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NVN1000, a novel nitric oxide-releasing compound, inhibits HPV-18 virus production by interfering with E6 and E7 oncoprotein functions

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1. Introduction

Human papillomaviruses (HPVs) are prevalent human pathogens that infect cutaneous or mucosal epithelia. The non-enveloped virion contains a double-stranded circular DNA genome of approximate 7900 base pairs. Infections by the mucosotrophic HPVs can lead to benign hyperproliferative lesions of the ano-genital and oropharyngeal epithelia. The infections are usually cleared by a healthy immune system. Those caused by the low-risk (LR) HPV types such as HPV-6 and -11 rarely progress. However, persistent infections by the high-risk (HR) alpha HPV genotypes (notably HPV-16, HPV-18 and closely related types) can develop into high-grade dysplasia and carcinomas (zur Hausen, 2009). HR-HPV types cause almost all cervical cancers, 80% of anal cancers, and some 30% of head and neck carcinomas (de Martel et al., 2017). Effective vaccines and early therapeutic intervention would reduce HPV-induced morbidity and cancer burden. The multivalent prophylactic vaccines Gardasil 4 and Gardasil 9 are safe and highly efficacious (Joura et al., 2015). However, vaccination has not been widely achieved, largely due to economic and social issues (National Vaccine Advisory, 2016; Roden and Stern, 2018). Molecular diagnostic tools for HPV infections are highly sensitive, but there are no FDA approved therapeutic agents to treat the lesions (AACR Cancer Progress Report, 2018, https://cancerprogressreport.org/Documents/AACR_CPR18.pdf). A few topical agents (Imiquimod, Trichloroacetic acid, cidofovir etc) are commercially available but none are highly effective (Lacey, 2005; Cengiz et al., 2016; Banerjee et al., 2018a,b). Accordingly, physical removal by various means is typically utilized. Thus, there is an urgent need to identify safe and effective therapeutic agents to treat premalignant lesions.

Nitric oxide (NO) is a naturally produced free radical which has numerous critical physiologic functions, including immunomodulation, antimicrobial roles, regulation of blood pressure and vascular tone. Within the epithelial tissues, host immune responses are inherently sensitive to the local endogenous concentration of NO and its temporal-spatial release properties (Bogdan, 2001). In cultured cells, nitric oxide inhibits the replication of DNA viruses including herpesviruses simplex, Epstein-Barr virus and the vaccinia virus (Bi et al., 1995; Croen, 1993; Karupiah et al., 1993). Nitrosylation and inactivation of viral and host proteins contribute to these activities (Colasanti et al., 1999). The potential of exogenous NO as a novel topical antimicrobial agent has been investigated based on its ability to diffuse readily through cell membranes (Weller, 2009).

Certain small molecule NO donors are not ideal for drug development because of instability and inability to tune the NO release rate (Shin and Schoenfisch, 2008). NVN1000 (berdazimer sodium, CAS. 1846565-00-1, Novan Inc.) is a novel macromolecular polysiloxane polymer which stably stores NO as a covalently bound N-diazeniumdiolate and complete NO release was achieved within 1 h in physiological solution (Stasko et al., 2018). Topical application of NVN1000 gel (SB206) was moderately effective in reducing genital and perianal warts caused by HPV-6 and -11 in a Phase 2 clinical trial (Tyring et al., 2018). To investigate the molecular basis for this outcome, we examined the effects of topical application of NVN1000 to a three-dimensional epithelial tissue culture system, which supports the complete productive program of the oncogenic HPV-18.

HPV infection initiates upon epithelial tissue abrasion exposing the basal cells. The viral E6 and E7 oncoproteins facilitate the viral productive phase in post-mitotic, differentiated cells. Briefly, the HPV

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oncoprotein E7 promotes S-phase reentry in differentiated keratinocytes by destabilizing p130, a pocket protein related to the tumor suppressor, the retinoblastoma susceptibility protein pRB (Cheng et al., 1995; Genovese et al., 2008). E7 also induces a DNA damage response (DDR) (Banerjee et al., 2011; Chow and Broker, 2013; Moody and Laimins, 2009). Both activities are critical for efficient viral DNA amplification. The tumor suppressor p53 is stabilized by E7 (Demers et al., 1994; Eichten et al., 2002; Jian et al., 1998), but the E6 protein inactives p53 or destabilizes p53 in conjunction with E6AP, a host encoded ubiquitin ligase (Howie et al., 2009). This E6 function is essential to overcome p53 inhibition of viral DNA amplification (Kho et al., 2013; Wang et al., 2009). Upon viral DNA amplification, viral capsid proteins are expressed in the superficial cells whereupon progeny virions are assembled in the nuclei. Mature virions are shed with the acellular tissue remnants that slough off the surface of the epithelium.

Organotypic cultures of primary human keratinocytes (PHKs) and NIKS cells, a spontaneously immortalized epithelial cell line, allow for progressive squamous differentiation over a period of 2 weeks (Allen-Hoffmann et al., 2000; Wilson et al., 1992). Low-to-moderate levels of HPV DNA amplification have been achieved in NIKS cells transfected with HPV DNA excised from recombinant plasmids (Egawa et al., 2017; Flores et al., 1999; Lambert et al., 2005). Using PHK raft cultures, we have recapitulated a robust HPV-18 productive program yielding high titers of infectious virions (Wang et al., 2009). In this system, PHKs are transfected with recombinant DNA plasmids from which HPV-18 replicons are generated via Cre-LoxP recombination in vivo. This productive experimental system allows for in-depth characterization of inhibitors of the infection cycle.

Herein, we demonstrate that a 1-h exposure of HPV-18 infected raft cultures to 2 mg/ml of NVN1000 on six consecutive days compromized E6 and E7 activities, significantly reduced HPV-18 DNA amplification and abrogated progeny virus production and the inhibitory effects are durable. These results explain the efficacy of SB206 in the phase 2 trial to treat HPV infections.

2. Materials and methods

Isolation of PHKs from neonatal foreskins, generation of PHKs containing replication competent HPV-18 plasmids (hereafter called HPV-18 infected PHKs) and preparation of organotypic raft cultures were described (Wang et al., 2015). Modifications introduced for topical application of NVN1000 were described in Supplemental Information. NVN1000 was dissolved to 0.75, 1.0, 1.5 or 2.0 mg/ml concentration in 50 mM phosphate buffer (1.3 g m anhydrous potassium dihydrogen phosphate, 10.85 g m di-sodium hydrogen phosphate hepta-hydrate, and 8.74 g m sodium chloride in 1.0 L, pH 7.4). 300 µl of freshly prepared solutions were delivered topically to the epithelial tissue on days specified for each experiment. Following 1 h at 37 °C in the incubator with 5% CO_2, the solution was aspirated off. The culture media were refreshed and the assemblies returned to the incubator. In parallel, control cultures were topically exposed to the vehicle solvent. Cultures were harvested on different days as described for each experiment. Six to 12 h prior to harvest, 5-bromo-2′-deoxyuridine (BrdU, cat. B5002, MilliporeSigma) was added at 100 µg/ml to the culture medium in one raft culture of each test condition to mark cells in S-phase. BrdU-treated cultures were fixed in 10% buffered formalin and embedded in paraffin (FFPE) for in situ analyses, while parallel replicate cultures were flash frozen in liquid nitrogen and stored at −80 °C for biochemical analyses. Assay details are provided in Supplemental Information. Repeat experiments were conducted with different batches of PHKs.

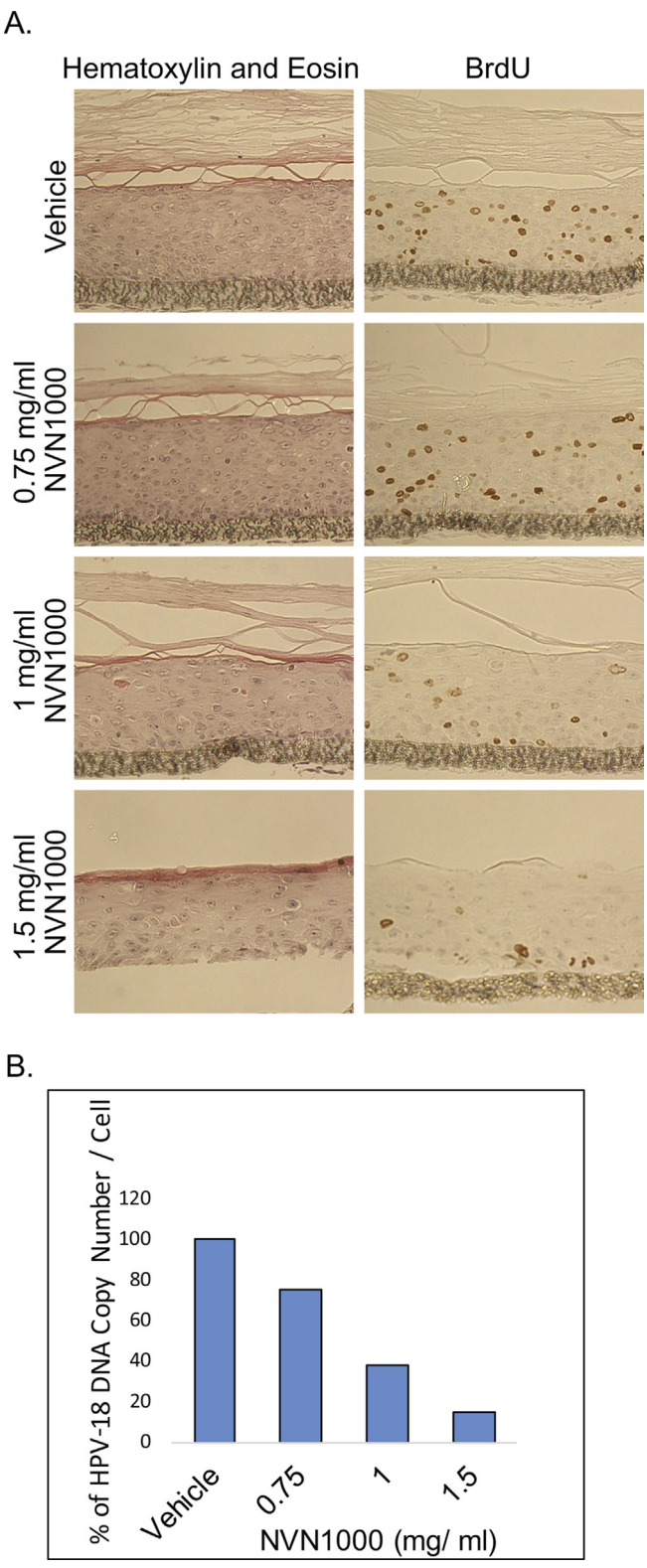

Fig. 1. Inhibitory effect on HPV-18 DNA amplification requires a minimal of 1 mg of NVN1000. Vehicle or NVN1000 was topically applied for 1 h daily to HPV-18 infected PHK raft cultures on days 7–12. BrdU was added 12 h prior to harvest on day 13. A. Histology (left panels) and immunohistochemistry to probe for BrdU incorporation into cellular DNA (right panels, reddish brown staining) induced by HPV. B. Relative viral DNA copy number per cell as determined by qPCR from parallel cultures from the same experiment. This set of experiments was conducted once to approximate the effective concentration of the test compound. Microscope objective lens = 20× magnification.

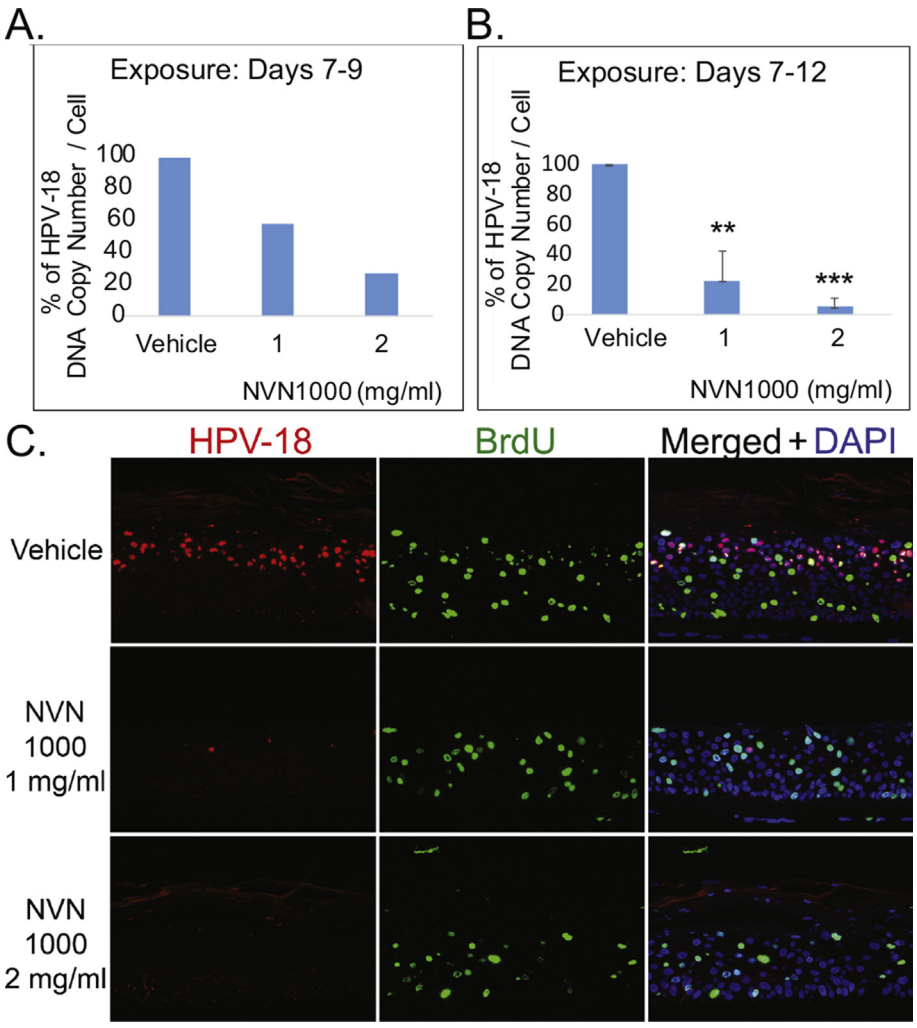

Fig. 2. Six daily applications of 2 mg/ml NVN1000 is highly effective in inhibiting HPV-18 DNA amplification. NVN1000 was delivered topically for 1 h on days 7–9 (A) or days 7–12 (B, C). BrdU was added 6 h prior to harvest on day 13. A and B. Relative HPV DNA copy number/cell as determined by qPCR. C. In situ assays of sections treated on days 7–12. Viral DNA amplification was revealed by FISH (red). BrdU incorporation was detected by indirect IF (green) with antibodies. Nuclei were stained with DAPI (blue). Microscope objective lens = 20× magnification. Data in panel A were obtained from one set of raft cultures. Data in Panel B were derived from three independent experiments. Images were obtained from one of the 3 independent experiments. ** and *** indicate p values at < 0.05 and < 0.005.

3. Results

3.1. Conditions of exposure to NVN1000

Because viral DNA amplification occurs between days 9/10 and days 13/15 in the productive HPV-18 raft cultures, we selected daily 1 h topical application on days 7–12 and analyzed the day 13 cultures. First, we determined that vehicle alone applied on this schedule did not affect the histology of infected or uninfected tissues, nor the productive viral program. We then evaluated the effects of NVN1000. S-phase cells were marked by BrdU added to the culture medium immediately prior to tissue harvest. Increasing concentrations of the test compound (0.75, 1.0 and 1.5 mg/ml) reduced suprabasal BrdU incorporation as revealed by immunohistochemistry of FFPE tissue sections (Fig. 1A). Viral DNA amplification was also reduced as determined by real time qPCR (Fig. 1B). Six daily 1 h applications (days 7–12) were much more effective than three daily applications (days 7–9) (Fig. 2A). Based on these results, we selected 1 and 2 mg/ml NVN1000 for 6 daily 1 h exposure for further experimentation.

Quantitative analysis of three independent experiments confirmed the initial observations. NVN1000 at 2 mg/ml reduced HPV-18 DNA copy number per cell by 95% ($p < 0.002$) (Fig. 2B). Using FFPE tissue sections, we performed HPV DNA fluorescence in situ hybridization (FISH) to detect amplified HPV-18 DNA. In the vehicle-treated raft cultures, FISH generated strong signals in numerous nuclei in the upper differentiated strata. In contrast, few or no HPV-18 positive nuclei were detected in cultures treated with 1 mg/ml or 2 mg/ml NVN1000 (Fig. 2C).

3.2. Impairment of S-phase progression in differentiated strata

S-phase reentry in differentiated keratinocytes was detected by indirect immunofluorescence (IF) with an antibody to BrdU. In the HPV-18 infected cultures, exposed to vehicle alone or to 6 daily treatment with 1 mg/ml NVN1000, many basal and suprabasal strata exhibited strong BrdU signals (Fig. 2C). Upon treatment with 2 mg/ml NVN1000, nuclei with strongly BrdU signals decreased, though many nuclei with weaker signals were detected (Fig. 2C). For quantitative assessments, we used the cellSens program to count the total number of nuclei and those positive for BrdU in 3 non-overlapping regions of serial sections of three independent day 13 raft cultures. The results revealed a reduction of the total number of nuclei in the treated cultures relative to the vehicle-treated control, but the percentages of BrdU-positive nuclei did not significantly decline (Fig. 3A and B). Box and Whisker plots (Fig. 3C) depicted the range, mean and medians of BrdU incorporation efficiency in individual nuclei, as measured by gray scale signal intensity. The data revealed that the signal intensities were significantly reduced ($p < 0.0018$) in 2 mg/ml NVN1000-treated cultures. We conclude that host DNA replication during S-phase progression were significantly impaired.

CellSens counting also confirmed the reduction of HPV-Positive nuclei in the NVN1000-treated cultures.

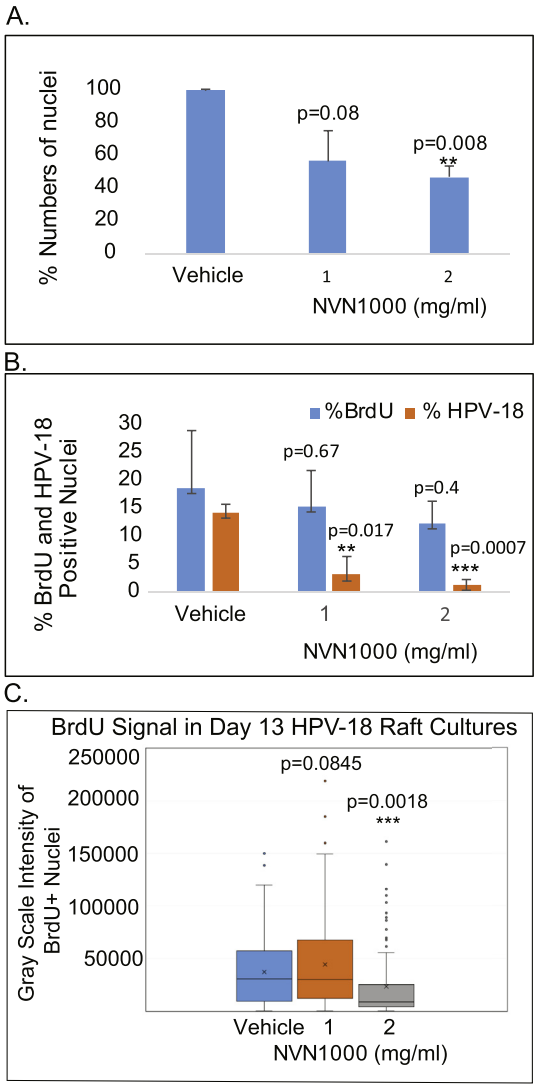

Fig. 3. NVN1000 reduces total cell number and impaired suprabasal S phase progression, but not S phase reentry in HPV-infected cultures The cellSens program of the fluorescence microscope was used to quantify total nuclei, HPV-18 DNA-positive nuclei and BrdU-positive nuclei as well as the BrdU signal intensities in raft culture tissue sections. Three non-overlapping areas per slide from 3 independent experiments are summarized. A. relative cell number of vehicle- or NVN1000-treated raft culture cultures. Cell numbers were derived by counting DAPI positive nuclei. B. Relative percentages of HPV-positive- and BrdU-positive nuclei that were also DAPI-positive. C. Box and Whisker plots represent the relative intensities of BrdU positive nuclei in rafts cultures treated with vehicle or NVN1000. Each color box contain middle 50% of nuclei. The horizontal line within the box is median and x indicate average. Dots outside the whiskers are individual outliers. Tissue sections of vehicle treated HPV-18 infected cultures were used as reference in each case. Statistical significance (p value) was indicated by ** (< 0.05) and *** (< 0.005). Microscope objective lens = 20× magnification.

3.3. Reduction of E6 and E7 proteins and activities

We next analyzed the effects of NVN1000 on the levels of HPV-18 E6 and E7 proteins by immunoblots of raft culture lysates (Fig. 4A, B, D). Six daily exposures to 1 mg/ml of NVN1000 reduced viral proteins slightly relative to the vehicle-treated cultures. In contrast, six applications of 2 mg/ml NVN1000 diminished both viral proteins significantly. We also examined the host proteins targeted by the viral oncoproteins. Uninfected PHK cultures had high levels of p53, whereas no p53 was detectable in vehicle-treated HPV-18 raft cultures (Fig. 4B). As expected from the reduced levels of E6 protein in cultures exposed to NVN1000, p53 protein became elevated relative to vehicle-treated cultures. E6AP, the ubiquitin ligase responsible for p53 destabilization in association with E6, was also reduced in 2 mg/ml treated cultures. Moreover, consistent with the reduced E7 protein levels upon exposure to NVN1000, immunoblots showed that E7-induced proliferating cell nuclear antigen (PCNA) and cyclin B1 were greatly decreased relative to vehicle-treated HPV-18 raft cultures (Fig. 4B and C). Indirect IF assays also showed stabilized p53 and reduced cyclin B1 in NVN1000-treated cultures (Fig. 5). Uninfected PHK cultures exhibited higher levels of pRB and p130 than did HPV-18 infected raft cultures (Fig. 4A). Interestingly, levels of these proteins did not increase; rather, both were decreased, in response to NVN1000 exposure despite of reduced E7 protein levels. We conclude that this low E7 protein level was nonetheless sufficient to effect adequate p130 destabilization to permit S-phase reentry in the differentiated cells (Figs. 2C and 3B).

3.4. Durable inhibition of HPV-18 DNA replication

To examine the durability of the inhibitory effects of NVN1000, the cultures were harvested on day 13 or 18 after exposure to NVN1000 on days 7–12. As before, qPCR revealed that NVN1000 at 1 or 2 mg/ml reduced the HPV-18 DNA copy number/cell to 15% or 4% of the vehicle-treated day 13 culture, respectively. After a 6-day compound-free chase (day 13–18), DNA copy number increased to 16.5% or 19% (Fig. 6) and the intensity of suprabasal BrdU signals also increased (Fig. 7A). This small increase in HPV DNA copy number was not evident by HPV DNA-FISH analyses (Fig. 7A). In accordance with our previous studies, L1 capsid protein signal was weak in day 13 vehicle-treated cultures, but was more abundant in day 18 cultures (Fig. 7B, left panels). In cultures treated with 2 mg/ml NVN1000, L1 protein was not detected in day 13 or day 18 cultures, consistent with the relatively low viral DNA copy number (Fig. 7B).

3.5. Induction of DNA damage, apoptosis and incomplete differentiation in HPV-18 raft cultures

Compared to uninfected epithelium (see Fig. 10), the histology of vehicle-treated HPV-18 infected raft cultures showed slightly hyperplastic growth and increased thickness of the squamous epithelium (Fig. 8). After 6 daily applications of 2 mg/ml NVN1000, the day 13 raft cultures were thinner and devoid of superficial granulocytes indicative of a lack terminally differentiation. The reduction in cell number (Fig. 3A) can be explained in part by the inhibition of S-phase entry by basal cells. In addition, retarded S-phase progression could lead to DNA damage and possibly apoptosis. Indeed, we observed occasional empty spaces or cells devoid of nuclei, suggestive of intraepithelial cell loss. Interestingly, many cells in the upper strata had highly condensed nuclei. They might represent apoptotic cells or incompletely differentiated superficial cells that were undergoing ineffective programmed cell death (Fig. 8, upper row).

To test these hypotheses, we probed for differentiation markers and for DNA damage and apoptosis by indirect IF. Relative to vehicle-treated cultures, cytokeratin 10 (CK10), an early differentiation marker was not affected, whereas loricrin, a marker for terminal differentiation, was absent in 2 mg/ml treated cultures (Fig. 8, middle row). The vehicle-treated cultures were negative for γ-H2AX, a marker of double-stranded DNA breakage, and for TUNEL, which is commonly used to detect apoptotic cells with extensively degraded nuclear DNA. Suprabasal γ-H2AX signals were detected in cultures treated with NVN1000 (Fig. 8, lower panels). Enumeration revealed that only few cells in 1 mg/ml NVN1000-treated cultures accumulated high DNA damage marker. At 2 mg/ml much higher percentage of cells were γ-H2AX positive, but with low signals, possibly due to loss of apoptotic cells with extensive DNA damage (Fig. 8). Live suprabasal strata in cultures treated with 2 mg/ml NVN1000 had only occasional TUNEL signals

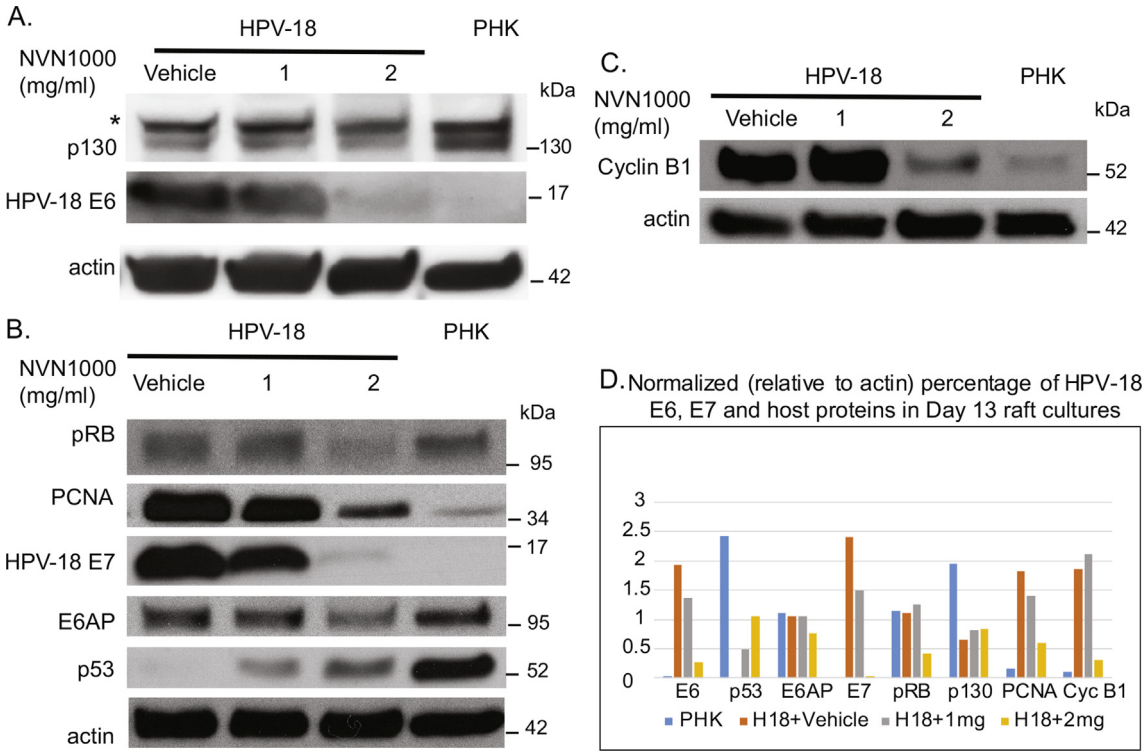

Fig. 4. Immunoblot analyses of raft culture lysates reveal that 2 mg/ml NVN1000 significantly reduced HPV-18 E6 and E7 proteins and their abilities to modulate the host target proteins. Raft cultures were treated for 1 h daily with topical application of the agent from day 7 to day 12 and harvested on day 13. The lysates were derived from parallel cultures from one of the three independent experiments described above. Panels A, B and C each represent a separate blot, with actin serving as a loading control. Panel D is densitometric quantification of viral and host protein bands. In panel A, * indicates a nonspecific cross-reactive protein.

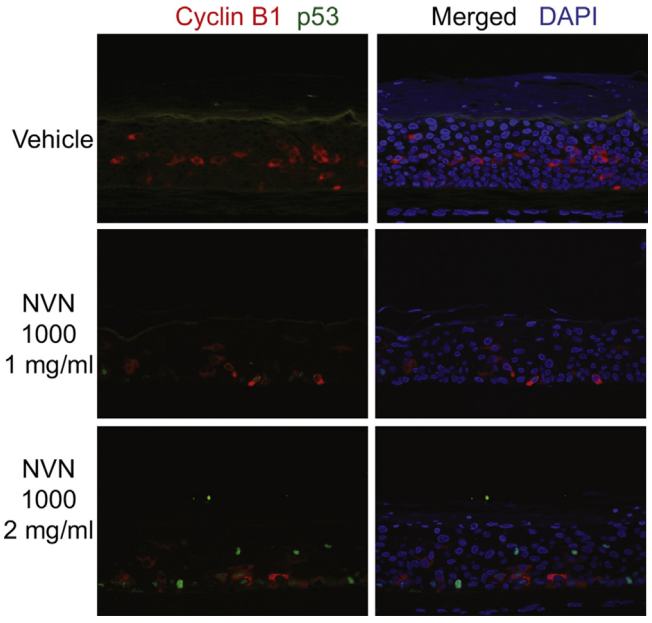

Fig. 5. Indirect IF confirms that exposure to NVN1000 stabilized p53 while reducing cyclin B1. Vehicle or NVN-treated infected cultures were probed with p53 (green) and cyclin B1 (red). Nuclei were stained with DAPI. These observations are representative of 3 independent experiments. Microscope objective lens = 20× magnification.

indicative of apoptosis. Rather, many superficial cells were TUNEL positive. We suggest that at least some of these signals could have originated from partially degraded nuclear DNA due to ineffective programmed cell death of incompletely differentiated cells. Immunoblots also showed that 2 mg/ml NVN1000 for 6 days induced

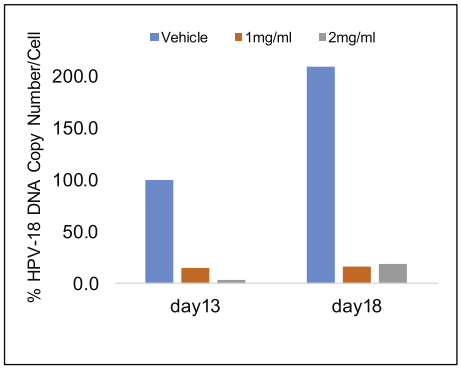

Fig. 6. The inhibitory effects of NVN1000 on HPV-18 DNA amplification is durable as revealed by a 6-day compound-free chase. HPV-18 infected raft cultures were treated daily with 1 h topical exposure on days 7–12. Parallel cultures were harvested on day 13 or on day 18 without further NVN1000 application. BrdU was added to the culture media 6 h prior to harvest. Relative HPV-18 DNA copy number per cell on day 13 and day 18, determined by real time qPCR. The chase experiment was performed once.

elevated γ-H2AX (Fig. 9A, C) and cleaved caspase 3, a marker of early apoptosis (Fig. 9B, D).

3.6. Effects of NVN1000 on uninfected PHK raft cultures

In two independently conducted experiments, uninfected PHK raft cultures were examined along with the HPV-18 infected raft cultures for cytopathic effects upon exposure to NVN1000. Data from one representative experiment are presented. Histology of vehicle-treated cultures revealed proper squamous differentiation. No residual nuclei was observed in stratum corneum, demonstrating a normal and complete programed cell death. CK10 was detected in suprabasal cells and

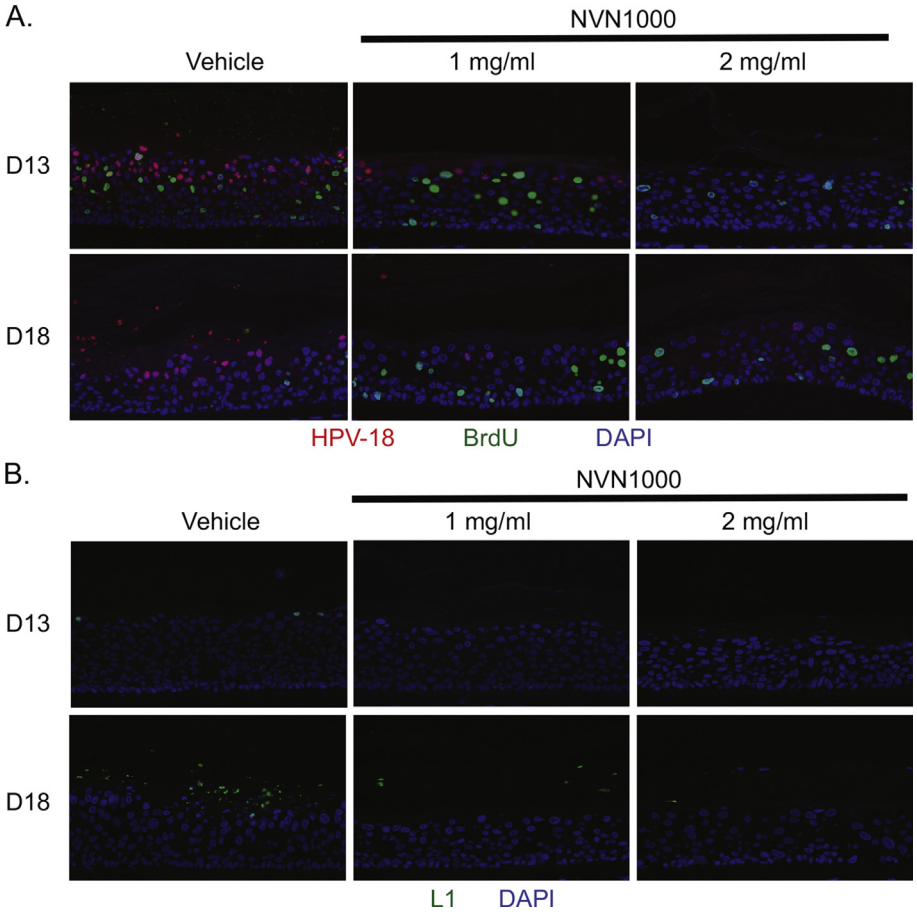

Fig. 7. In situ analyses of the chase experiment confirm NVN1000 abrogate HPV-18 productive infection. A. Dual detection of amplified viral DNA by DNA-FISH (red) and of BrdU incorporation by indirect IF (green) to assess host DNA replication. B. Indirect IF detection of the L1 capsid protein (in green) in raft cultures harvested on day 13 and on day 18. Nuclei were detected with DAPI staining. Microscope objective lens = 20× magnification.

loricrin was detected in the superficial granulocytes (Fig. 10 left panels). In contrast, NVN1000 treated cultures had thin spinous strata, but thick stratum corneum with condensed residual nuclei (Fig. 10, upper panels). CK10 was detected only in the compacted stratum corneum (Fig. 10, 2nd row), while loricrin was not detected, indicative of a lack of terminal differentiation. The presence of residual DAPI-positive nuclei present in the stratum corneum is suggestive of ineffective programmed cell death (Fig. 10, 2nd row).

In vehicle-treated cultures, cellular DNA replication occurred only in the basal stratum as revealed by BrdU incorporation. Exposure to NVN1000 reduced BrdU incorporation into basal cell nuclei (Fig. 10, 3rd row). Thus, basal cell proliferation slowed down or ceased. Indeed, immunoblot analysis revealed that cyclin B1 was reduced relative to vehicle-treated cultures (Fig. 11A and B). In addition, NVN1000 stabilized p53, which can also prevent S-phase entry. The vehicle-treated PHK raft cultures were negative for γ-H2AX or TUNEL (Fig 10, 4th row, left panel). Following 6 applications of 2 mg/ml NVN1000, we detected γ-H2AX in basal as well as suprabasal cells, (Fig. 10, 4th row, right panels). However, TUNEL signals were primarily detected within the residual nuclei in stratum corneum (Fig. 10, 4th row, right panels).

Collectively, NVN1000 impaired basal cell proliferation and reduced squamous differentiation. Importantly, these results indicate that NO or NO carrier penetrated down to the basal stratum.

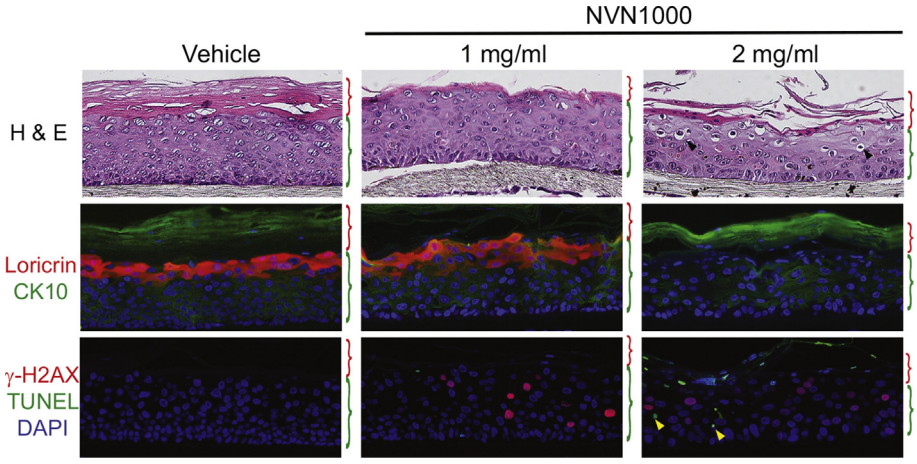

Fig. 8. NVN1000 inhibits terminal differentiation and induces DNA damage and TUNEL signals in HPV-18 infected raft cultures. In situ assays of vehicle-treat and NNV1000 treated cultures. Top panels, histology (H&E staining) revealed cytopathic effects. Some of the abnormal nuclei were indicated with black arrowheads. Middle panels, indirect IF detected no loricin when treated with 2 mg/ml test compound but the expression CK10 appeared unaltered. Lower panels. NVN1000 induced γ-H2AX (red) and TUNEL signals (green). An atypical area in 1 mg/ml NVN1000 treated culture shows strong γ-H2AX signals. Yellow arrowheads point to some apoptotic cells within spinous strata. Nuclei were stained with DAPI. Parentheses } (red) and } (green) to the right of each panel indicates stratum corneum and live tissue compartments, respectively. Microscope objective lens = 20× magnification.

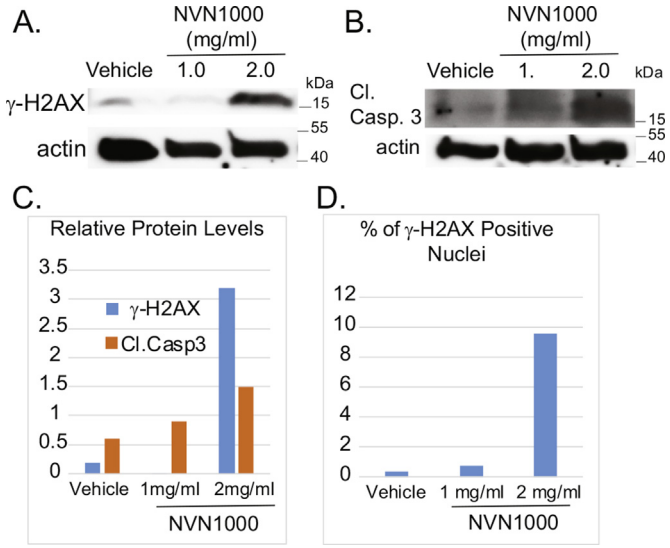

Fig. 9. Immunoblot assays confirm that NVN1000 induces γ-H2AX and cleaved caspase 3 in infected cultures. A and B. Parallel cultures of those shown in Fig. 8 were lysed and immunoblotted. Actin from the same blots served as the respective loading control. B. Densitometric quantification of γ-H2AX and cleaved caspase 3 protein bands, relative to actin. C. Percentages of γ-H2AX positive nuclei. Data are from one set of raft cultures, representative of 3 independent experiments.

4. Discussion

NO inhibits DNA replication by inactivating ribonucleoside diphosphate reductase (Lepoivre et al., 1991). S-Nitrosylation also inhibit DNA polymerase delta activity in vitro (Ding et al., 2013). These properties are in agreement with the antiviral properties of ectopic NO on large DNA viruses, but the effects on the productive HPV infection has not been described. Using organotypic epithelial raft cultures harboring HPV-18 genomes, we demonstrated that 6 daily 1 h topical applications of NVN1000 inhibit productive HPV-18 DNA amplification. Capsid protein synthesis was not detected (Fig. 1A). The inhibitory effect is durable as demonstrated by a 6-day compound-free chase. Our results would account for the phase 2 trial in which NVN1000 (in SB206) moderately reduced genital and perianal warts (Tyring et al., 2018). These encouraging observations in vivo and in vitro suggest that NO-releasing compounds should be further tested or developed to achieve a more durable efficacy in inhibiting or possibly abrogating HPV infection. Notably, high viral load is a risk factor for lesion progression to high grade dysplasia or carcinomas (Ylitalo et al., 2000). By significantly reducing the viral DNA, NO treatment could be anticipated to slow or eliminate progression.

Immunoblots revealed that viral E6 oncoprotein and the host encoded E6AP were reduced resulting in p53 stabilization. The increase in p53 could also contribute to the reduction of viral DNA amplification (Kho et al., 2013; Wang et al., 2009). Interestingly, NO has been reported to stabilize p53 through S-nitrosylation of C77 to inhibit its interaction with HDM2 (Schonhoff et al., 2002). In the uninfected cultures, NO reduced basal S phase entry resulting in much thinner epithelium. NO also reduced viral oncoprotein E7 levels, leading to reduced S phase progression based on the significant reduction in BrdU incorporation and in the accumulation of cyclin B1, the G2 cyclin. Because HPV DNA amplifies during G2-phase, the inability to complete S-phase and progress to G2 would be one of the major impediments for viral DNA amplification. We also suspect that viral DNA replication is impaired as is host DNA replication. The incompletely replicated viral DNA could be quickly lost, contributing to the dramatic reduction of viral DNA copy number per cell.

NVN1000 exposure leads to impaired S-phase progression and DNA damage. It also prevented proper differentiation. Interestingly, TUNEL signals were infrequently observed only in the live epithelium of infected cultures. Rather, the signals were primarily detected in the stratum corneum of both infected and uninfected cultures. We suggested the majority of the TUNEL signals might be attributed to residual nuclear DNA when ineffective programmed cell death occurred in incompletely differentiated cells. Despite of the toxicity in uninfected normal raft cultures, in a clinical trial NVN1000 (Trial agent name SB206), topical application to external genital and perianal wart was generally well tolerated without scar formation (Tyring et al., 2018). Presumablly, in vivo, the basal cells or surrounding normal tissues can regenerate a healthy epithelium.

Our finding is different than an earlier report which described that ectopic DETA-NO increased E6 and E7 mRNA expression, reduced pRB and p53 and induced mutations in submerged monolayer cultures of W12E or CIN-612 9 E, immortalized human cervical CIN–I derived cell lines harboring HPV-16 or HPV-31 plasmids, respectively (Wei et al., 2009). These cultures support neither viral DNA amplification nor progeny virus production. DETA-NO is a small molecule that contains primary amines, known to be toxic to cells. The half-life of DETA-NO is very long and delivers low amounts of NO for days. In contrast,

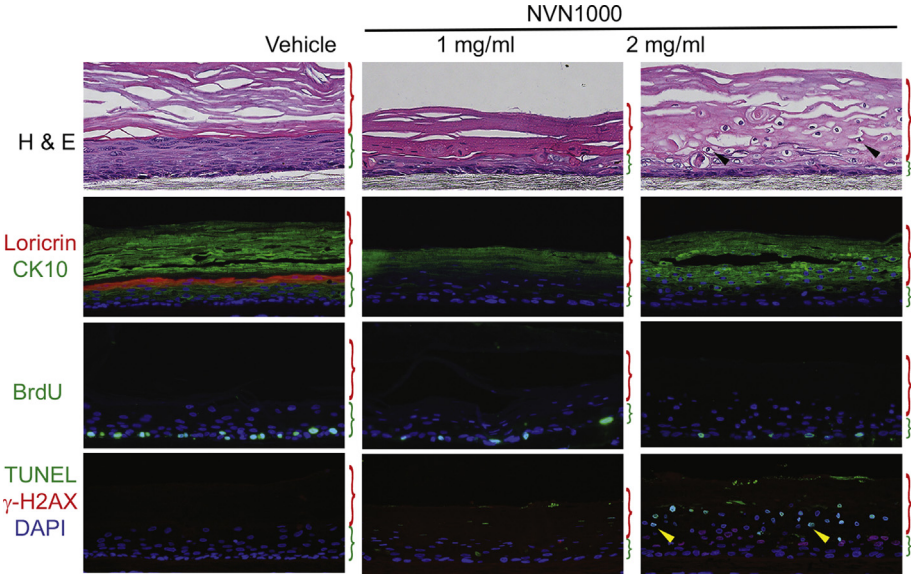

Fig. 10. NVN1000 reduces basal cell DNA replication and impairs differentiation in uninfected PHK raft cultures. The in situ assays were performed on day 13 cultures that were vehicle or NNV1000 treated cultures (days 7–12). Nuclei were stained with DAPI (blue). First row, H&E staining revealed increased strata corneum and reduced spinous strata in NVN1000-treated cultures. Black arrowheads indicate some of the residual nuclei in stratum corneum. Second row, NVN 1000 exposure inhibited CK 10 (green) and loricin (red) expression in live epithelium. Third row, NVN1000 reduced BrdU (green) incorporation in the basal stratum. Fourth row, γ-H2AX (red) was detected in live epithelium while TUNEL signals were primarily localized to the residual nuclei in stratum corneum (yellow arrow heads). Nuclei are stained with DAPI. Results are reproducible in two independent experiments. Parentheses } (red) and } (green) indicate stratum corneum and live tissue compartments, respectively. Microscope objective lens = 20× magnification.

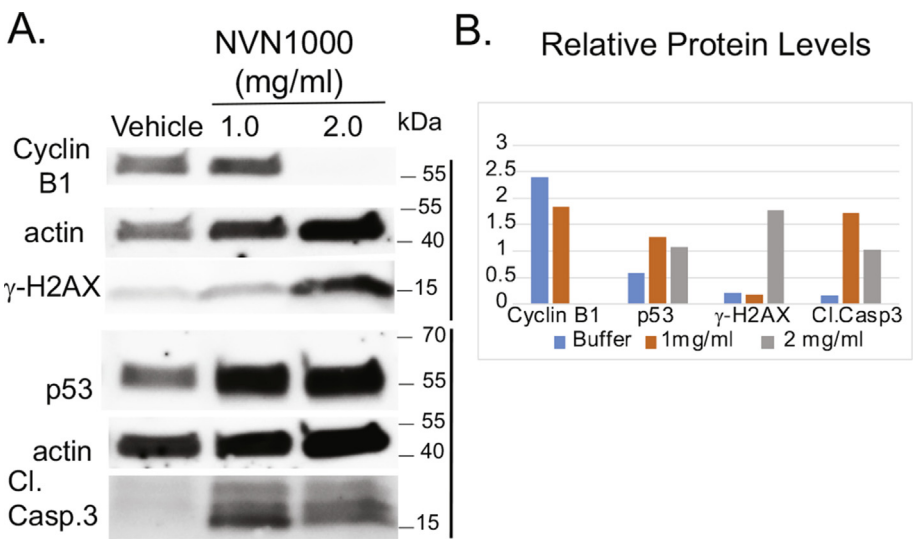

Fig. 11. Immunoblot probing reveals a reduction in steady state levels of cyclin B1 and increase of γ-H2AX, p53 and cleaved caspase-3 proteins. A. Immunoblot. B. Densitometric quantification of protein bands of cyclin B1, p53, γ-H2AX and cleaved caspase 3 proteins, each normalized to actin. This experiment was performed using lysates from one set of raft cultures.

NVN1000 does not contain primary amines and quickly releases NO in an aqueous environment at neutral pH. These differences illustrate the importance of both the release rate on pharmacodynamics of NO and the experimental model systems used.

In conclusion, the three-dimensional raft culture is a highly informative and relevant system to assess anti-HPV agents. The agents can be supplied through the liquid medium (Banerjee et al., 2018a, 2018b) or applied topically, as in this study. Our results indicate that NVN1000 offer new possibilities for topical treatment of premalignant HPV lesions and are worthy of further investigation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104559.

References

Allen-Hoffmann, B.L., Schlosser, S.J., Ivarie, C.A.R., Meisner, L.F., O'Connor, S.L., Sattler, C.A., 2000. Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. J. Investig. Dermatol. 114, 444–455.

Banerjee, N.S., Moore, D.W., Broker, T.R., Chow, L.T., 2018a. Vorinostat, a pan-HDAC inhibitor, abrogates productive HPV-18 DNA amplification. Proc. Natl. Acad. Sci. U.S.A. 115, E11138–E11147.

Banerjee, N.S., Wang, H.K., Beadle, J.R., Hostetler, K.Y., Chow, L.T., 2018b. Evaluation of ODE-Bn-PMEG, an acyclic nucleoside phosphonate prodrug, as an antiviral against productive HPV infection in 3D organotypic epithelial cultures. Antivir. Res. 150, 164–173.

Banerjee, N.S., Wang, H.K., Broker, T.R., Chow, L.T., 2011. Human papillomavirus (HPV) E7 induces prolonged G2 following S phase reentry in differentiated human keratinocytes. J. Biol. Chem. 286, 15473–15482.

Bi, Z., Quandt, P., Komatsu, T., Barna, M., Reiss, C.S., 1995. IL-12 promotes enhanced recovery from vesicular stomatitis virus infection of the central nervous system. J. Immunol. 155, 5684–5689.

Bogdan, C., 2001. Nitric oxide and the immune response. Nat. Immunol. 2, 907–916.

Cengiz, F.P., Emiroglu, Nazan , Su, Ozlem , Onsun, Nahide, 2016. Effectiveness and safety profile of 40% trichloroacetic acid and cryotherapy for plantar warts. The Journal of Dermatology 43 (9), 1059–1061. https://doi.org/10.1111/1346-8138.13370.

Cheng, S., Schmidt-Grimminger, D.C., Murant, T., Broker, T.R., Chow, L.T., 1995. Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. Genes Dev. 9, 2335–2349.

Chow, L.T., Broker, T.R., 2013. Human papillomavirus infections: warts or cancer? Cold Spring Harb. Perspect. Biol. 5.

Colasanti, M., Persichini, T., Venturini, G., Ascenzi, P., 1999. S-nitrosylation of viral proteins: molecular bases for antiviral effect of nitric oxide. IUBMB Life 48, 25–31.

Croen, K.D., 1993. Evidence for antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. J. Clin. Investig. 91, 2446–2452.

de Martel, C., Plummer, M., Vignat, J., Franceschi, S., 2017. Worldwide burden of cancer attributable to HPV by site, country and HPV type. Int. J. Cancer 141, 664–670.

Demers, G.W., Foster, S.A., Halbert, C.L., Galloway, D.A., 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. Proc. Natl. Acad. Sci. 91, 4382.

Ding, S.-J., Liu, M., Talmadge, J., 2013. P23: global S-nitrosylation analysis provides insight into tumor-induced immune suppression. Nitric Oxide 31, S22–S23.

Egawa, N., Wang, Q., Griffin, H.M., Murakami, I., Jackson, D., Mahmood, R., Doorbar, J., 2017. HPV16 and 18 genome amplification show different E4-dependence, with 16E4 enhancing E1 nuclear accumulation and replicative efficiency via its cell cycle arrest and kinase activation functions. PLoS Pathog. 13, e1006282.

Eichten, A., Westfall, M., Pietenpol, J.A., Münger, K., 2002. Stabilization and functional impairment of the tumor suppressor p53 by the human papillomavirus type 16 E7 oncoprotein. Virology 295, 74–85.

Flores, E.R., Allen-Hoffmann, B.L., Lee, D., Sattler, C.A., Lambert, P.F., 1999. Establishment of the human papillomavirus type 16 (HPV-16) life cycle in an immortalized human foreskin keratinocyte cell line. Virology 262, 344–354.

Genovese, N.J., Banerjee, N.S., Broker, T.R., Chow, L.T., 2008. Casein kinase II motif-dependent phosphorylation of human papillomavirus E7 protein promotes p130 degradation and S-phase induction in differentiated human keratinocytes. J. Virol. 82, 4862–4873.

Howie, H.L., Katzenellenbogen, R.A., Galloway, D.A., 2009. Papillomavirus E6 proteins. Virology 384, 324–334.

Jian, Y., Schmidt-Grimminger, D.C., Chien, W.M., Wu, X., Broker, T.R., Chow, L.T., 1998. Post-transcriptional induction of p21cip1 protein by human papillomavirus E7 inhibits unscheduled DNA synthesis reactivated in differentiated keratinocytes. Oncogene 17, 2027–2038.

Joura, E., Bautista, O., Luxembourg, A., 2015. A 9-valent HPV vaccine in women. N. Engl. J. Med. 372, 2568–2569.

Karupiah, G., Xie, Q.W., Buller, R.M., Nathan, C., Duarte, C., MacMicking, J.D., 1993. Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. Science 261, 1445–1448.

Kho, E.Y., Wang, H.K., Banerjee, N.S., Broker, T.R., Chow, L.T., 2013. HPV-18 E6 mutants reveal p53 modulation of viral DNA amplification in organotypic cultures. Proc. Natl. Acad. Sci. U. S. A. 110, 7542–7549.

Lacey, C.J.N., 2005. Therapy for genital human papillomavirus-related disease. Journal of Clinical Virology 32, 82–90. https://doi.org/10.1016/j.jcv.2004.10.020.

Lambert, P.F., Ozbun, M.A., Collins, A., Holmgren, S., Lee, D., Nakahara, T., 2005. Using an immortalized cell line to study the HPV life cycle in organotypic "raft" cultures. Methods Mol. Med. 119, 141–155.

Lepoivre, M., Fieschi, F., Coves, J., Thelander, L., Fontecave, M., 1991. Inactivation of ribonucleotide reductase by nitric oxide. Biochem. Biophys. Res. Commun. 179, 442–448.

Moody, C.A., Laimins, L.A., 2009. Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. PLoS Pathog. 5, e1000605.

National Vaccine Advisory, C., 2016. Overcoming Barriers to Low HPV Vaccine Uptake in the United States: Recommendations from the National Vaccine Advisory Committee: Approved by the National Vaccine Advisory Committee on June 9, 2015, vol. 131. Public health reports, Washington, D.C., pp. 17–25 1974.

Roden, R.B.S., Stern, P.L., 2018. Opportunities and challenges for human papillomavirus vaccination in cancer. Nat. Rev. Cancer 18, 240.

Schonhoff, C.M., Daou, M.C., Jones, S.N., Schiffer, C.A., Ross, A.H., 2002. Nitric oxide-

mediated inhibition of Hdm2-p53 binding. Biochemistry 41, 13570–13574.
Shin, J.H., Schoenfisch, M.H., 2008. Inorganic/organic hybrid silica nanoparticles as a nitric oxide delivery scaffold. Chem. Mater. 20, 239–249 : a publication of the American Chemical Society.
Stasko, N., McHale, K., Hollenbach, S.J., Martin, M., Doxey, R., 2018. Nitric oxide-releasing macromolecule exhibits broad-spectrum antifungal activity and utility as a topical treatment for superficial fungal infections. Antimicrob. Agents Chemother. 62 e01026-01017.
Tyring, S.K., Rosen, T., Berman, B., Stasko, N., Durham, T., Maeda-Chubachi, T., 2018. A phase 2 controlled study of SB206, a topical nitric oxide-releasing drug for extragenital wart treatment. J. Drugs Dermatol. JDD : J. Drugs Dermatol. JDD 17, 1100–1105.
Wang, H.K., Broker, T.R., Chow, L.T., 2015. Robust HPV-18 production in organotypic cultures of primary human keratinocytes. Methods Mol. Biol. 1249, 93–109.
Wang, H.K., Duffy, A.A., Broker, T.R., Chow, L.T., 2009. Robust production and passaging of infectious HPV in squamous epithelium of primary human keratinocytes. Genes Dev. 23, 181–194.
Wei, L., Gravitt, P.E., Song, H., Maldonado, A.M., Ozbun, M.A., 2009. Nitric oxide induces early viral transcription coincident with increased DNA damage and mutation rates in human papillomavirus-infected cells. Cancer Res. 69, 4878–4884.
Weller, R.B., 2009. Nitric oxide–containing nanoparticles as an antimicrobial agent and enhancer of wound healing. J. Investig. Dermatol. 129, 2335–2337.
Wilson, J.L., Dollard, S.C., Chow, L.T., Broker, T.R., 1992. Epithelial-specific gene expression during differentiation of stratified primary human keratinocyte cultures. Cell Growth Differ. 3, 471–483.
Ylitalo, N., Sørensen, P., Josefsson, A.M., Magnusson, P.K.E., Andersen, P.K., Pontén, J., Adami, H.-O., Gyllensten, U.B., Melbye, M., 2000. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. The Lancet 355, 2194–2198.
zur Hausen, H., 2009. Papillomaviruses in the causation of human cancers — a brief historical account. Virology 384 (2), 260–265. https://doi.org/10.1016/j.virol.2008.11.046. http://www.sciencedirect.com/science/article/pii/S0042682208007721.