In Vitro and In Vivo Efficacy of Nitric Oxide-Releasing Antiviral Therapeutic Agents

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ABSTRACT

The efficacy of topical nitric oxide-releasing therapeutics to inhibit viral replication was demonstrated in vitro and in vivo. NVN1000 or NVN4000 drug substance, dissolved in phosphate buffered saline, was applied 1 hour/day for 6 days to organotypic cultures of primary human keratinocytes containing HPV-18 genomic replicons. Preliminary results indicate a dose responsive reduction of viral DNA copy number to less than 20% of the untreated cultures. At the highest concentrations applied (1.5 mg or 2 mg/ml), no cytotoxicity was detected, as judged from hematoxylin and eosin staining of normal or HPV-18 containing raft cultures. The NVN1000 drug substance was formulated into SB206 Gel and the ability of a daily topical drug product (5 doses/week for 8 weeks) to inhibit papilloma growth in vivo was assessed in the Cottontail Rabbit Papillomavirus (CRPV) model. Dosing of SB206 Gel was initiated two weeks after inoculation of CRPV viral DNA into the scarified skin sites. Topical SB206 Gel treatment inhibited papilloma formation in a dose dependent fashion with the highest dose, 10% SB206 Gel, achieving 73% inhibition compared to the Placebo control group. Blinded histological analysis of biopsies from 10% SB206-treated animals exhibited mild hyperplasias, with an absence of viral infection as evidenced by a lack of intra-nuclear basophilic inclusions. Qualitative assessment of inflammation and quantitative cytokine gene expression was similar across all dose groups, suggesting immune activation did not account for the efficacy observed in the CRPV model and supports a direct anti-viral effect of Novan's nitric oxide-releasing therapeutic agents. SB206 Gel is currently being evaluated as a topical therapy for external genital warts in a Phase 2 clinical trial.

MATERIALS & METHODS

Keratinocyte Raft Cultures: This study was performed by collaborators at the University of Alabama-Birmingham. Primary human keratinocytes were seeded onto a dermal equivalent and kept submerged for two days to establish cell monolayers. At day 0, the epithelial raft culture was lifted to the air:liquid interface. On days 7 through 12, 400 µl of NVN1000 or NVN4000 at varying concentrations (diluted in 50 mM PBS) were applied to the upper surface of the raft cultures and incubated for 1 hour. The solution was then gently aspirated from the surface of the rafts and the raft culture media was refreshed. Control cultures were treated with 50 mM PBS using the same protocol. Twelve hours prior to culture harvest on Day 13, all cultures were incubated with 100 µg/mL BrdU as a marker of cellular DNA synthesis. Raft cultures were then formalin-fixed and paraffin-embedded. Sections were probed with BrdU antibodies to determine patterns and intensity of DNA replication. HPV-18 copy number was determined by qPCR and was reported as a ratio to the copy number of control cultures treated with 50 mM PBS (set to 100%).

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 four back sites with CRPV viral DNA using the delayed scarification protocol. 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 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 drug product 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 SB206 Gel (NVN1000 Gel and hydrogel) 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 an eight week duration. **Additional Observations:** The papillomas were measured weekly in three axes (length, width, height) in order to determine the geometric mean diameter. 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. At the end of in-life, animals were sacrificed and the papilloma, or area where a papilloma should have developed, were bisected and two tissue samples were obtained. One tissue sample was stored in RNAlater for subsequent inflammatory cytokine analysis and the second tissue sample was fixed for blinded histological analysis by an independent investigator.

RESULTS

Figure 1. In vitro Nitric Oxide Release Profiles of Drug Substance (NVN1000 or NVN4000) in PBS (37 °C, pH 7.4) Nitric oxide-releasing particles can be designed with **A**) different instantaneous fluxes of nitric oxide while maintaining **B)** the same total amount of nitric oxide released over time.

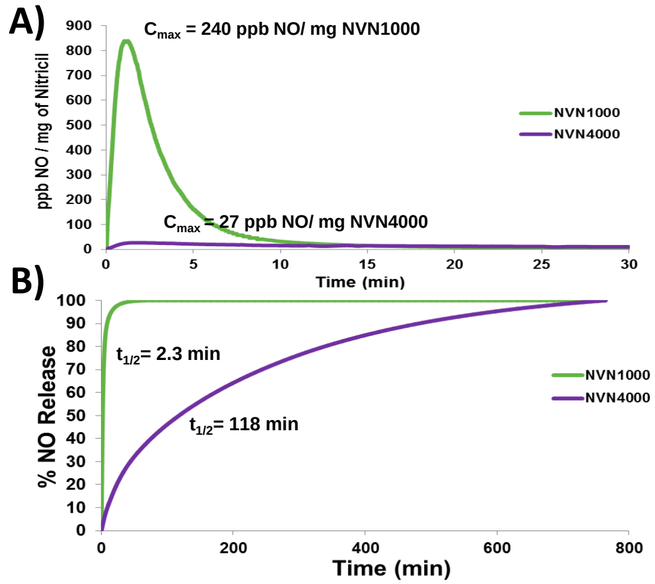


Figure 2. Dose Responsive Effect of Nitric Oxide Exposure on HPV-18 replication in the keratinocyte raft culture model. A) BrdU immunohistochemistry of raft cultures and **B)** HPV-18 viral DNA copy number via qPCR analysis following six days of daily 1 hr drug substance exposure.

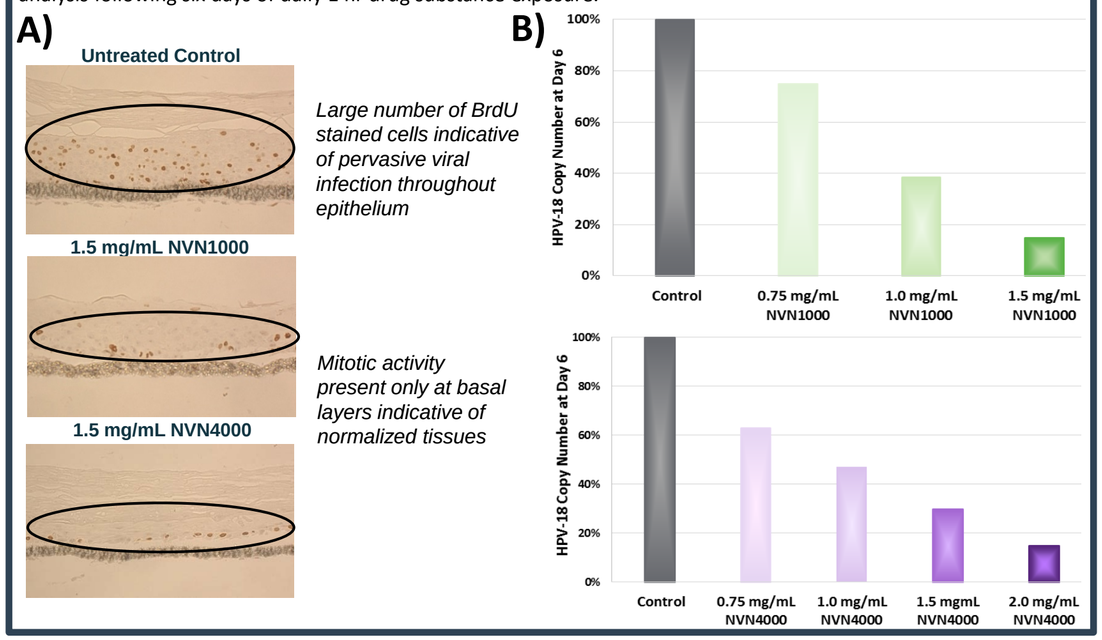
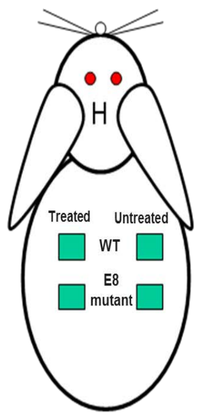


Figure 3. Experimental Design of the CRPV Study. Animals were inoculated with both wild-type (WT) and E8 mutant (E8) CRPV DNA at study initiation. N=5 rabbits per treatment group. At study end the papilloma sites were excised and bisected for qualitative histology and semi-quantitative inflammatory cytokine analysis.

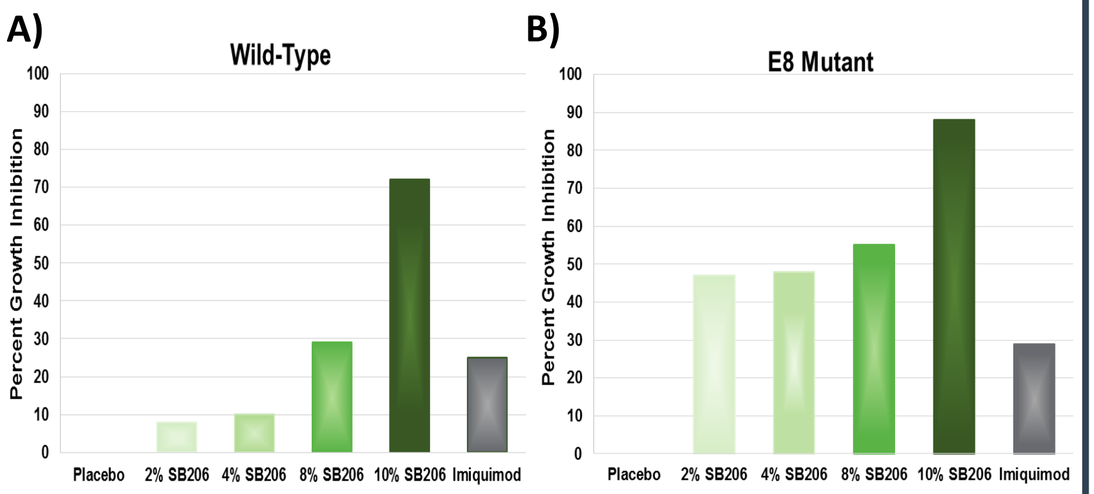


Day -3: Rabbits are scarified
Day 0: Rabbits receive viral DNA inoculation*
Days1-14: Virus infects and replicates
Day 15: Treatment begins
Rabbits receive once daily treatments (5x/week)
Day 70: 8-week treatment regimen concludes

*WT virus and mutant virus (E8 knockout) are 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. Dose Responsive Effect of SB206 (NVN1000) treatment in the CRPV Model. Animals were inoculated with both **A)** wild-type (WT) and **B)** E8 mutant (E8) CRPV DNA and topical treatment began two weeks following inoculation. Treatment occurred once daily (5 doses/week) for eight weeks. Percent inhibition of papilloma growth was calculated versus the mean Placebo control papilloma size at study termination.



CONCLUSIONS

- **In virally-infected organotypic cultures, HPV-18 viral replication was significantly inhibited following 6 days of daily 1 hr topical treatment with nitric oxide-releasing macromolecules as evidenced by BrdU staining as well was qPCR. Treatment with either the slow- or fast-releasing macromolecules resulted in comparable inhibition of viral replication.**
- **Topical treatment with SB206 (NVN1000) Gel demonstrated a dose responsive pharmacological effect against both WT and E8 mutant papillomavirus in the Cottontail Rabbit Model.**
  - Blinded histological analysis of biopsies from animals treated with 10% SB206 Gel exhibited mild hyperplasias, with an absence of viral infection as evidenced by a lack of intra-nuclear basophilic inclusions as compared to biopsies from animals treated with Placebo, Imiquimod, or less effective doses of SB206 Gel (2%).
  - Quantitative cytokine gene analysis was similar across all dose groups, suggesting immune activation did not account for the efficacy observed in the CRPV model and supports a direct anti-viral effect of nitric oxide therapy.

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