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Antiviral Efficacy of Nitric Oxide-Releasing Drug Candidates In Vivo Utilizing the Cottontail Rabbit Papillomavirus Model

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ABSTRACT

Background: Human papillomaviruses (HPVs) are epitheliotropic DNA viruses that infect either mucosal or cutaneous epithelium and may lead to the development of warts and other benign lesions. Certain strains have a higher propensity to progress to malignancies of infected tissues types including cancer of the cervix, vagina, penis, anus, and oral cavities. Nitric oxide (NO), a key component of the host immune response, has previously been demonstrated to inhibit the replication of several viruses (e.g. HSV, HIV) but the lack of pharmaceutically acceptable drug products has prevented NO's clinical utility. Novan has developed a novel approach for the treatment of HPV infection by stably storing the gaseous species as an engineered macromolecule that can be applied to the skin as a topical product allowing for controlled release of NO. **Methods:** In a Cottontail Rabbit Papillomavirus (CRPV) model, several NO-releasing drug candidates were evaluated to determine the effect of release rate on the inhibition of papilloma formation after inoculation of CRPV (wild-type and E8 mutant) DNA into scarified wounds on the back of rabbits. Two weeks following viral inoculation, topical treatment was initiated with daily treatment occurring five times per week for five weeks. **Results:** Results of the study demonstrated near complete inhibition (85%) of wild-type papilloma formation with NVN1000, a drug delivering a high, rapid burst of NO. Sites inoculated with E8 mutant DNA developing slower-growing papillomas were completely inhibited with NVN1000 (100%) compared to vehicle and untreated controls. **Conclusions:** The complete cessation of wart growth upon topical application suggests direct anti-viral activity. Furthermore, in this model we demonstrated that the modulation of NO dose and release rate, collectively influence anti-viral efficacy. Lower concentrations of drug and formulations with slower NO release rates showed limited efficacy. The data presented herein demonstrate for the first time inhibition of papilloma growth in vivo following topical NO treatment in an animal model. This novel treatment approach may be beneficial for the treatment of extra genital warts and may reduce the incidence of HPV associated cancers.

MATERIALS & METHODS

Inoculation of rabbits with CRPV viral DNA: The study was reviewed and approved by the Penn State, College of Medicine IACUC, and the PSU Biological and Recombinant DNA Committee. Rabbits were inoculated at 4 back sites with CRPV viral DNA using the delayed scarification protocol (Cladel, 2008). Upper back sites were inoculated with wild-type CRPV DNA and two lower back sites were inoculated with E8 knockout mutant DNA. The E8 knockout mutant genome develops papillomas that are substantially smaller and slower-growing which better mimic the clinical tumor mass and size of human warts. Papillomas grown from wild-type inoculated sites can reach a diameter of 15-20 cm in 8-10 weeks (Hu, 2002) and propose a significant challenge to antiviral treatment.

Treatments: Topical treatments were performed daily for 5 days per week by applying compound to only the papilloma sites established on the left side. Skin tattoo spots next to the sites of infection were used as guides to locate the initial site of viral infection for early treatments and to positionally identify sites of cure. Dual chamber pumps containing active pharmaceutical ingredient (NVN1000 or NVN4428) and hydrogel (Figure 1) were depressed a single time to deliver 300 µl of admixture to a sterile weigh boat. The two phases were then combined by brief mixing with a sterile spatula. The admixture was then divided into two equal parts and transferred to the two papilloma sites on the left side of the animal. Treatments began at week two following inoculation of viral DNA and continued for a five week duration.

Additional Observations: The papillomas were measured weekly in three axes (length, width, height) in order to determine the geometric mean diameter. Papillomas were additionally measured 1 week following the end of treatment. The body weights of all animals were recorded weekly in order to determine that topical treatment did not adversely affect the overall health of treated animals. Additionally, photographic images of the animals backs were taken weekly to visually follow the efficacy of treatment over the timecourse of treatment.

Statistical Analyses: Students *t*-test were performed on mean papilloma sizes for treated versus untreated sites for both wild-type CRPV-infected and E8 knockout mutant-infected sites at weekly timepoints to determine whether significant ($p<0.05$) differences in papilloma size were observed.

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Figure 1. Composition of Drug Candidates.

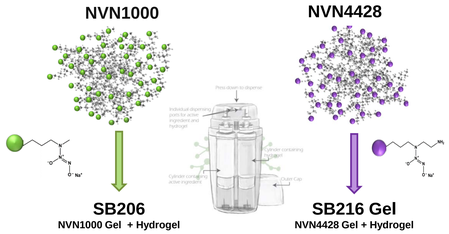
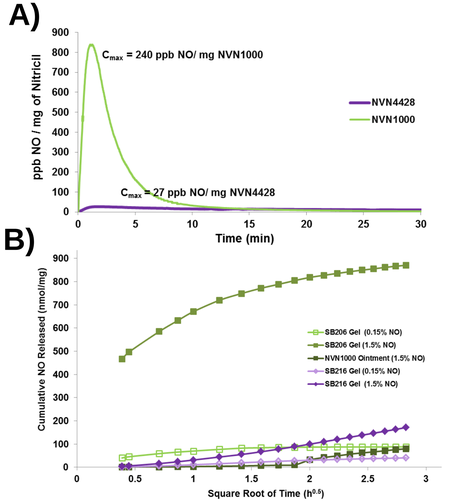
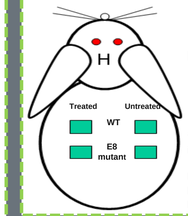


Figure 2. In vitro Nitric Oxide Release Profiles of (A) Drug Substance in PBS (37 °C, pH 7.4) and (B) Drug Candidates following admixture at 32°C.



RESULTS

Figure 3. Experimental Design of CRPV Study. Each treatment group consisted of n = 4 animals.



Day -3: Rabbits are scarified
Day 0: Rabbits receive viral DNA inoculation*
Days1-14: Virus infects and replicates
Day 15: Treatment Begins
Rabbits receive once daily treatments (5x/week)
Day 50: 5-week treatment regimen concludes

*both WT virus and mutant virus (E8 knockout) will be inoculated into scarified sites.

E8 knockout virus infection generates smaller, slower-growing papillomas that are thought to be more clinically similar to human papillomavirus infections.

Figure 4. Efficacy of Nitric Oxide Drug Candidates Against Wild-type CRPV. Animals were treated with SB206 Gel (A) or SB216 Gel (B). Papillomas were measured in three axes (length, width, height) and the geometric mean diameter was calculated. The mean ± SEM for each treatment group is plotted.

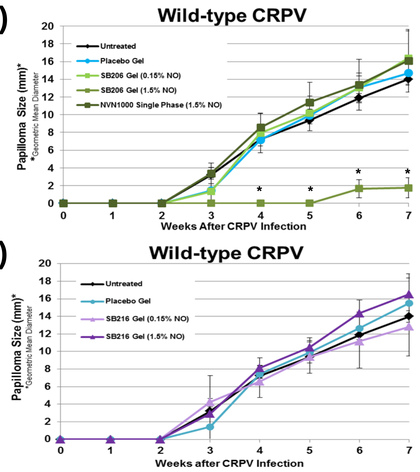


Figure 5. Efficacy of Nitric Oxide Drug Candidates Against E8 Mutant CRPV. The mean ± SEM for each treatment group is plotted.

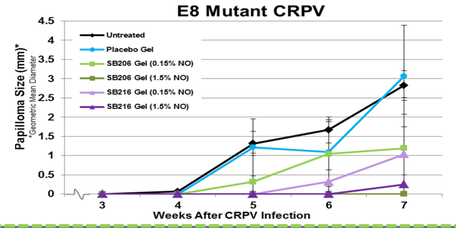


Table 1. Number of Animals Demonstrating Complete Cessation of Papilloma Growth. Papillomas were measured weekly during the course of treatment and one week following treatment completion. The number of animals per treatment group demonstrating clearance of palpable papillomas is reported.

WT	Week 7 Measurement (Last Treatment)	Week 8 Measurement (1 wk post-dose)
SB206 Gel (0.15% NO)	0/4	0/4
SB206 Gel (1.5% NO)	2/4	1/4
SB216 Gel (0.15% NO)	0/4	0/4
SB216 Gel (1.5% NO)	0/4	0/4
Mutant	**Week 7 Measurement (Last Treatment)**	**Week 8 Measurement (1 wk post-dose)**
SB206 Gel (0.15% NO)	1/4	0/4
SB206 Gel (1.5% NO)	**4/4**	**4/4**
SB216 Gel (0.15% NO)	3/4	2/4
SB216 Gel (1.5% NO)	3/4	3/4

REFERENCES

Cladel, N., Hu, J., Balogh, K., Mejia, A., and Neil Christensen. 2008. Wounding Prior to Challenge Substantially Improves Infectivity of Cottontail Rabbit Papillomavirus and Allows for Standardization of Infection. *J. Virol. Methods*. 148(1-2):34-39.

Hu, J., Han, R., Cladel, N., Pickel, M., and Neil Christensen. 2002. Intracutaneous DNA vaccination with the E8 gene of cottontail rabbit papillomavirus induces protective immunity against virus challenge in rabbits. *J. Virol.* 76:6453-6459.

Figure 6. Representative Images from CRPV Study. WT CRPV-infected sites 1 week post-dosing with Placebo Gel (**A**), high dose SB216 Gel (1.55% NO) (**B**), and high dose SB206 Gel (1.5% NO) (**C**).

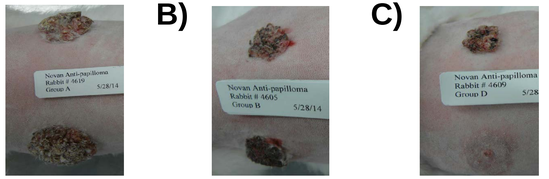
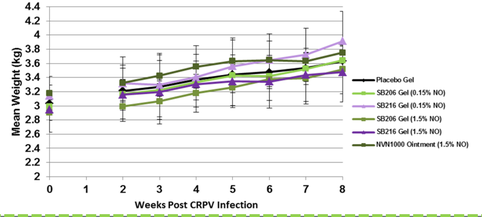


Figure 7. Weekly Body Weights of All Animals in Treatment Groups. The mean ± SEM for each treatment group is plotted.



CONCLUSIONS

- All topical treatments, regardless of formulation or dosage, were well tolerated by all animals in this study.
- The results of this study demonstrated in vivo inhibition of papilloma growth in a well characterized (CRPV) animal model.
  - Statistically significant inhibition of both WT (88%) and E8 mutant (100%) papilloma growth at the end of treatment with SB206 (1.5% NO)
  - Inhibition of E8 papilloma growth at the end of treatment with SB216 (1.5% NO) (93% inhibition) and SB216 (0.15% NO) (67% inhibition).
- Treatment with both fast and slow NO-releasing formulations could inhibit papilloma growth, however the faster release kinetics of SB206 were more efficacious, particularly against the robust WT strain.
- The complete cessation of papilloma growth at sites inoculated with E8 mutant CRPV DNA following topical treatment with SB206 Gel (1.5% NO) suggests direct antiviral activity against the more clinically relevant slower-growing papillomas generated by this mutant.