A novel immunotherapy for the treatment of HPV+ tumors based on a chimeric Virus-like Drug Conjugate

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ABSTRACT

Introduction: HPV virus-like particles (VLPs) bind a wide variety of tumor types via modified tumor-associated glycosaminoglycans (GAGs) found on the tumor cell surface. This finding led to the development of the virus-like drug conjugate (VDC) belzupacap sarotalocan (bel-sar), an HPV-derived VLP conjugated to a light-activated cytotoxic payload. When activated by near-infrared light, this VDC has been shown to induce rapid tumor cell necrosis resulting in pro-immunogenic cell death, release of tumor neoantigens and long-term anti-tumor immunity. When HPV16 E6 and E7 expressing TC-1 mouse tumors are treated with bel-sar, we detect E7-specific T-cells in the absence of provided tumor antigens. We are now developing a novel chimeric VDC (cVDC) in which E6 and E7 are fused to the L2 capsid protein as a means to further enhance this anti-tumor response. This cVDC could allow for the targeted cytotoxicity of HPV+ tumors in addition to the release of supplemental tumor antigens E6 and E7 within the now pro-immunogenic tumor milieu potentially leading to a long-term anti-tumor response.

Methods: The detoxified sequences of E6 and E7 were engineered as one fusion polypeptide on the C-terminus of L2. Both L2-E6-E7 and L2-E7-E6 protein expression vectors were generated to determine if the order of the proteins impacted L2's ability to co-assemble with L1. The plasmids were co-expressed alongside L1 using the mammalian 293TT expression system. The chimeric VLPs (cVLPs) were conjugated with the light-activated payload and their binding and potency were measured using HPV16+ human tumor cell lines.

Results: Both the L2-E6-E7 and L2-E7-E6 fusion proteins were expressed and co-assembled with L1 into cVLPs. Fusion protein expression was validated by western blots for L2, E6 and E7, and VLPs were confirmed by electron microscopy. cVDCs were capable of binding and killing HPV16+ tumor cells in vitro. Binding and potency were comparable to wild-type L2-containing VDCs and they retained their tumor-associated GAG targeting specificity.

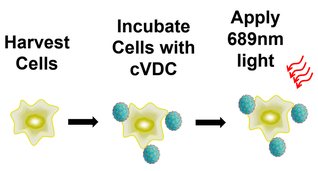
Conclusions: Preliminary data indicate that cVDCs containing E6 and E7 can successfully be generated using the 293TT mammalian expression system. Studies evaluating the cytotoxicity and E6 and E7 immunogenicity of the cVDCs, as well as the impact on tumor targeting, are underway using the TC-1 murine tumor model.

STUDY GOAL

Generate near infrared (NIR) dye conjugated chimeric VLPs (cVDC) containing capsid protein L1 and capsid protein L2 expressed as a fusion polypeptide with the HPV16 "detoxified" tumor antigens E6 and E7 (rendered no longer oncogenic). The goal is to use the cVDC as a means of delivering the tumor antigens, E6 and E7, to HPV16+ tumors to further enhance the anti-tumor immunity generated by cVDC binding and light activation.

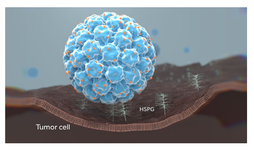
METHODS

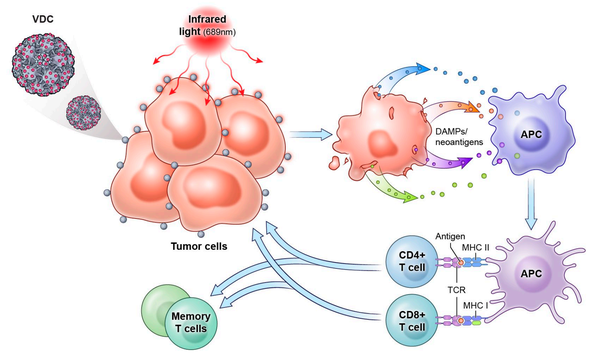
- Generate detoxified polypeptide sequences of L2-E6-E7 and L2-E7-E6 and confirm that, when co-expressed with L1, VLPs can form, and the full fusion polypeptide is incorporated within the chimeric VLP (cVLP).
- Conjugate the cVLP with a NIR photoactivatable dye and ensure tumor binding specificity and cytotoxicity is retained.
- *In vitro* binding and cytotoxicity of cVDCs was assessed using a panel of HPV16+ human cancer cell lines and TC-1, a murine tumor line expressing HPV16 E6 and E7. Binding and potency EC_{50} values were generated and tumor-associated modified GAG targeting was assessed by inclusion of heparin in the binding assay.



BACKGROUND

- Human papillomavirus virus-like particles preferentially target tumor cells via specifically modified glycosaminoglycans on the tumor cell surface.[1]
- Belzupacan saratocalan (bel-sar) is an investigational virus-like drug conjugate composed of a modified HPV VLP and a NIR activatable small molecule.[2]
- Upon activation with NIR light, bel-sar has been shown to cause acute tumor cytotoxicity *in vitro* and *in vivo.*
- Bel-sar mediated tumor cell death has been shown to be highly inflammatory and leads to an upregulation of markers of immunogenic cell death. Strong anti-tumor immunity against tumor neoantigens (e.g., E6, E7) is capable of generating long-term protection from tumor rechallenge.[2,3]



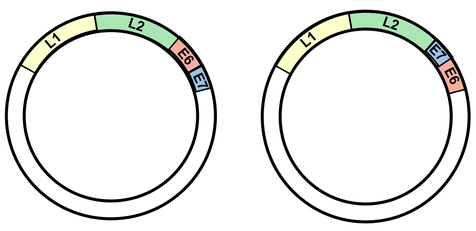


RESULTS

1. Cloning strategy:

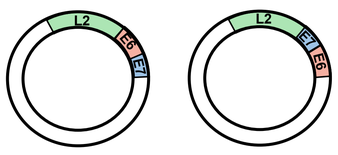
- Use full length HPV16L2 (L2wt) and remove stop codon.
- Add detoxified E6E7 or E7E6 to the C-terminus of L2.
- The HPV16 E6 and E7 genes were codon optimized[4,5] and the following mutations/deletions were made for the purposes of rendering the oncoproteins inert (detoxified, dx)[6]:
  - 16dxE6 **C63G** and **C106G** (two zinc finger domains can't degrade p53, prevents immortalization; prevents telomerase activation); C-terminal 5 residues deleted (Δ147-151; PDZ domain).
  - 16dxE7 **C24G** and **E26G** (Rb binding) and **C91G** (Zinc finger/immortalization/HDAC, c-jun, BRA1).
- Bicistronic (L1/L2) and L2 only constructs were generated

Bicistronic (L1/L2)



L2 only

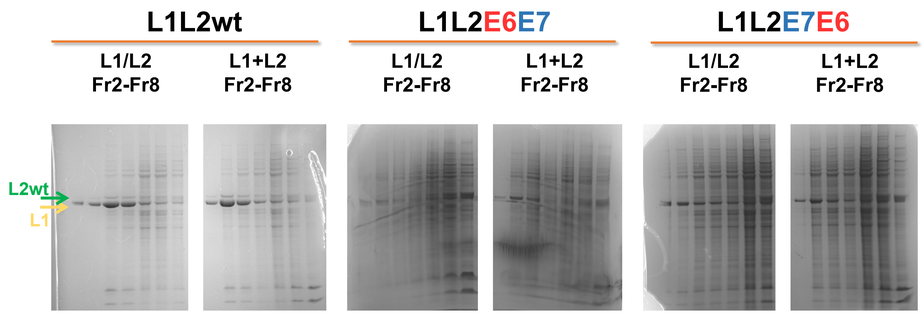
allows for adjusting L1:L2 ratio with L1 plasmid co-transfection



2. Protein expression and characterization:

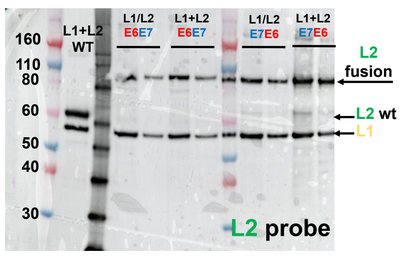
- 293TT cells were transfected with bicistronic plasmids (L1/L2) or L1 and L2 plasmids (L1+L2).
- Cell lysates were purified over density gradient and the collected fractions were screened by SDS-PAGE
- Samples were tested by western blot to determine if E6 and E7 were expressed and incorporated in the cVLP.
- Electron microscopy to verify VLP formation

SDS-PAGE to screen density gradient fractions



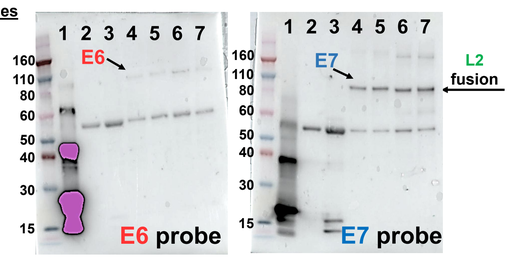
Western blots of fractions 3 and 5 to detect L2, E6 and E7 proteins

L2 – 55 kDa (runs ~60-70kDa)
Fusion protein – 85 kDa (~90-110 kDa)

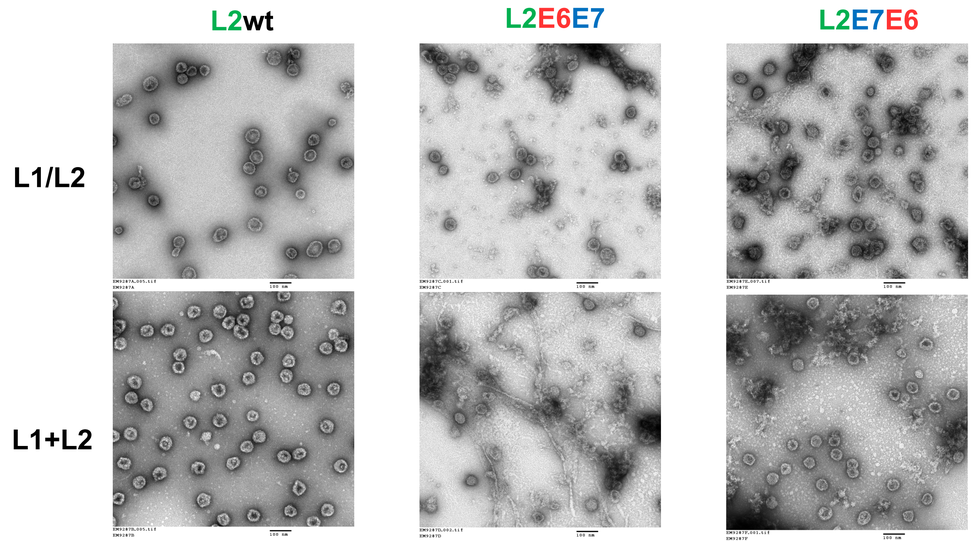


E6 – 19 kDa
E7 – 11 kDa (runs ~14-20kDa)

E6 and E7 blots lanes
1. E6 or E7 protein
2. L1/L2 wt
3. L1+L2 wt
4. L1/L2 E6E7
5. 5. L1+L2 E6E7
6. 6. L1/L2 E7E6
7. 7. L1+L2 E7E6

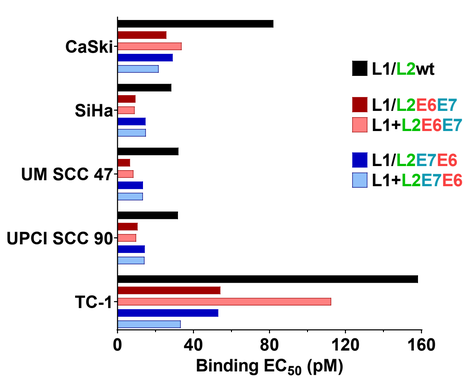


Electron microcopy confirming VLP formation

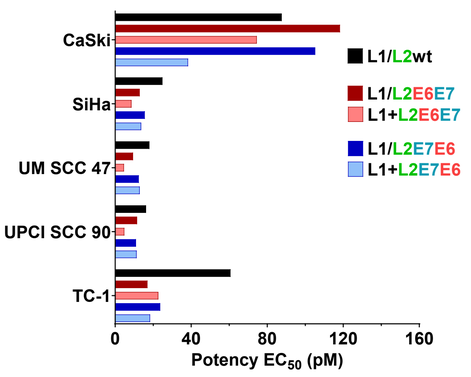


3. Binding and cytotoxicity of cVDCs

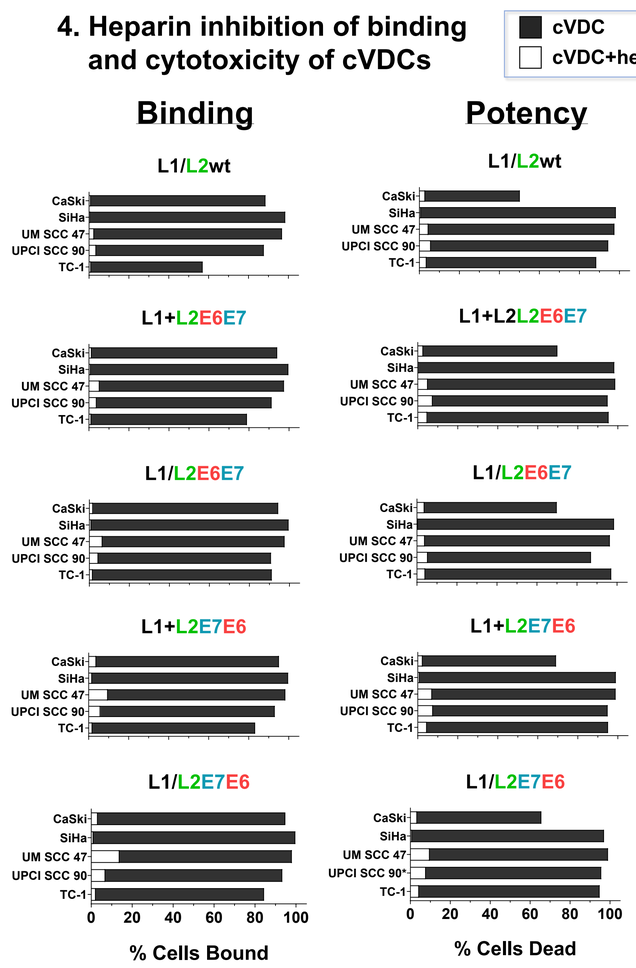
EC_{50} of cVDC binding to HPV16+ tumor cell lines



EC_{50} of cVDC potency after NIR activation on HPV16+ tumor cell lines



4. Heparin inhibition of binding and cytotoxicity of cVDCs



CONCLUSIONS

- Virus-like particles containing the fusion polypeptides of L2-E6-E7 and L2-E7-E6 could successfully be generated using the 293TT mammalian cell expression system.
- The presence of the fusion polypeptides was confirmed by western blot and the VLPs were visualized using electron microscopy.
- The chimeric VLPs were successfully labeled with the NIR photoactivatable molecule and were comparable to L2wt VLPs.
- The chimeric virus-like drug conjugates (cVDCs) bound a panel of HPV16+ human tumor cell lines, as well as the E6/E7+ murine tumor line, TC-1. Upon activation with NIR light, the cVDCs demonstrated comparable potency to the L2wt VDC.
- Binding and potency of the cVDCs was heparan sulfate dependent, as both were inhibited by heparin indicating that, 1) incorporation of the polypeptide L2-E6-E7 or L2-E7-E6 into the VLP and 2) labeling with the photosensitizer did not alter the tumor-associated modified GAG tropism of the VLP.

FUTURE DIRECTIONS

- Estimate L2 occupancy in the cVLP preparations and alter the ratio of L1 and L2 plasmid in order to achieve full occupancy of the cVLP.
- Examine immunogenicity of the cVLPs and cVDCs *in vivo* to determine if they are able to generate E6 and E7 immune responses
- Use the cVDCs to treat TC-1 tumors *in vivo* and compare their efficacy to the L2wt VDCs to determine if the delivery of tumor antigens, E6 and E7, improves overall survival, enhanced E6 and E7 T-cell responses, and long-term tumor protection.

REFERENCES

1. Kines, R. and Cerio, R., et al. *Int. J Cancer*, 138(4):901-11, 2016.
2. Kines, R., et al. *Mol Cancer Ther*, 17(2):565-574, 2018.
3. Kines, R., et al. *Can Immunol Res* 9(6):693-706, 2021.
4. Pastrana, D., et al. *Virology* 321(2):205-16, 2004.
5. https://github.com/BUCK-LCO-NCI/Codmod_as_different_as_possible
6. Peng, S., et al. *Mbio* 12(1): e03224-20, 2021.