OPTIMIZATION OF A SUICIDE GENE VECTOR FOR THE GEN2 CANCER IMMUNOTHERAPY CLINICAL TRIAL

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ABSTRACT

The optimization of a suicide gene which serves to unmask neoantigens within tumor cells and generate immune activation was undertaken using a series of criteria focused on more effective distribution of the suicide gene within the tumor cells and better selectivity for the prodrug, ganciclovir (GCV).

GEN2 is a highly engineered non-replicating Moloney Murine Leukemia Viral (MLV) vector encoding for a modified Herpes Simplex Virus Thymidine Kinase (HSV-TK) suicide gene. HSV-TK is an enzyme that locally converts an orally administered prodrug activator (valganciclovir) into a toxic cellular metabolite that ultimately induces cell death in transduced and nearby cancer cells, release of neoantigens into the tumor microenvironment and activation of local and system immune responses. These responses include increased uptake of tumor cell antigens by dendritic cells, maturation of various other antigen presenting cells for enhanced antigen capture, more cross-priming of T cells, and increased tumor infiltration by, and activation of, cytotoxic T cells.

In order to enhance the cell killing activity of HSV-TK, three strategies were used.

1. The distribution of HSV-TK was enhanced in the cytosol of the targeted cancer cells by:
   a. modification of nuclear localization signal (NLS) which results in uniform distribution of HSV-TK in the nucleus and cytosol;
   b. insertion of a nuclear export signal (NES) at the 5' terminus of the HSV-TK sequence to relocate HSV-TK into the cytoplasm.

Distribution of the HSV-TK enzyme to the cytosol enhances the cellular efficiency of conversion of the GCV to its active toxic metabolite and also the availability of this metabolite to affect surrounding cancer cells through bystander effect.

2. Increasing selectivity of HSV-TK for GCV over thymidine was achieved through the mutation of the substrate binding domain.
3. The optimization of the coding sequence for the HSV-TK was achieved through:
   a. codon optimization to remove rare and low use codons to improve HSV-TK protein expression;
   b. lowered GC content within the newly codon optimized gene to avoid gene synthesis and other gene expression problems;
   c. removal of known splice acceptor and splice donor sequences within the optimized gene along removal of polypyrimidine tracts that may be involved in splicing;
   d. to further optimize expression of HSV-TK protein, a strong Kozak translation initiation sequence was introduced in front of the start codon while removing possible Kozak sequences within HSV-TK open reading frame.

Overall, the combination of all modifications renders GEN2 more potent in cancer cell killing compared to all other vectors tested.

GEN2 (HSV-TK-GMCSF) is currently being evaluated in a Phase I clinical trial (NCT04313868).

INTRODUCTION

GEN2 is a replication-incompetent therapeutic gene transfer vector, with an amphotropic envelope protein that binds to the PiT-2 receptor and possesses high transduction gene transfer activity. The core of the vector contains an RNA genome which codes for an optimized mutant HSV-TK gene which when transduced into a tumor cell renders it sensitive to the lethal effects of the pro-drug GCV. HSV-TK phosphorylates GCV to activate the pro-drug allowing it to eventually incorporate into the host cell DNA to act as a chain terminator of DNA replication and trigger apoptotic cancer cell death. Tumor cells without HSV-TK adjacent to those transduced may be affected i.e. killed due to the transfer of the toxic forms of GCV into those cells through gap junctions, a process known as the bystander effect. Various mutants were developed to ascertain the one with the optimal profile of localization, expression, and selectivity to the prodrug. Tumor bearing animal experiments were undertaken to investigate the effect of the mutant HSV-TK/VCGV on tumor eradication and to determine the protection against tumor re-challenge *in vivo* as well as the ability of harvested splenocytes to kill tumor cells *in vitro*. The vector genome also codes for the human GM-CSF gene which when transduced into a cancer cell causes the cell to produce and secrete GM-CSF. The local expression of GM-CSF may augment an immunotherapeutic effect by enhancing tumor associated antigens (TAA) presentation to the local immune effector cells. The vector is manufactured at high active titers with a low impurity profile and is being evaluated in a Phase I clinical trial.

METHODS AND MATERIALS

Methods and materials are briefly described in the figure legends.

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GenVivo Inc. is a clinical-stage gene therapy company headquartered in San Marino, CA, currently advancing a cancer immunotherapy enveloped vector product candidate.

RESULTS

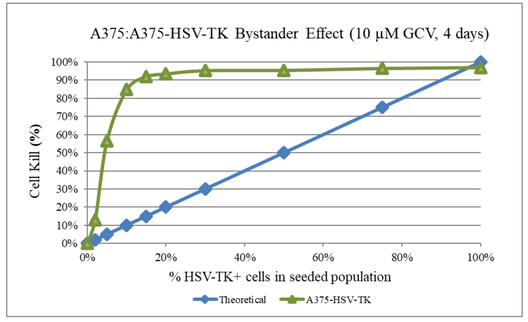
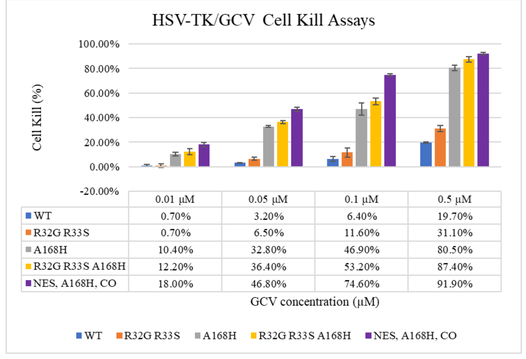


Figure 1. Bystander effect of HSV-TK/GCV on A375 cell kill activity.

The bystander assay cell kill curve was demonstrated with an A375 human melanoma parental cell line mixed with an A375/HSV-TK expressing clonal cell line by the PrestoBlue cell viability assay. The HSV-TK cell line was singly cloned after the drug selection. Cell mixtures of increasing HSV-TK cell percentages were exposed to 10 μM GCV for 4 days and percentages of the cell kill graphed accordingly. The theoretical line is the expected cell kill to mix of cells based on killing only the TK containing cells, without any further killing of the non-TK expressing cells if no bystander effect occurs. The results show a highly effective bystander activity in A375 cells with HSV-TK (NES, A168H, CO).



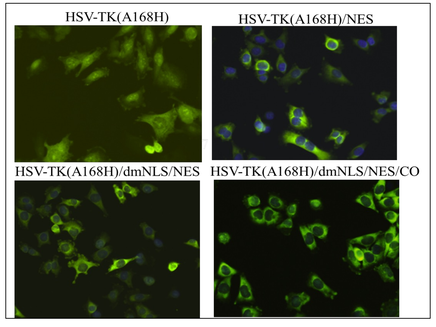
	0.01 μM	0.05 μM	0.1 μM	0.5 μM
WT	0.70%	3.20%	6.40%	19.70%
R32G R33S	0.70%	6.50%	11.60%	31.10%
A168H	10.40%	32.80%	46.90%	80.50%
R32G R33S A168H	12.20%	36.40%	53.20%	87.40%
NES, A168H, CO	18.00%	46.80%	74.60%	91.90%

Figure 2. Cell killing activity of various mutant HSV-TK constructs.

Cell kill activities were measured by PrestoBlue assay at different concentrations of GCV treatment for 3 days in A375 human melanoma cells after cells were transfected with various HSV-TK mutant constructs as indicated. The results show an increased cell kill activity with each modification.

Figure 3. Subcellular localization of wildtype HSV-TK and NLS/A168H mutant.

A375 human melanoma cells were visualized under a fluorescent microscope after cells were transduced with the vector expressing either a wildtype HSV-TK fused with GFP or the NLS/A168H mutant fused with RFP as indicated. The results show the redistribution of the HSV-TK mutant to include in the cytoplasm vs. wildtype distribution limited to the nucleus.



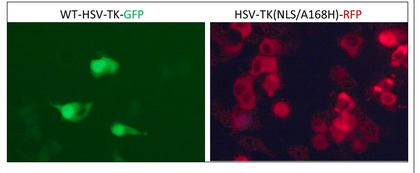


Figure 4. Subcellular localization of various HSV-TK mutants.

A375 human melanoma cells were visualized under a fluorescent microscope after cells were transduced with the vectors expressing different HSV-TK mutant constructs as indicated. Transduced cells were stained for HSV-TK with FITC labelled anti-HSV-TK antibody. The results show that all mutations except for A168H led to the localization of the HSV-TK in the cytoplasm.

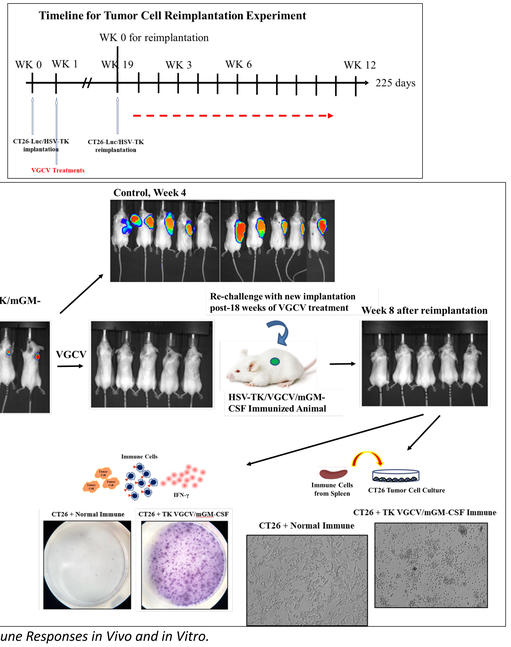


Figure 5. Anti-Tumor Immune Responses in Vivo and in Vitro.

CT26-Luciferase(Luc)-cells, stably expressing luciferase, enhanced mutant HSV-TK and mGM-CSF (mouse GM-CSF) genes were subcutaneously implanted in BALB/c mice and grown for 1 week before VGCV treatment given for three consecutive days using oral gavage at a concentration of 2 mg/mL and administered at 5 mL/kg body weight. Imaging of implanted tumors were visualized by an IVIS Imaging System. VGCV treatment caused tumor regression and significantly prolonged the survival of those animals compared to the control animals without VGCV treatment. When the immunized animals reached 18 weeks post-VGCV treatment, they were rechallenged with new tumor cells (CT26-Luc/HSV-TK). The immunized animals rejected the re-implanted tumor and survived an additional 12 weeks until the end of the experiment. Splenocytes isolated at 8 weeks from spleens of the immunized animals were co-incubated with naïve CT26 cells and showed anti-tumor responses in vitro by killing naïve CT26 cells and demonstrated increased IFN-γ production as shown by the ELISpot assay compared to splenocytes from untreated animals.

CONCLUSIONS

The selected enhanced mutant HSV-TK suicide gene was optimized at several levels, demonstrating localization of the protein into the cytoplasm and increased levels of cell killing activity and bystander effect activity. CT26 mouse colorectal tumors transduced with the enhanced mutant HSV-TK and mGM-CSF genes regressed after the addition of VGCV. No tumor was visible after 18 weeks of VGCV compared to control animals who developed tumors by 4 weeks and required euthanasia. Moreover, re-challenge of these animals post 18 weeks of VGCV with CT26 cells resulted in a failure to implant with no subsequent development of tumor. Splenocytes derived from these animals 8 weeks after the attempted reimplantation killed naïve CT26 cells in culture with a significant ELISpot response to interferon-γ. The *in vivo* and *in vitro* data indicate that an optimized suicide gene along with a gene encoding GM-CSF can stimulate immune responses directed against the tumor and lead to tumor eradication and a durable immunological response against a tumor.

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