REVIEW ARTICLE

A review of the development of nitric oxide as a topical treatment for cervical intraepithelial neoplasia caused by high-risk human papillomavirus infection

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Disclosure
DAR, TMC, EM, and CG are all employees and stockholders of Novan, Inc.

Abstract

Persistent human papillomavirus (HPV) infection, especially of the high-risk (HR) HPV-16 and HPV-18 types, is linked to anogenital and oropharyngeal cancer. In women, HR-HPV infections of the cervix lead to growth of precancerous cervical intraepithelial neoplasias (CIN) before onset of invasive cervical cancer. Low incidence of widespread, preventive HPV vaccination and the disadvantages of surgical CIN excision (e.g., recurrence rates, pain, and risk of complications in future pregnancies) highlight the need for novel treatments to target HPV infection. Nitric oxide (NO) is a small molecule gaseous species with potent antiviral activity. This review of the preclinical and clinical development of nitric oxide-based topical treatments by Novan, Inc and others supports that NO delivery to the site of infection has the therapeutic potential to manage HPV infection and CIN.

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1. Introduction

1.1 Human Papillomavirus and Cervical Intraepithelial Neoplasia

Genital human papillomavirus (HPV) infection is one of the most common sexually transmitted infections within the United States, and the CDC estimates that nearly all sexually active people will contract HPV at some point during their lifetime.[1] The HPV infection occurs at basal epithelial cells that can be exposed post-trauma or abrasion to the skin during sexual intercourse. Once the infected cells differentiate, viral replication and amplification ensues. The majority of HPV infections are cleared by the immune response, but persistent infections can lead to development of precancerous lesions of the mucosal epithelial tissue. These hyperproliferative lesions can further progress to cancer if left untreated.[2]

Human papillomaviruses are nonenveloped double-stranded DNA viruses on the order of 8 kilobases long.[3] The *Papillomaviridae* family of viruses is broad and encompasses numerous genera, of which the *Alphapapillmoavirus* genus is the largest.[4-6] Within this genus, the HPVs can be further classified into low-risk and high-risk types based on their propensity to cause cancer.[3] Persistent high-risk HPV (HR-HPV) infections have been conclusively linked to the development of malignant lesions; including cervical, vaginal, vulvar, penile, anal and oropharyngeal cancers.[7] Current estimates attribute HPV as the cause of approximately 30,000 cancers in the United States annually, with cervical cancer being the most common HPV-associated cancer in women.[8] The fourteen HR-HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.[9] The HR-HPV types are characterized by elevated levels of E6 and E7 viral protein expression that is essential for the development of malignant lesions.[3, 10] Recently, the prevalence of any genital HPV infection among adult women aged 18-59 was estimated to be 39.9% while the prevalence of HR-HPV specifically was estimated at 20.4% in the same demographic.[9] Nearly all cervical cancer can be attributed to HR-HPV infection of some type; however, the most critical types are HPV-16 and HPV-18. Indeed, 66% of invasive cervical cancer has been attributed to HPV-16 and HPV-18 collectively.[11] Furthermore, HPV-16 has been estimated to account for only 20% of infections yet 50% of cervical cancer, underscoring the link between HR-HPV type and the progression to cancer.[12]

The premalignant lesions caused by persistent HR-HPV infection in the cervix are termed cervical intraepithelial neoplasias (CIN), or dysplasia.[2, 12] Cervical intraepithelial neoplasia is classified histologically by grade, based on the extent or depth of the morphological changes of the epithelial tissue of the cervix ranging from the lower parabasal layers of the squamous epithelium up to the whole thickness of the epithelium, as depicted in Figure 1.[13] The classifications of CIN 1 and 2 correspond to mild and moderate dysplasia, respectively. Both severe dysplasia and carcinoma *in situ* are encompassed within CIN 3.[14] Formation of CIN 1 is usually transient and resolves without intervention within 1 or 2 years.[15] However, high grade CIN can progress to invasive cervical cancer if left untreated.[2, 12] There are estimated to be ~200,000 diagnosed cases of high grade CIN in the US annually, with rates highest in women aged 24-29 years old.[8] Due to the clear progression from higher grade CIN to invasive cervical cancer, current recommendations for CIN grade 2/3 are ablative or excisional treatment to remove the CIN and underlying HR-HPV

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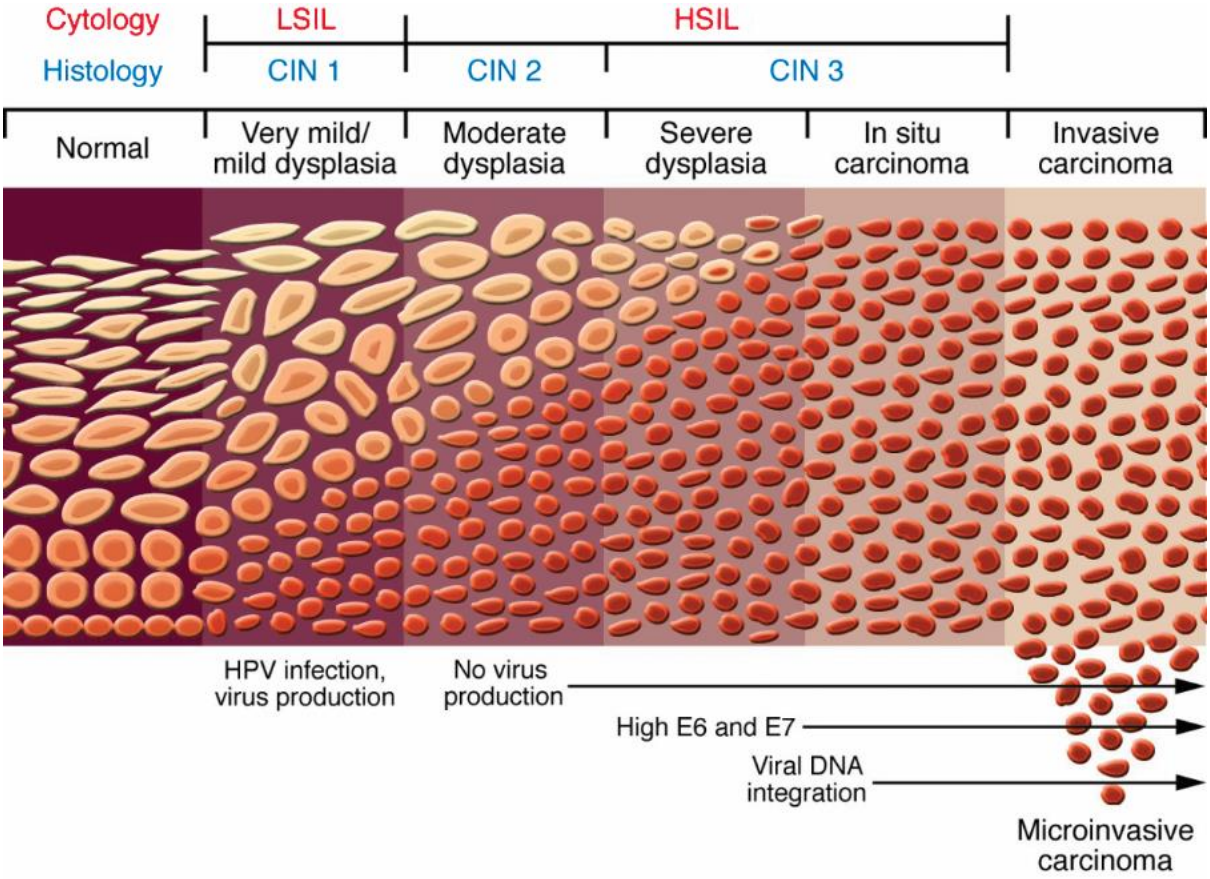
infection.[16] The most recent information from the CDC demonstrates that nearly 13,000 women in the US will be newly diagnosed with cervical cancer and more than 4,000 will die from cervical cancer annually.[17]

Figure 1: The Classification of Cervical Intraepithelial Neoplasia Grades. Cytology classifications of dysplasia consist of low-grade squamous intraepithelial lesion (LSIL) or high-grade intraepithelial lesion (HSIL). Reproduced with permission from Lowy DR and Schiller JT. Prophylactic human papillomavirus vaccines. *J Clin Invest.* 2006;116(5):1167-1173. https://doi.org/10.1172/JCI28607



Currently, the progression from HPV infection to cervical cancer is assumed to occur on average over 12-15 years.[14] The well-established linear model of disease progression highlights critical opportunities to intervene such as prevention of HPV infection (i.e., via vaccination), treatment of an active infection, or treatment of the precancerous CINs (e.g., via ablasion or excision)[16] Prophylactic vaccines, (e.g., Gardasil and Cervarix), protect against up to nine HR-HPV types and are currently recommended for ages 9 to 26 in both females and males.[18] However, a US national survey conducted in 2018 found that only 53.7% of girls aged 13–17 years had received all recommended doses in the series, and the percentage for boys was lower at 48.7%.[19] From a global perspective, it's estimated that an average of only 6.1% of females aged 10 to 20 years old have received the full course of vaccination with estimated vaccination rates varying widely by geographical region and income level.[20] Additionally,

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preventative vaccines were only introduced in recent years and have no direct therapeutic effect on treating patients already infected with HPV. The standard of care for CIN can include "watch-and-wait" for cells to heal on their own or surgical procedures to excise the abnormal cells. However, invasive procedures (e.g., conization, loop excision) are often associated with pain and increased risk of pre-term birth in future pregnancies.[21] Standard of care does not directly address the underlying HPV infections and may result in recurrence. Indeed, detection of a persistent HPV infection has been found to occur in 10% of cases at 24 months post-CIN treatment.[16] Furthermore, studies indicate that 15% of women treated for high-grade neoplasias, experience recurrence as CIN 2 or 3 or invasive cervical cancer, most of which occur within 2 years of treatment.[22]

Research into alternative treatments for CIN and cervical HPV infection have been investigated to overcome the lack of widespread HPV vaccination and the disadvantages of invasive surgical procedures. For example, photodynamic therapy and the topical administration of biologics have both shown promise and potential.[21, 23] However, there are no currently FDA-approved therapeutics indicated to treat HR-HPV infections or cervical neoplasias that offer a safe and efficacious way to target the site of infection.

1.2 Nitric Oxide

Nitric oxide is a gaseous, free radical signaling molecule that is endogenously produced via nitric oxide synthase (NOS). The three isoforms of the NOS enzyme found throughout the body are neuronal (nNOS, NOS1), inducible (iNOS, NOS2) and endothelial (eNOS, NOS3).[24, 25] The production of NO from nNOS and eNOS are constitutively expressed, calcium-dependent and on the order of pico to nanomoles. Arguably, the most well-known property of NO is as the endothelium-derived relaxation factor that controls smooth muscle relaxation and vascular tone. The iNOS isoform is distinguished from both eNOS and nNOS by being calcium-independent and as the name implies, induced or stimulated in cells as part of the immune response. Additionally, the levels of NO produced from iNOS are substantially higher with reports approaching µmoles.[26]

The role of NO in the immune response to infection has spurred a large body of research into the antimicrobial activity of NO against fungal, bacterial, and viral infections.[27-29] The reactivity of NO with multiple targets via redox chemistry and radical-radical recombination imparts a breadth of mechanism of actions that NO can invoke as a broad-spectrum antimicrobial agent. Specifically, NO can react with superoxide and oxygen to form peroxynitrite and myriad other reactive nitrogen species (e.g., nitrogen dioxide and dinitrogen trioxide), respectively. The nitrosative stress and the propensity for these reactive nitrogen species to react with thiol moieties on cysteine residues of critical proteins to form *S*-nitrosothiols is at the crux of the antiviral activity of NO.[30]

2 Nitric Oxide and Treatment of HPV-infections

While iNOS and elevated levels of NO have been implicated in the pathophysiology of many viral infections,[31] including being associated with persistent HPV infection,[32] research still concurs that S-nitrosylation of viral proteins is beneficial in combating viral infections.[30, 31] Researchers have demonstrated a benefit from application of exogenous NO or stimulation of endogenous NO to induce the *S*-nitrosylation of viral proteins critical to HPV infections.[33] Yu et al

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have shown that application of a small molecule NO donor, (Z)-1-[2-(2-aminoethyl)-N-(2- ammonioethyl) amino] diazen-1-ium-1, 2-diolate (DETA-NO), resulted in inhibited transcription of the HPV-16 E6 gene in SiHa cells in a dose-dependent manner. This result is critical and promising for NO-based therapy as E6, along with E7, is an oncogene linked to the progression of HPV to CIN. Specifically, E6 initiates degradation of the tumor suppressor protein p53. In their study, Yu et al postulated that the efficacy in reducing E6 transcription was tied to modulation of the mitogen-activated protein kinase (MAPK) pathway. This report in conjunction with others have shown the potential of exogenous NO treatment for HR-HPV and associated cancers (e.g., cervical cancer).[34]

To date, the majority of NO-releasing therapies have been classified as small molecule NO donors such as *N*-diazeniumdiolates (e.g., DETA-NO). *N*-Diazeniumdiolates are a well-characterized class of NO donor functional group that covalently bind two equivalents of NO per amine backbone.[35] Subsequent proton-initiated decomposition releases the NO and regenerates the parent amine. The pH-dependent trigger and release of NO position this NO donor class as therapeutically attractive. However, low molecular weight NO donor compounds are disadvantaged by having limited reservoirs of NO, lack the ability for targeted and controllable NO delivery, and may suffer from potential toxicity concerns. Macromolecular NO-releasing scaffolds represent an advancement in the field of NO-based therapies due to larger doses of NO that can be delivered and the ability to utilize the chemistry of the scaffold to enhance how the drug is formulated.[36] For example, Shin and Schoenfisch have demonstrated that silica particles can be synthesized with *N*-diazeniumdiolate NO donors embedded throughout.[37] These NO-releasing polysiloxane materials represent druggable solid active pharmaceutical ingredients (API) that can be formulated into various products. Novan, Inc (Morrisville, NC) is a biotechnology company which has advanced this technology in the fields of dermatology and virology by creation of numerous topical drug products that contain the active ingredient, berdazimer sodium.

Berdazimer sodium (CAS 1846565-00-1, company code NVN1000) is a polysiloxane-based macromolecule with *N*-diazeniumdiolate functional groups incorporated throughout the silica network of the polymer. The structure of berdazimer sodium is shown in

Figure 2. The antifungal and antibacterial properties of berdazimer sodium within formulated topical gel products have been demonstrated both preclinically and clinically.[38-40] Most recently, the efficacy of a topical gel containing berdazimer sodium against the molluscum contagiosum virus has been studied clinically.[41] Considering the supportive literature documenting the antiviral potential of NO in HPV infections, the antimicrobial activity of berdazimer sodium has been expanded to look at its antiviral activity against HPV.

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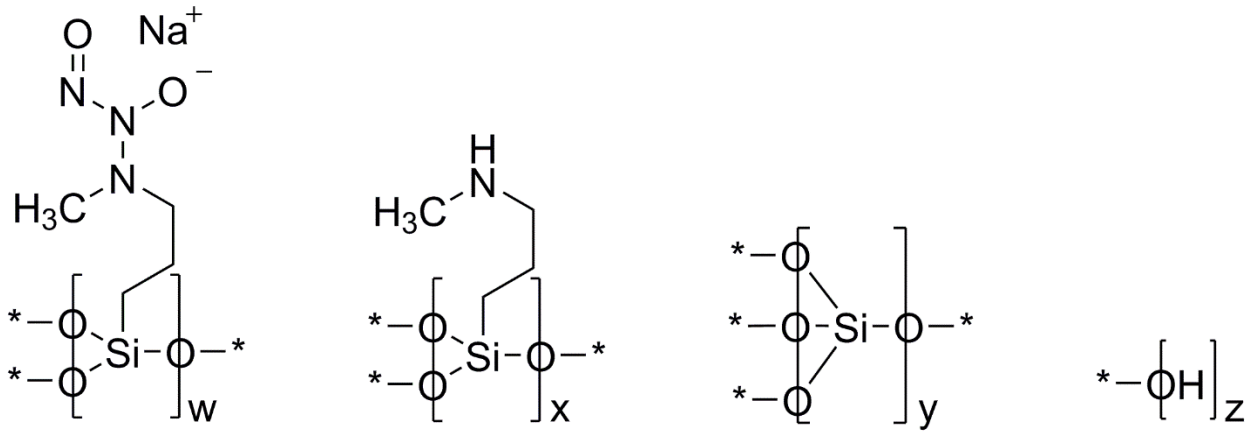
Figure 2: Structure of Berdazimer Sodium. *Denotes shared oxygen atom between bonded constituents; resulting bonds are Si-O-Si or Si-OH



Berdazimer sodium has been topically applied in a three-dimensional epithelial tissue culture model to mechanistically study the impact on the HPV infection cycle.[42] In this study, Banerjee et al. applied a 2 mg/mL dose of berdazimer sodium to primary human keratinocyte (PHK) raft cultures that had been previously infected with HPV-18. The treatment period with the NO-releasing compound consisted of 1-hour daily exposure for six consecutive days of treatment. Key findings were that this dosing regime was able to reduce HPV-18 DNA copy number per cell by 95% and decrease both E6 and E7 protein levels. Reduction of E6 and E7 activity was further corroborated by examination of the host proteins targeted by these viral oncoproteins. For example, levels of the tumor suppressor p53, which is destabilized by E6, were found elevated following berdazimer sodium treatment compared to vehicle. Additionally, after a 6-day wash out period following the 6-day treatment with berdazimer sodium, HPV-18 DNA copy numbers were quantified. Although HPV-18 DNA copy numbers increased, they were still inhibited compared to the control, illustrating the inhibition was to an extent sustained after treatment.

A similar study was conducted with Novan's NVN4000, an alternative NO-releasing macromolecular drug to berdazimer sodium. Longer sustained and lower levels of NO release are achieved with NVN4000 due to the structure of the parent amine and corresponding *N*-diazeniumdiolate incorporated into the polysiloxane network, underscoring the tunability in NO delivery that can be achieved with macromolecular APIs. Conclusions of this work echoed those of the berdazimer sodium study and demonstrated that NVN4000 could inhibit HPV-18 DNA amplification and reduce levels of E6 and E7.[43] Such *in vitro* studies support that topical treatment of NO represents an attractive therapy for HPV infections and CIN as reduction in viral load is beneficial to mitigate lesion progression to epithelial neoplasia.

The efficacy of NO-releasing macromolecular drugs as therapies for papillomavirus infections has also been evaluated in *in vivo* animal pharmacology models. McHale et al. has presented on the

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performance of berdazimer sodium and NVN4000 formulated as the topical gel drug products SB206 and SB216, respectively.[44, 45] In the disease model of the cottontail rabbit, papillomavirus (CRPV) infection was induced on to the back of rabbits by inoculation of either wild-type CRPV DNA or an E8 knockout mutant DNA. Viral inoculation with the E8 mutant DNA has shown to present in smaller, slower-growing lesions purported to be more clinically relevant to HPV infections. After daily treatment for 5-days per week for a period of 5 weeks, the fast releasing SB206 gel showed inhibition of papilloma growth for both the wild-type and the E8 mutant compared to vehicle gel treatment. However, the slower NO-releasing SB216 gel was only efficacious against papillomas caused by the E8 mutant and not the wild-type.[44] A dose response for inhibition was demonstrated, by increasing strengths of SB206, for both the wild-type and E8 mutant variant. SB206 containing 10% berdazimer sodium achieved 100% inhibition of the E8 mutant vs. vehicle control.

The *in vitro* and *in vivo* pharmacology and mechanistic understandings have led to the evaluation of SB206, the topical gel product containing berdazimer sodium API in the clinical setting.

2.1 SB206 for External Genital Wart (Condyloma Acuminata) Treatment

Genital warts (condyloma acuminata), mainly caused by HPV types 6 and 11, affect approximately 1% of sexually active adults in the United States and Europe.[46, 47] More than 1 million new cases of extragenital warts (EGW) and perianal warts (PAW) are diagnosed annually.[48-50] Although HPV 6 and 11 have low oncogenic potential,[51-53] other high-risk HPV types may be present concurrently with HPV 6 and 11 and may initiate the development of squamous cell carcinoma.[50, 54] Even after the successful removal or elimination of EGW/PAW, residual HPV is still a concern as it can lead to recurrence.

Tyring et al. has reported on a multicenter, double-blind, randomized, study to evaluate the tolerability, safety, and efficacy of topical SB206 at doses ranging from 4% to 12% compared with vehicle in patients with EGW/PAW. (Clinicaltrials.gov: NCT02462187).[55] The study design encompassed a total of 108 adult patients whom were randomly assigned to SB206 or vehicle in a 3:1 manner, starting with 4% SB206 twice daily (BID), and followed by 8% SB206 once daily (QD) and 12% SB206 QD. The primary efficacy endpoint was complete clearance of baseline EGW/PAW at or before Week 12. The percentage of patients with complete clearance of baseline EGW/PAW at or before Week 12 was higher for all of the SB206 groups than the vehicle groups (Figure 3), with the greatest difference between SB206 12% QD (33.3%; P=0.010) and vehicle QD (4.3%).

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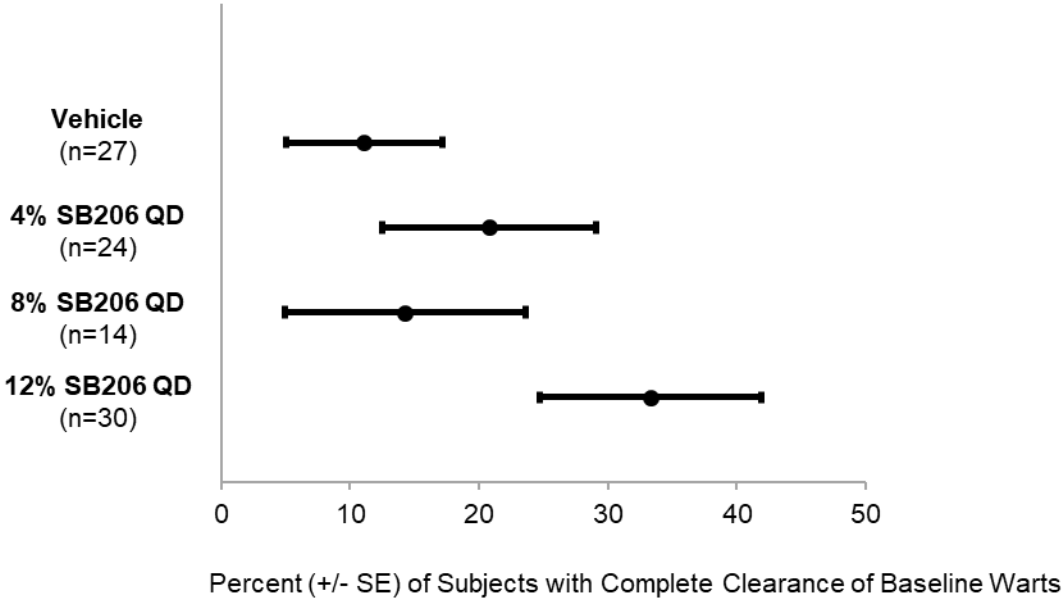
Figure 3: Complete Clearance of Baseline EGW/PAW at or before Week 12 (for the Intent-to-treat (ITT) Population)



Safety and tolerability were also assessed throughout the study and the majority of treatment emergent adverse event (TEAEs) reported were mild or moderate in intensity. The most commonly reported TEAEs were diarrhea (2.5%) and application site-related events. Application site-related TEAEs were reported for 50.0%, 26.1%, 7.1%, and 16.7% patients in the SB206 4% BID, 4% QD, 8% QD, and 12% QD groups, respectively. Due to the tolerability concern of BID dosing, 4% BID was switched to 4% QD. All doses of SB206 were well tolerated in the QD dosing schedule. None of the SAEs were considered by the investigator to be related to study drug, and no deaths were reported.

This study demonstrated that SB206 administered once daily for 12 weeks at doses ranging from 4% to 12% was effective, safe, generally well tolerated, and warrants further development to treat EGW and PAW. In theory, SB206 may also prevent recurrence of EGW and PAW due to its virucidal potential. Finally, its ease of patient-applied administration makes SB206 an appealing first-line treatment option for patients with EGW or PAW and may serve as adjunctive therapy to neutralize remaining virus after surgical removal of warts. While clinically proven to be efficacious for low-risk HPV types associated with EGW and PAW, the supporting pharmacology and mechanistic studies against HPV-16 and HPV-18 suggest that SB206 or a similar NO-releasing topical would be beneficial in the clinical treatment of HR-HPV and CIN.

3 Conclusions and the Future of the Field

The field of nitric oxide as a topical treatment for CIN is promising. The mechanistic pre-clinical evidence and the positive clinical trials to date for treatment of EGW with NO-releasing topical therapies emphasize the opportunity and benefit from future

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development of such treatments in the field of CIN caused by high-risk persistent HPV infections. The future of the field will benefit from technological advancements to new dosage forms specifically designed for vaginal delivery as well as further mechanistic understandings to elucidate the ideal NO release profile outfitted to deter HPV progression to CIN. Novan, Inc has recently expanded the therapeutic business units to Men's and Women's Health to fulfill this unmet need. Furthermore, Novan, Inc has recently been awarded both Department of Defense and National Institutes of Health federal funds to progress two potential product candidates. Novan, Inc is developing both a vaginal suppository as well as a vaginal gel for the treatment of CIN, labelled WH504 and WH602, respectively. Future drug development work in the specific intravaginal delivery of NO is poised to have a significant impact in the treatment of CIN and HR-HPV infections.

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