization AP20187. This inducer has previously been reported to effectively activate iCasp9 at a concentration of 10nM.[10] Therefore, we investigated the activation of the iCasp9 gene in the selected iCasp9-IL15 CAR-T cell population by applying AP20187 at concentrations of 5, 10, and 20nM.

The results indicated that AP20187 at concentrations of 10 nM and above could activate the iCasp9 suicide gene, resulting in the elimination of over 90% of iCasp9-IL15 CAR-T cells (Chart 3). However, a concentration of 20 nM was required to achieve near-complete eradication, with post-induction cell viability of only 2.14%. These results suggest that AP20187 at 20 nM can be effectively used to inactivate iCasp9-IL15 CAR-T cells in scenarios of severe treatment-related toxicity. Previous studies have demonstrated that the iCasp9 suicide gene can be activated rapidly within 30 minutes upon administration of a dimerization inducer.[11] The optimal concentration of the inducer identified in this study is higher than that reported in several earlier studies.[11,12] This variation may be attributed to differences in promoter selection or the number of transgenes linked via 2A peptide sequences driven by a single promoter construct.[13]

V. CONCLUSION

The results demonstrate that iCasp9-IL15 CAR-T cells can be efficiently and almost entirely ablated using the dimerization inducer AP20187 at a concentration of 20, providing an effective safety mechanism in cases of severe treatment-related toxicity.

Acknowledgements

We acknowledge the assistance and advice of the following in the study process: Department of Pathophysiology and Oncology Center, Military Hospital 103, Vietnam Military Medical University.

Conflicts of interest

All authors have no conflicts of interest to declare.

REFERENCES

1. Davila ML, Riviere I, Wang X, et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med*. 2014 Feb 19;6(224):224ra25.

2. June CH, Sadelain M. Chimeric antigen receptor therapy. *New England Journal of Medicine*. 2018;379(1):64-73.

3. Bangolo A, Amoozgar B, Mansour C, et al. Comprehensive Review of Early and Late Toxicities in CAR T-Cell Therapy and Bispecific Antibody Treatments for Hematologic Malignancies. *Cancers (Basel)*. 2025;17(2):282.

4. Teachey DT, Lacey SF, Shaw PA, et al. Identification of predictive biomarkers for cytokine release syndrome after chimeric

antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Cancer Discov*. 2016;6(6):664-679.

5. Hoyos V, Savoldo B, Quintarelli C, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia*. 2010;24(6):1160-1170.

6. Bento FM, Takeshita D, Sacramento CB, et al. Over expression of the selectable marker blasticidin S deaminase gene is toxic to human keratinocytes and murine BALB/MK cells. *BMC Biotechnol*. 2004;4:1-10.

7. Buck JM, Bártulos CR, Gruber A, et al. Blasticidin-S deaminase, a new selection marker for genetic transformation of the diatom Phaeodactylum tricornutum. *PeerJ*. 2018;6:e5884.

8. Asoshina M, Myo G, Tada N, et al. Targeted amplification of a sequence of interest in artificial chromosome in mammalian cells. *Nucleic Acids Res*. 2019;47(11):5998-6006.

9. Hanswillemenke A, Stafforst T. Protocols for the generation of caged guideRNAs for light-triggered RNA-targeting with SNAP-ADARs. *Methods in Enzymology*. Elsevier; 2019:47-68.

10. Zhou X, Di Stasi A, Brenner MKJGToSCM. iCaspase 9 suicide gene system. *Methods Mol Biol*. 2015:87-105.

11. Gargett T, Brown MPJFip. The inducible caspase-9 suicide gene system as a “safety switch” to limit on-target, off-tumor toxicities of chimeric antigen receptor T cells. *Front Pharmacol*. 2014;5:235.

12. Yu S, Yi M, Qin S, et al. Next generation chimeric antigen receptor T cells: safety strategies to overcome toxicity. *Mol Cancer*. 2019;18:1-13.

13. Liu Z, Chen O, Wall JBJ, et al. Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. *Sci Rep*. 2017;7(1):2193.