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TITLE: Topical Nitric Oxide Therapy to Treat Cervical Neoplasias and Prevent HPV-Associated Cancers

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14. ABSTRACT
The goal of this research was to develop a nitric oxide-releasing vaginal suppository for self-administration to treat cervical neoplasias, eradicating HPV-18 infection and inhibiting disease progression to cancer. The suppository contains our proprietary NO-releasing drug, NVN1000, with demonstrated in vitro antiviral efficacy against HPV-18. We developed five prototype formulations using excipients determined to be compatible with NVN1000 and appropriate analytical methods. Post 12-week stability data, a lead prototype was selected, although it requires refrigeration to maintain stability. Our collaborators at the University of Alabama Birmingham established an NVN1000 dose (4 mg/mL) and application frequency (every other day) that successfully inhibits HPV-18 replication in human raft cultures while minimizing cytotoxicity. Immunoblot data show increased p53 levels and p53 stabilization via phosphorylation, corroborating decreased E6 activity/levels post NVN1000-treatment. However, the exact mechanism of action (e.g., inhibition of association of E6 with p53) was not discerned. RNA sequence data show reduced transcription of E6 mRNA, but no increase in p53 transcription and overall support pleiotropic effects of NVN1000 on HPV-18-infected cells.

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TABLE OF CONTENTS

1. INTRODUCTION:

The goal of this research was to develop a nitric oxide-releasing vaginal suppository to be self-administered by female patients as a treatment for cervical neoplasias to eradicate latent HPV infection and inhibit disease progression to cancer. This goal was attempted to be reached by formulating a stable vaginal suppository with well-characterized physical chemical properties suitable for intravaginal administration and evaluating the effect of varying concentrations and treatment durations of NVN1000 against HPV-18 in human raft cell culture in vitro studies.

2. KEYWORDS:

Nitric oxide, Human Papillomavirus, HPV-18, Vaginal Suppository, Antiviral, Cervical Intraepithelial Neoplasias

3. ACCOMPLISHMENTS:

What were the major goals of the project?.

	Timeline (months)	**% Complete**
Specific Aim 1: Formulate a stable vaginal suppository with well-characterized physical chemical properties suitable for intravaginal administration.		
1.1 Formulation development of a nitric oxide releasing vaginal suppository.	12-14	75%
Deliverable 1: Selection of excipients compatible with NVN1000 following conduct of pre-formulation excipient compatibility studies.	1.5-2.0	100%
Deliverable 2: Creation of 5 prototype vaginal suppository formulations for evaluation of performance and stability.	2.0-2.5	100%
Deliverable 3: Development of a stability indicating chromatography analytical method development for the routine characterization of vaginal suppository at release and over time on stability. – Corresponding method development report.	2.0-3.0	100%
~~*Deliverable 4: Execution of stability testing program at 3 recommended ICH Climate conditions for up to 6 months in duration targeting at least one formulation having the minimum acceptable stability for clinical use. Corresponding stability report.*~~	7.0-8.0	0% (Deliverable abandoned)
2.1 In vitro dissolution testing of vaginal suppository.	5.0-6.0	0% (Aim abandoned)
~~*Deliverable 1: Development of an in vitro dissolution test method in simulated vaginal fluid utilizing the chemiluminescent nitric oxide analyzer employed in the characterization of other nitric oxide releasing drug products. – Corresponding method development report.*~~	1.5-2.0	
~~*Deliverable 2: Screening of the 5 prototype vaginal*~~	0.5-1.0	

~~*suppositories following dissolution in simulated vaginal fluid to determine acceptable loadings of NVN1000 for continued development based on appropriate pH thresholds.*~~		
~~*Deliverable 3: Generation of real time nitric oxide release kinetics of all of the lead prototype vaginal suppositories that have acceptable pH values upon dissolution.*~~	2.0-3.0	
3.1 Additional Performance Testing	4.0-5.0	0% (Aim abandoned)
~~*Deliverable 1: Execution of condom compatibility testing and identification of a lead prototype suppository that does not impact condom integrity.*~~	1.0-1.5	
~~*Deliverable 2: Execution of mucoadhesion testing in an in vitro perfusion model and identification of a lead prototype suppository that has greatest mucoadhesive performance.*~~	1.0-1.5	
~~*Deliverable 3: Publish a Pharmaceutical Development Report integrating pre- formulation, formulation, analytical characterization, and performance testing results to assist with the preparation of future regulatory submissions to the FDA.*~~	1.0-2.0	
Specific Aim 2: Evaluate the effect of varying concentrations and treatment durations of NVN1000 in HPV-18 infected human raft cell cultures.		
2.1 **Evaluation of NVN1000's antiviral activity across a higher range of concentrations in vitro**	10.0	100%
Deliverable 1: Regulatory review and approval by the USAMRMC Human Research Protection Office (HRPO)	2.0-4.0	100%
Deliverable 2: Established minimally effective dose response of NVN1000 required to inhibit viral replication >90% qPCR. Data at 3, 4, 5 and 6 mg/ml administered to uninfected PHKs and HPV-18 infected PHK raft cultures.	4.0-6.0	100%
2.2 **Optimization of NVN1000 frequency of administration to HPV-18 infected cultures**	6.0	100%
Deliverable 1: Data for application once every other day	2.0-3.0	100%
Deliverable 2: Data for application once every three days	2.0-3.0	100%
Deliverable 3: Final report on minimally effective dose and frequency of application to inhibit >90% viral replication.	2.0-3.0	100%
Deliverable 4: Establish recovery and rebound of HPV DNA replication once NVN1000 challenge is removed. Data on drug-free chase experiments.	2.0-3.0	100%

2.3 **Further the understanding of nitric oxide's mechanism of action against E6 and E7 oncoproteins.**	6.0	100%
Deliverable 1: Data from in situ assays	1.0-2.0	100%
Deliverable 2: Data from biochemical assays	1.0-2.0	100%
Deliverable 3: Data from RNA-sequencing assays	2.0-2.5	100%
Deliverable 4: Final report and Manuscript on the impact of higher concentrations and longer exposure duration of NVN1000 on E6 and E7 oncoprotein activity, DNA damage, and markers of apoptosis.	1.0-2.0	100% (Manuscript drafted and planned for submission)

What was accomplished under these goals?

Specific Aim 1: Formulate a stable vaginal suppository with well-characterized physical chemical properties suitable for intravaginal administration.

Specific Aim 1.1: Formulation development of a nitric oxide releasing vaginal suppository

Specific Aim 1.1, Deliverable 1: Selection of excipients compatible with NVN1000 following conduct of pre-formulation excipient compatibility studies.

In preliminary experiments, MedPharm and Novan confirmed that exposure to heat during manufacturing greatly impacts the stability of NVN1000. Learnings from these experiments were leveraged to increase the stability of NVN1000 in suppository prototypes by focusing on formulations with lower melt temperatures, rather than single excipients. Additionally, due to initial challenges related to the development of an extraction/quantitation method for NVN1000 in the suppository excipients, the excipient-NVN1000 compatibility data was initially found to be highly variable. The analytical method was optimized as described in Specific Aim 1, Deliverable 3 below, and formulation work using this optimized method was re-initiated.

Following the optimization of the extraction method, excipient selection and formulation was restarted by MedPharm. Initially, vehicle formulations were developed and systems with fast melting times were prioritized due to the requirement for a fast release of NO from NVN1000 for an efficacious product. Several iterations of vehicles were manufactured (using a generic manufacturing method of 40 minutes of mixing at 70 °C), building on the data generated from the previous round of development. The compositions of the developed vehicles are presented in Table 1, Table 2, and Table 3, with their melting profiles presented in Table 4. Also presented in Table 4 are the melting profiles of several single excipients.

Several individual excipients were first assessed for their melting profile to inform the development of binary systems (first 9 rows in Table 4). Suppocire AM, Ovucire WL3264, Ovucire 3460 and the high molecular weight (MW) polyethylene glycols (PEGs) were the most promising bases due to their low melt time compared to the other individual excipients. Using these excipients, binary prototypes were developed with the goal of obtaining a range of acceptable melt times and maximizing miscibility between excipients.

Table 1. Compositions (% w/w) of the first set of developed vehicle formulations, and the time until full melt at 37 °C.

Excipient	Compositions (% w/w)						
	S01	S02	S03	S04	S05	S06	S07
Ovucire WL3264	70.00	-	-	60.00	-	-	70.00
Ovucire 3460	-	-	-	-	50.00	-	-
PEG 1000	-	-	-	-	-	50.00	-
PEG 1450	30.00	-	-	-	-	-	-
Suppocire AM	-	-	70.00	-	-	50.00	-
Suppocire CM	-	80.00	-	-	-	-	-
Suppocire D	-	-	-	-	-	-	30.00
Petrolatum	-	20.00	30.00	40.00	50.00	-	-
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Justification	The excipients used and the ratios were selected based on MedPharm's experience, the formulations were designed to better understand immiscibilities that may be present between the excipients.						
Approximate melt time	15 mins	55 mins	35 mins	55 mins	65 mins	25 mins	30 mins

Table 2. Compositions (% w/w) of the second set of developed vehicle formulations, justifications for development and the time until full melt at 37 °C.

Excipient	Compositions (% w/w)							
	S08	S09	S10	S11	S12	S13	S14	S15
Ovucire WL3264	80.00	90.00	-	-	-	-	-	-
PEG 1000	-	-	70.00	90.00	-	-	-	-
Suppocire AM	-	-	-	-	70.00	90.00	-	-
Suppocire DM	-	-	-	-	-	-	50.00	50.00
Petrolatum	-	-	-	-	-	-	50.00	25.00
Mineral oil	20.00	10.00	-	-	30.00	10.00	-	25.00
PEG 400	-	-	30.00	10.00	-	-	-	-
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Justification	Binary system with mineral oil to lower melting time of an example Ovucire		Binary system with low MW PEG to lower melting time of an example PEG suppository		Binary system with mineral oil to lower melting time of an example Suppocire		Investigation of a previously used model system	Including mineral oil to lower melting time of S14
Approximate melt time	15 mins	15 mins	25 mins	15 mins	10 mins	15 mins	>65 mins	>65 mins

Table 3. Compositions (% w/w) of the third round of developed vehicle formulations, justifications for development and the time until full melt at 37 °C.

Excipients	Composition (% w/w)					
	S16*	S17	S18*	S19*	S20	S21
Ovucire WL3264	70.00	90.00	-	-	-	-
Ovucire 3460	-	-	-	-	70.00	80.00
PEG 1450	-	-	-	-	30.00	-
Suppocire AM	-	-	70.00	90.00	-	-
Mineral oil	-	-	-	-	-	20.00
PEG 400	30.00	10.00	30.00	10.00	-	-
Total	100.00	100.00	100.00	100.00	100.00	100.00
Justification	S01 but with a lower MW PEG to reduce melting time		Investigation of a Suppocire: PEG 400 binary system		S01 but with an alternative Ovucire to minimize risk of incompatibilities with NVN1000	S08 but with an alternative Ovucire to minimize risk of incompatibilities with NVN1000
Approximate melt time	15 mins	15 mins	30 mins	25 mins	20 mins	10 mins

(*) PEG 400 was not fully miscible in these systems.

Table 4. Melting profile assessment of several excipients and the developed vehicle formulations. Cells highlighted in red indicate the system had not begun to melt, those in orange indicate it was melting, and green indicates full melt.

System	Melting time (mins)														
	0	2	5	10	15	20	25	30	35	40	45	50	55	60	65
Suppocire D*															
Suppocire CM															
Suppocire AM															
Suppocire A															
Suppocire NB															
Ovucire WL3264															
Ovucire 3460															
PEG 1450															
PEG 1000															
S01															
S02															
S03															
S04															
S05															
S06															
S07															
S08															
S09															
S10															
S11															
S12															
S13															
S14															
S15															
S16															
S17															
S18															
S19															
S20^															
S21															

(*) – Only assessed up to 30 mins as no melting had been observed. (^) – heterogeneity observed.

Based on the results of vehicle formulation development, the vehicles detailed in Table 5 were selected for progressing into active formulation development. With some exceptions, melt times ≤ 20 min were prioritized. The compositions of the vehicle formulations are detailed in Table 6. Additional details about the rationale for the selections are summarized below:

- Three individual excipients (Suppocire AM, Ovucire WL3264, and Ovucire 3460) were selected for progression as single excipient formulations, without modification, based on their short melting time.

- S01 and S08 were selected based on their short melting time and because they are both Ovucire-based formulations with PEG and mineral oil, respectively.
- S06 was selected due to a slightly longer melt time (25 minutes) and can be compared to S12, which also contains Suppocire AM, and S11 which also contains PEG 1000.
- S07 was selected due to a longer melt time (30 minutes) and as a comparator to S01, S08, S09 and S17, which also contain Ovucire WL3264.
- S09 was selected as an alternative to S08 with different ratio of excipients. It also can be a comparator to S17 for determining the impact of mineral oil and PEG 400 on NVN1000 stability.
- S11 was selected based on the short melt time and 100% PEG composition.
- S12 was selected based on the short melt time and Suppocire:mineral oil composition.
- S17 was selected based on the short melt time and PEG 400:hard fat composition.
- S21 was selected as a formulation alternative to S08 where Ovucire 3460 was used in place of Ovucire WL3264 (in S08) to evaluate the effect of Ovucire composition on the stability of NVN1000.

The minimum melt temperatures of the selected prototypes were all approximately 36 °C (Table 5). Accordingly, 36 °C was selected as the melt temperatures that would be utilized on vehicle formulations prior to addition of NVN1000 during active formulation development.

Table 5. Selection of vehicles for active development, along with a summary of the data generated to date and justification for selection.

Selected system	Melt time at 37 °C (mins)	Melt temperature (°C)	Justification for selection
Suppocire AM	20	36	Fast melt time
Ovucire WL3264	10	36	Fast melt time
Ovucire 3460	10	35	Fast melt time
S01	15	33	Ovucire: PEG system
S06	25	Not Determined at the time	Longer melt time and as a comparison to S12 and S11
S07	30	Not Determined at the time	Longer melt time and as a comparison to S01, S08, S09 and S17
S08	15	35	Ovucire: mineral oil system
S09	15	35	As S08 with alternative ratio, also provides a direct comparison to S17
S11	15	36	100% PEG system, which did not start to melt immediately but completely melted after 15 minutes.
S12	10	37	Suppocire: mineral oil system
S17	15	35	Ovucire: PEG 400 system
S21	10	35	As S08 with alternative Ovucire

Table 6. Compositions (% w/w) of the vehicle formulations recommended for active formulation development.

Excipient	Compositions (% w/w)								
	S01	S06	S07	S08	S09	S11	S12	S17	S21
Ovucire WL3264	70.00	-	50.00	80.00	90.00	-	-	90.00	-
Ovucire 3460	-	-	-	-	-	-	-	-	80.00
PEG 1000	-	50.00	-	-	-	90.00	-	-	-
PEG 1450	30.00	-	-	-	-	-	-	-	-
Suppocire AM	-	50.00	-	-	-	-	70.00	-	-
Suppocire D			50.00						
Mineral oil	-	-	-	20.00	10.00	-	30.00	-	20.00
PEG 400	-	-	-	-	-	10.00	-	10.00	-
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Specific Aim 1.1, Deliverable 2: Creation of 5 prototype vaginal suppository formulations for evaluation of performance and stability:

Following on from the development of vehicle formulations with melting times of <30 mins at 37 °C (detailed above), MedPharm incorporated 1.0% w/w NVN1000 into the selected vehicle formulations to evaluate the interaction of excipients with NVN1000 and further narrow the selection of formulation candidates. Individual aliquots were prepared and whole aliquot extractions performed to determine if any immediate incompatibilities were present. The active formulations were characterized for NVN1000 content and purity, melting profile, macroscopic appearance, and microscopic appearance as detailed below.

(a) NVN1000 content and purity

Individual aliquots were extracted using the improved extraction method, and the percentage recovery and percentage peak purity of NVN1000 are shown in Table 7. The defined criteria for a chemically stable formulation was a percentage peak purity of NVN1000 of >90% area of the main band, based

on the purity of a standard solution of NVN1000. Consistent percentage peak purity data was generated, with NVN1000 appearing to be stable during the manufacture of most formulations (the percentage peak purity of NVN1000 in a standard solution is typically ca. 92% area). The mean percentage peak purities of NVN1000 in Ovucire 3460 and S21 were 78.65 and 75.82% area, respectively. Furthermore, the % recovery of NVN1000 in most samples was consistent, the exceptions being Ovucire 3460, S12, S17 and S21 for which some extent of variability between replicates was observed (% CV of 9% - 24%). The difference in the % recovery and % peak area of NVN1000 in Ovucire 3460 as compared to previous results obtained in the confirmatory extractions for this excipient might suggest differences in the sample preparation.

Mass balance between percentage recovery and percentage peak purity data was not achieved for many of the formulations, potentially due to undetected impurities, or because the extraction procedure is not optimized for all of the formulations.

As NVN1000 was stable following the manufacture of most formulations, MedPharm did not recommend the investigation of manufacture in an inert atmosphere or the inclusion of an overage at this stage of development. It should be noted that the manufacturing method employed is not necessarily representative of a larger scale manufacture and therefore inert atmosphere manufacture and the use of overage could still be investigated upon formulation scale-up.

Table 7. Percentage recovery (% compared to the theoretical) and % peak purity (% area) of NVN1000, in the developed formulations.

System	Percentage recovery (%) from the theoretical concentration			Percentage peak purity (% area)	Sum of impurities > 0.045% a/a
	Replicates	Mean	%CV		
Suppocire AM	93.56	91.00	3.27	89.41 (87.84 - 90.76)	11.86 (10.18 - 13.83)
	87.73				
	91.70				
Ovucire WL3264	95.28	91.62	6.70	89.94 (87.10 - 92.09)	11.24 (8.58 - 14.81)
	84.53				
	95.04				
Ovucire 3460	69.18	64.86	23.76	78.65 (70.33 - 85.72)	27.99 (16.65 - 42.17)
	77.64				
	47.74				
S01	87.46	92.29	4.58	89.92 (89.12 - 90.39)	11.21 (10.63 - 12.20)
	94.08				
	95.32				
S06	98.22	93.81	4.58	90.70 (89.24 - 92.11)	10.26 (8.55 - 12.06)
	89.64				
	93.58				
S07	90.81	94.68	3.54	91.56 (90.72 – 91.96)	9.19 (8.62 – 10.23)
	96.72				
	96.52				
S08	91.65	94.66	3.02	92.97 (92.95 - 93.00)	7.55 (7.52 - 7.57)
	95.00				
	97.33				

Table 7 (cont.): Percentage recovery (% compared to the theoretical) and % peak purity (% area) of NVN1000, in the developed formulations.

System	Percentage recovery (%) from the theoretical concentration			Percentage peak purity (% area)	Sum of impurities > 0.045% a/a
	Replicates	Mean	%CV		
S09	92.84	90.03	2.71	92.97 (92.95 - 92.99)	7.55 (7.53 - 7.57)
	88.41				
	88.85				
S11	95.22	91.82	5.82	88.85 (88.80 - 88.93)	12.50 (12.39 - 12.57)
	94.58				
	85.66				
S12	95.36	91.92	9.06	91.47 (90.28 - 92.14)	9.33 (8.54 - 10.76)
	98.76				
	81.64				
S17	97.70	90.55	13.93	92.76 (92.60 - 92.86)	7.79 (7.65 - 7.99)
	97.96				
	75.98				
S21	67.19	75.10	22.23	75.82 (65.26 - 89.96)	34.26 (11.15 - 53.23)
	94.28				
	63.84				

(b) Melting profile

The melting profiles of the active formulations containing 1% w/w NVN1000 were assessed at 37 °C, to determine if the presence of NVN1000 in the formulation had an impact, as compared to the vehicle formulations. A summary of the conclusions is below and data are shown in Table 8:

- All the active formulations fully melted within 10 – 35 min. Ovucire 3460, S11, S12 and S21 took ca. 10 min to fully melt, whereas S07 was the formulation that took the longest to melt (35 min), as expected. Prototype S07 is the only system composed of Suppocire D which was one of the two excipients that did not start melting within 30 min.
- In addition, the melting profiles of the active formulations were similar to those of their corresponding vehicles (Table 4). In some cases, a difference of ca. 5 min in the time for full melt was noted between active and vehicle formulations. However, no trend was observed. The slight variation observed might result from the variability inherent to the visual assessment of this parameter.

Table 8. Melting profile assessment of the developed active formulations. Cells highlighted in red indicate the system had not begun to melt, those in orange indicate it was melting, and green indicates full melt. Vehicle data presented here from Table 4 for reference.

System		Melting time (min)								
		t=0	t=2	t=5	t=10	t=15	t=20	t=25	t=30	t=35
Suppocire AM	Active									
	Vehicle									
Ovucire WL3264	Active									
	Vehicle									
Ovucire 3460	Active									
	Vehicle									
S01^	Active									
	Vehicle									
S06^	Active									
	Vehicle									
S07	Active									
	Vehicle									
S08	Active									
	Vehicle									
S09	Active									
	Vehicle									
S11	Active									
	Vehicle									

^ Heterogeneity observed.

Table 8 (cont.): Melting profile assessment of the developed active formulations. Cells highlighted in red indicate the system had not begun to melt, those in orange indicate it was melting, and green indicates full melt. Vehicle data presented here from Table 4 for reference.

System		Melting time (min)								
		t=0	t=2	t=5	t=10	t=15	t=20	t=25	t=30	t=35
S12	Active									
	Vehicle									
S17	Active									
	Vehicle									
S21	Active									
	Vehicle									

(c) Macroscopic appearance

The macroscopic appearance of the active formulations has been detailed in Table 9. All the active formulations had a slight yellow coloration as compared to the vehicles. The formulations with a darker coloration were Ovucire 3460, S07, S12 and S21. While a correlation between the dark yellow color and a lower % purity of NVN1000 was observed in Ovucire 3460 and S21, this was not observed in S07 and S12. All the formulations were described as opaque and solid. Heterogeneity was observed in S01 and S06 actives, however this was not observed during vehicle development and therefore it is likely that this was caused by the manufacturing procedure in use for these samples, rather than an immiscibility between the excipients.

Table 9. Macroscopic images of the active formulations (and vehicles for comparison) following manufacture.

Suppocire AM	Ovucire WL3264
	
Ovucire 3460	**S01**
	
S06	**S07**
	

Table 9 (cont.): Macroscopic images of the active formulations (and vehicles for comparison) following manufacture.

S08	S09
	
S11	S12
	
S17	S21
	

(d) Microscopic appearance

The microscopic appearance of the active and vehicle formulations is detailed in Table 10. It was challenging to identify drug particulates in the microscopy due to the large number of excipient particulates present in the formulations. This was expected since the formulations were largely composed of excipients which are solid at room temperature.

Table 10. Microscopic appearance of the active and vehicle formulations following manufacture.

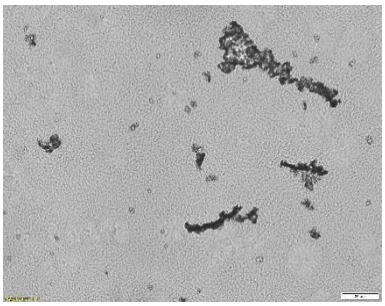
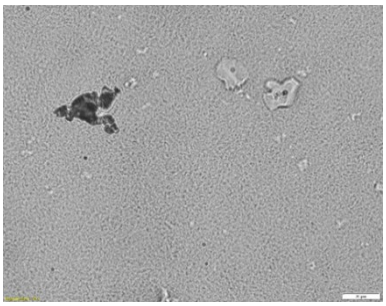
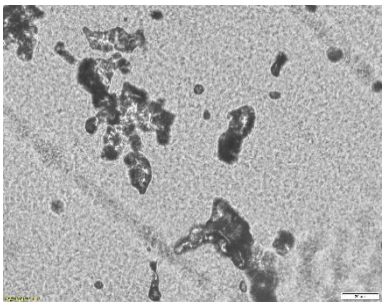
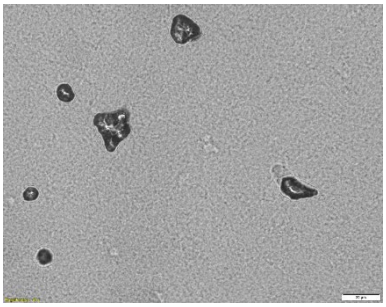
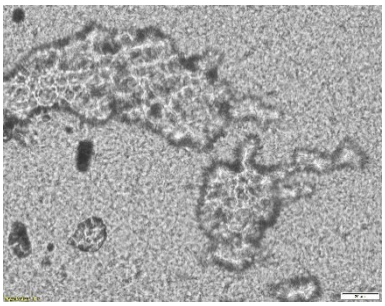
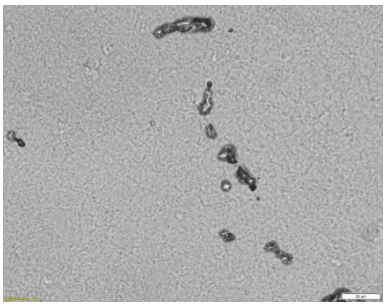
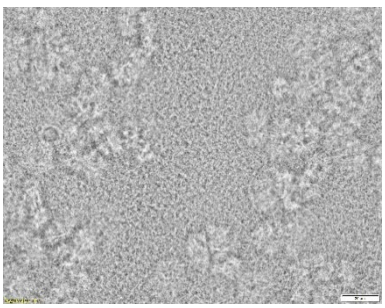
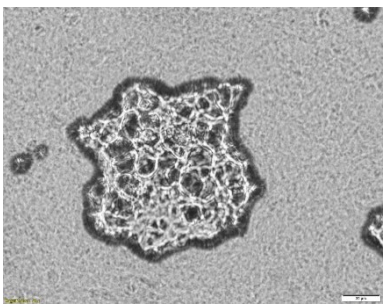
System	Microscopic appearance	
	Vehicle	Active
Suppocire AM		
Ovucire WL3264		
Ovucire 3460		
S01		

Table 10 (cont.): Microscopic appearance of the active and vehicle formulations following manufacture.

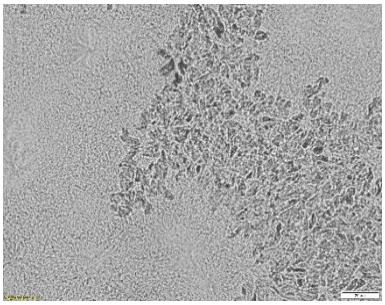
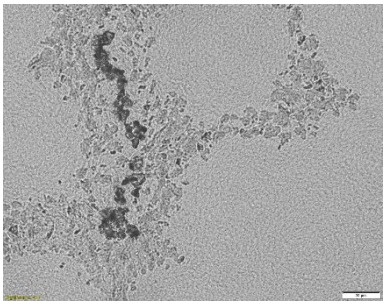
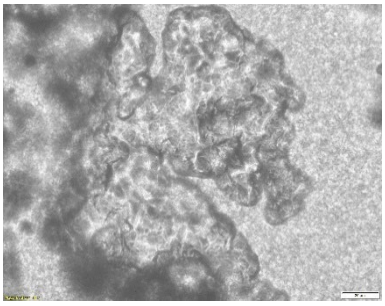
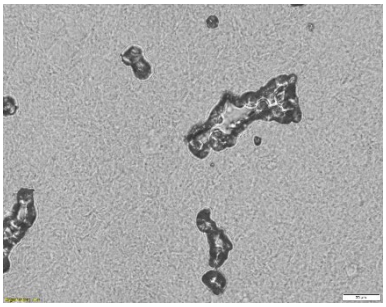
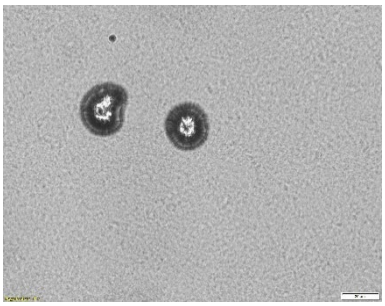
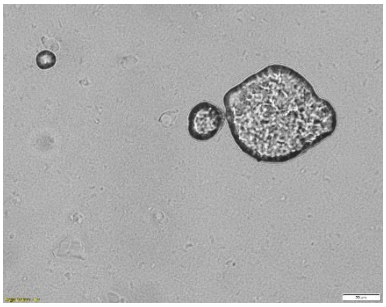
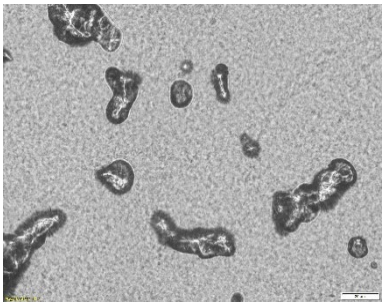
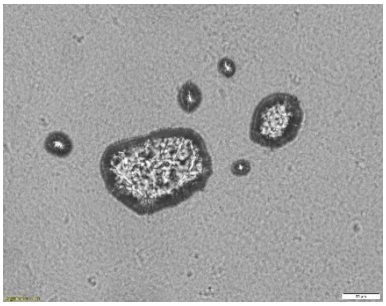
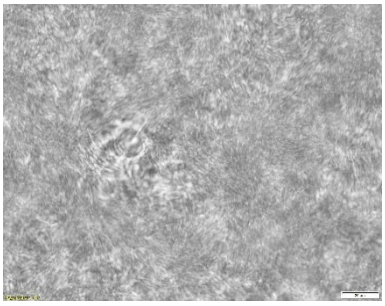
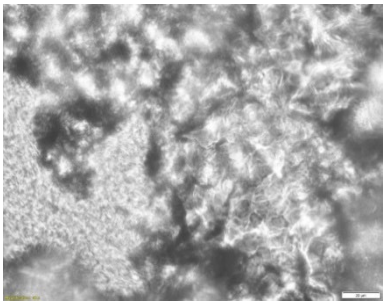
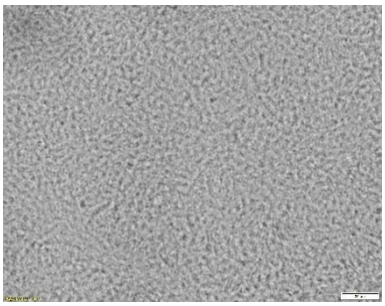
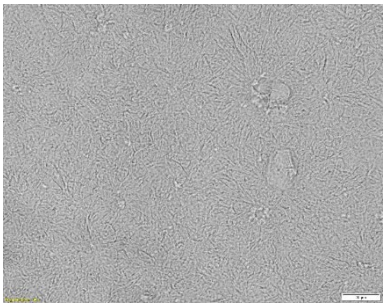
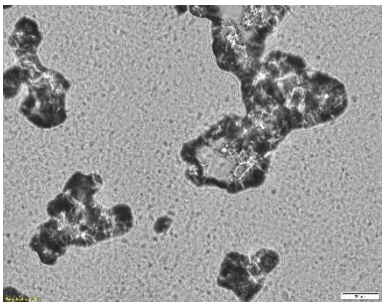
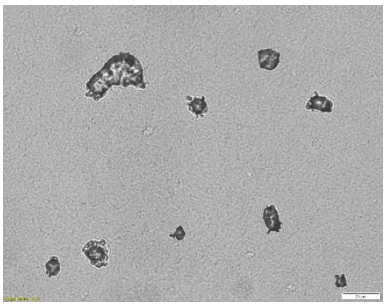
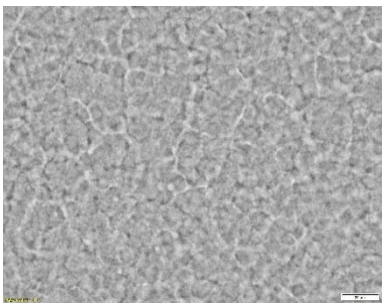
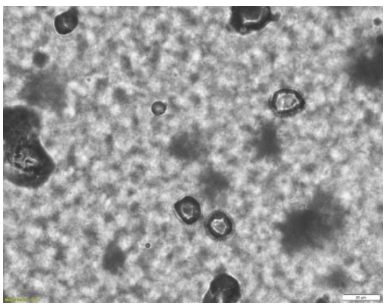
System	Microscopic appearance	
	Vehicle	Active
S06		
S07		
S08		
S09		
S11		

Table 10 (cont.): Microscopic appearance of the active and vehicle formulations following manufacture.

System	Microscopic appearance	
	Vehicle	Active
S12		
S17		
S21		

(e) Selection of formulations for short-term stability

Based on the results from the active formulation development, a decision matrix was prepared to aid the selection of formulations for short-term stability assessment (Table 12 and Table 13). The parameters and criteria assessed are presented in Table 11. A short-term stability assessment was conceived to de-risk and inform the design of the 6 month stability study originally planned under Specific Aim 1.1; Deliverable 4.

Priority was given to shorter melt times. Percentage peak purity (% area) was prioritized over percentage recovery since a fully optimized extraction method had not been developed for each formulation. MedPharm recommended the selection of the formulations colored in green (S06, S07, S08, S12 and S17) for short-term stability (Table 12 and Table 13), with that in orange being potential option, based on the criteria assessed in this decision matrix and the compositions of the formulations.

Table 11: Parameters and criteria assessed in the decision matrix.

Parameter	Criteria	
Melting time (min)	Up to 10 min	
	10-15 min	
	15-20 min	
	20-35 min	
Purity (% peak area) of NVN1000	Group 1	>90%
	Group 2	80 – 90%
	Group 3	<80%

Table 12: MedPharm's recommendations for short-term stability and justifications.

Formulation	Justification for recommendation
S06	• Ranked Group 1 in purity of NVN1000 • Only formulation amongst those selected for stability that had a full melt time of 15 – 20 min. • Only formulation comprised of Suppocire AM and PEG 1000. • Heterogeneity was observed in the active formulation, however not the vehicle and therefore it was likely caused by manufacturing process rather than an immiscibility.
S07	• Ranked Group 1 in purity of NVN1000 • Only formulation that had a full melt time of 20 – 35 min. • Only formulation composed of Suppocire D.
S08	• Ranked Group 1 in purity of NVN1000 • One of two formulations selected for stability with a full melt time of 10 – 15 min. • Selected over S09 due to the higher level of mineral oil in S08.
S12	• Ranked Group 1 in purity of NVN1000 • Only formulation selected for stability that had a full melt time of up to 10 min. • Only formulation composed of Suppocire AM and mineral oil.
S17	• Ranked Group 1 in purity of NVN1000 • One of two formulations selected for stability that had a full melt time of 10 – 15 min. • Only formulation composed of Ovucire WL3264 and PEG 400.

Table 13: Decision matrix to aid in the selection of formulations for short-term stability testing.

Formulation	Melting time at 37 °C (min)	Purity (% peak area) of NVN1000	Recommended for selection
Suppocire AM	15 – 20	2	(Y)
Ovucire WL3264	10 – 15	2	(Y)
Ovucire 3460	Up to 10	3	N
S01 (Ovucire WL3264: PEG 1450 70: 30 w/w)	15 – 20	2	(Y)
S06 (Suppocire AM: PEG 1000 50: 50 w/w)	15 – 20	1	Y
S07 (Ovucire WL3264: Suppocire D 70: 30 w/w)	20 – 35	1	Y
S08 (Ovucire WL3264: mineral oil 80: 20 w/w)	10 – 15	1	Y
S09 (Ovucire WL3264: mineral oil 90: 10 w/w)	10 – 15	1	(Y)
S11 (PEG 1000: PEG 400 90: 10 w/w)	Up to 10	2	(Y)
S12 (Suppocire AM: mineral oil 70: 30 w/w)	Up to 10	1	Y
S17 (Ovucire WL3264: PEG 400 90: 10 w/w)	10 – 15	1	Y
S21 (Ovucire 3460: mineral oil 80: 20 w/w)	Up to 10	3	N

Y: Yes; N: No; (Y): Potential

(f) Short-term stability of selected formulations

Following on from the development of suppository formulations for NVN1000, MedPharm manufactured S06, S07, S08, S12 and S17 for assessment of prototype stability. The compositions of the manufactured formulations are shown in Table 14. As NVN1000 was present as a suspension, and no development into the manufacturing process had been performed at this stage, each aliquot was prepared as a separate manufacture from a bulk of vehicle. This was performed to avoid taking samples from a potentially heterogenous bulk of formulation. The formulations were manufactured and placed on stability at 2-8 and 25 °C for 12 weeks, and analyzed for NVN1000 content and purity, melting profile, macroscopic appearance, and microscopic appearance.

Table 14: Compositions (% w/w) of the formulations manufactured for short-term stability testing.

Excipient	Compositions (% w/w)									
	S06		S07		S08		S12		S17	
	Active	Placebo	Active	Placebo	Active	Placebo	Active	Placebo	Active	Placebo
NVN1000	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-
Ovucire WL3264	-	-	69.30	70.00	79.20	80.00	-	-	89.10	90.00
Suppocire D	-	-	29.70	30.00	-	-	-	-	-	-
Suppocire AM	49.50	50.00	-	-	-	-	69.30	70.00	-	-
PEG 1000	49.50	50.00	-	-	-	-	-	-	-	-
Mineral oil	-	-	-	-	19.80	20.00	29.70	30.00	-	-
PEG 400	-	-	-	-	-	-	-	-	9.90	10.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

(g) NVN1000 content and purity during short-term stability

Refrigerated (2-8 °C) and room temperature (25 °C) stability at 4, 8, and 12 weeks was performed for the 5 selected formulation prototypes. The week 12 timepoint is highlighted below in blue. The percentage recovery (%) and percentage peak purity (% area) of NVN1000 in formulations was assessed at t=0 and following t=4, 8 and 12 weeks at 2-8 and 25 °C, and the results have been presented in Table 15 and Table 16, respectively.
The mean recoveries for all formulations at t=0 was within 90 – 110% NVN1000, indicating all were successfully extracted, and that no substantial drop in the content of NVN1000 in the formulations was observed. The results for the percentage peak purity of NVN1000 in the formulations was in alignment with this, where no notable drop in NVN1000 purity was observed compared to a standard of ca. 400 µg/mL NVN1000. At this stage of the project MedPharm included the relative response factors (RRFs) provided by the Sponsor for the known impurities of NVN1000 (N-nitroso-MAP3, N-nitro-MAP3, nitrite and nitrate) in the calculations for NVN1000 percentage peak purity. This resulted in higher purities (ca. 95% area compared to ca. 92% area), although most importantly they trended consistently across formulations.

Following t=4 weeks of storage at 2-8 °C, minimal NVN1000 degradation occurred in S06, with ca. 5% area drop, and then little change was observed at the subsequent timepoints (ca. 91% area). Additionally, only a slight decrease in the percentage peak purity of NVN1000 was noted in S17 (95.97% area at t=0, compared to 93.59% area following t=4 weeks at 2-8 °C). Following t=8 weeks of storage, a substantial drop (ca. 25% area) in the percentage peak purity of NVN1000 in S17 was observed, with this continuing at the t=12-week timepoint (40% area) and the variability between replicates also increased.

For the remaining formulations, degradation of >30% area was observed following t=12 weeks at 2-8 °C While at 25 °C, degradation of >75% area was noted in all formulations following t=12 weeks. It should be noted that there was a high level of variability between replicates for the percentage peak purity and the percentage recovery of NVN1000. Due to the consistency of the data at t=0, and during formulation development, it is hypothesized that the variability is not due to inefficient extractions. However, it is possible that the variability between replicates was a result of heterogeneously distributed NVN1000 within each sample due to the fact NVN1000 is present as a suspension and the generic manufacturing method employed for all prototypes. This may have caused some samples to have a larger surface area of NVN1000 particulates in contact with the formulation vehicle, potentially causing an increased rate of degradation compared to others.

Formulation S06 is the most promising formulation when stored at 2-8 °C indicating that NVN1000 may be more stable in PEG 1000 than the hard-fat excipients. Regardless, data suggest that refrigeration of the final product will be required due to the sensitivity of NVN1000 to heat.

Table 155. Percentage recovery (%) of NVN1000 in the developed formulations at t=0 and following up to t=12 weeks at 2-8 and 25 °C.

Formulation	Percentage recovery (%) of NVN1000						
	T=0	T=4 weeks		T=8 weeks		T=12 weeks	
		2-8 °C	25 °C	2-8 °C	25 °C	2-8 °C	25 °C
S06	96.10 (91.19 - 99.29)	88.47 (79.76 - 94.92)	57.11 (53.17 - 60.07)	89.85 (83.59 - 98.35)	25.70 (13.79 - 34.35)	97.90 (91.81 - 101.78)	13.58 (0.00 - 21.92)
S07	100.08 (96.45 - 102.97)	75.35 (67.72 - 79.59)	11.99 (6.05 - 16.14)	55.72 (52.80 - 60.73)	10.95 (0.65 - 24.26)	33.94 (28.93 - 40.77)	0.44 (0.00 - 1.32)
S08	98.58 (96.77 - 99.71)	76.98 (74.66 - 80.44)	26.52 (9.40 - 37.69)	64.61 (39.26 - 96.02)	1.09 (0.49 - 1.59)	54.09 (36.82 - 70.85)	0.30 (0.00 - 0.90)
S12	93.27 (91.25 - 94.84)	78.77 (45.42 - 96.26)	59.12 (30.40 - 76.28)	41.90 (33.04 - 49.50)	35.77 (9.06 - 50.71)	70.34 (13.66 - 99.09)	14.43 (1.30 - 29.04)
S17	92.51 (86.69 - 97.32)	92.97 (90.12 - 94.74)	1.26 (0.68 - 1.72)	66.62 (30.47 - 94.37)	1.39 (0.04 - 3.60)	40.19 (19.89 - 64.09)	0.00 (0.00 - 0.00)

Table 16. Percentage peak purity (% area) of NVN1000 in the developed formulations at t=0 calculated with and without RRFs and following up to t=12 weeks at 2-8 and 25 °C calculated with the RRFs, n=3.

Formulation	Percentage peak purity (% area) of NVN1000							
	T=0		T=4 weeks		T=8 weeks		T=12 weeks	
	Without RRFs	With RRFs	2-8 °C	25 °C	2-8 °C	25 °C	2-8 °C	25 °C
QC2 (ca. 400 µg/mL NVN1000)	92.29*	96.00*	N/A					
S06	91.55 (91.13 - 91.92)	95.45 (95.18 - 95.67)	90.92 (87.97 – 93.24)	66.94 (63.33 - 69.43)	90.70 (87.38 - 93.77)	37.63 (26.25 - 47.28)	91.01 (90.56 - 91.28)	19.32 (0.00 - 30.67)
S07	92.08 (91.96 - 92.20)	95.74 (95.66 - 95.82)	83.24 (78.17 - 85.99)	23.78 (13.84 - 30.21)	71.32 (68.80 - 75.69)	16.23 (1.59 - 29.73)	46.61 (42.01 - 54.73)	0.95 (0.00 - 2.86)
S08	92.84 (92.84 - 92.85)	96.27 (96.27 - 96.28)	86.23 (83.35 - 91.79)	37.40 (21.35 - 47.20)	74.99 (59.39 - 91.19)	2.81 (1.26 - 4.02)	59.77 (51.54 - 67.49)	0.71 (0.00 - 2.12)
S12	91.83 (91.71 - 92.02)	95.58 (95.47 - 95.79)	83.47 (63.42 - 93.80)	69.00 (50.66 - 79.73)	59.08 (51.75 - 64.71)	51.32 (20.31 - 72.22)	68.39 (24.22 - 90.57)	22.93 (3.05 - 41.61)
S17	92.35 (92.31 - 92.40)	95.97 (95.90 - 96.03)	93.59 (92.38 - 94.20)	1.84 (1.04 - 2.52)	70.00 (37.61 - 94.14)	1.86 (0.12 - 4.67)	40.00 (22.42 - 63.34)	0.00 (0.00 - 0.00)

RRFs – Relative Response Factors
(*) – n=1

(h) Melting profile during short-term stability

The melting profile of the vehicle formulations at 37 °C was assessed at t=0 and up to t=12 weeks at 2-8 and 25 °C. The time until full melt was observed and is detailed in Table 17. The 12-week timepoint is highlighted in blue below. Only vehicle formulations were assessed, as previous experiments showed that there was no material difference between the melt profile of active and vehicle formulations.

At t=0, the melt profile of the formulations was comparable to those determined during formulation development, with slight variations to be expected from this subjective assessment. The exception to this was S07, where full melt was not observed following t=60 mins, while a melt time of 30 – 35 mins was observed during formulation development. During assessment it was noted that the outer layer of the suppository did melt following

t=30 mins, with no further change after this point. It is possible that this suppository was non-homogenous.

No material changes in the melting profile of any of the formulations was noted following t=12 weeks at 2-8 or 25 °C with the exception of S07. As S07 did not melt following t=60 mins at t=0, this formulation was further assessed for up to 2 hours at the subsequent timepoints. Following t=60 mins, the vials were periodically agitated to aid melting, and high levels of variability between timepoints and temperatures were observed. These results are not in alignment with formulation development, and it is unlikely to be due to heterogeneity due to the consistently long melt times for this formulation. Further investigations would be required to inform a hypothesis, and these could comprise multiple set ups with some containing different batches of each excipient to determine if the variability in the manufacturing method has caused the change in melting profile. However, based on the NVN1000 content and purity data which showed S07 as unlikely to be the lead formulation due to substantial NVN1000 degradation no investigations were conducted.

Table 17. Melting time of the developed vehicle formulations at t=0 and following up to t=12 weeks at 2-8 and 25 °C.

Formulation	Melting time (min)						
	T=0	T=4 weeks		T=8 weeks		T=12 weeks	
		2-8 °C	25 °C	2-8 °C	25 °C	2-8 °C	25 °C
S06	35	20	25	20	25	20	25
S07	>60	80*	110*	60	105*	50	70*
S08	20	20	25	20	25	20	20
S12	10	10	10	10	10	10	10
S17	20	25	20	25	25	20	25

(*) Following t=60 mins the vial was periodically agitated to aid melting.

(i) Macroscopic appearance during short-term stability

The macroscopic appearance of the formulations was assessed at t=0 and following up to t=12 weeks at 2-8 and 25 °C, and the results have been presented in Table 18, with representative images for t=0 and week 12 in Table 19 and Table 20, respectively. The 12 week timepoint is highlighted in blue below in Table 18. All formulations were described as opaque solids, with all actives being described as faint yellow, except S06 ACT and S08 ACT which were white. This faint yellow coloration for active formulations was also observed during formulation development. S06 was also the exception to the trend for the placebos, where all other placebos were described as white, while the placebo for S06 was faint grey.
Following up to t=12 weeks of storage at 2-8 and 25 °C, there was no material change in the macroscopic appearance of the majority of the formulations. The exceptions to this were the active samples of S06 which had turned faint yellow following t=4 weeks at 25 °C, and at both temperatures following t=8 and 12 weeks. This color change also occurred in S08 following t=4 and 8 weeks at 25 °C, and at both temperatures following t=12 weeks. S06 placebo had also turned white at 2-8 and 25 °C.. Additionally, two layers were observed in S06 active at every timepoint at 25 °C, where the top appeared more yellow than the bottom. It is possible that this is due to separation of the excipients during initial formulation

processing, however further investigation would be required to confirm if this is the case, and alternative manufacturing methods may resolve this issue. Additionally, this more dominant yellow discoloration at the top of the sample indicates that it is possible NVN1000 was not homogenously distributed throughout the sample, and degradation of the API has caused the yellow discoloration.

Table 18. Macroscopic appearance of the developed formulations at t=0 and following up to t=12 weeks at 2-8 and 25 °C.

Formulation	Macroscopic appearance						
	T=0	T=4 weeks		T=8 weeks		T=12 weeks	
		2-8 °C	25 °C	2-8 °C	25 °C	2-8 °C	25 °C
S06 ACT	White, opaque, solid	No obvious change from t=0	Faint yellow, opaque, solid (two layers observed)	Faint yellow, opaque, solid	Faint yellow, opaque, solid (two layers observed)	Faint yellow, opaque, solid	Faint yellow, opaque, solid (two layers observed)
S06 PBO	Faint grey, opaque, solid	White, opaque, solid					
S07 ACT	Faint yellow, opaque, solid	No obvious change from t=0					
S07 PBO	White, opaque, solid						
S08 ACT	White, opaque, solid	No obvious change from t=0	Faint yellow, opaque, solid	No obvious change from t=0	Faint yellow, opaque, solid	Faint yellow, opaque, solid	
S08 PBO		No obvious change from t=0					
S12 ACT	Faint yellow, opaque, solid	No obvious change from t=0					
S12 PBO	White, opaque, solid						
S17 ACT	Faint yellow, opaque, solid	No obvious change from t=0					
S17 PBO	White, opaque, solid						

Table 16: Macroscopic images of the developed formulations at t=0.

System	Macroscopic images
S06	
S07	
S08	

Table 19 (cont.): Macroscopic images of the developed formulations at t=0.

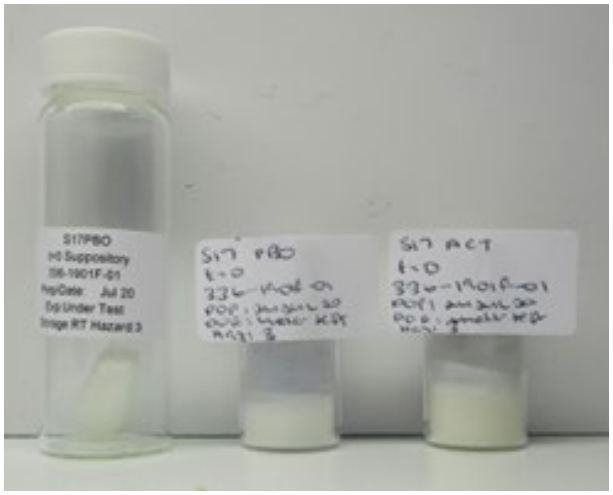
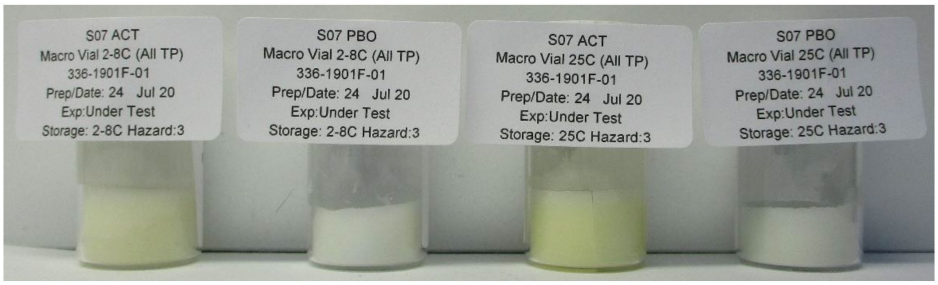
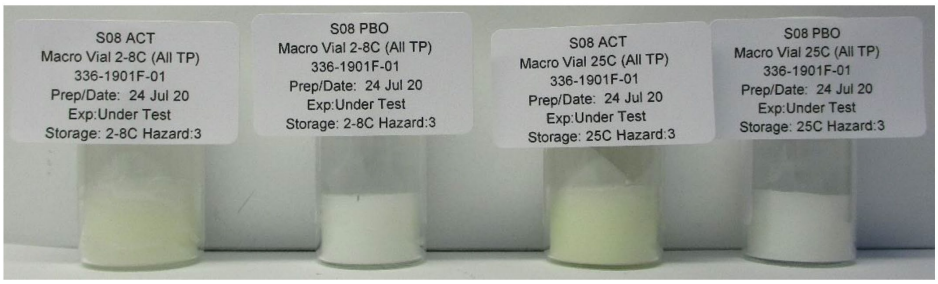
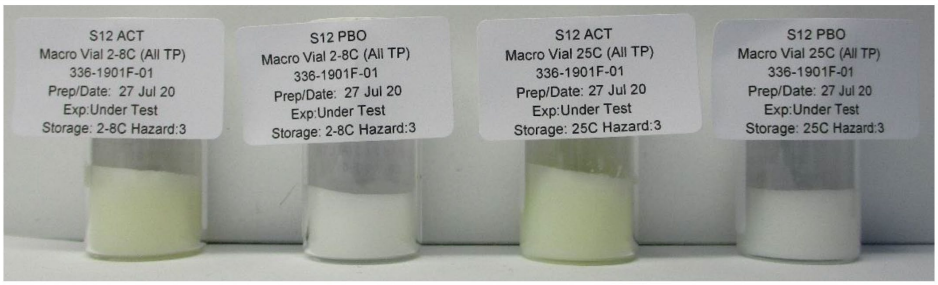
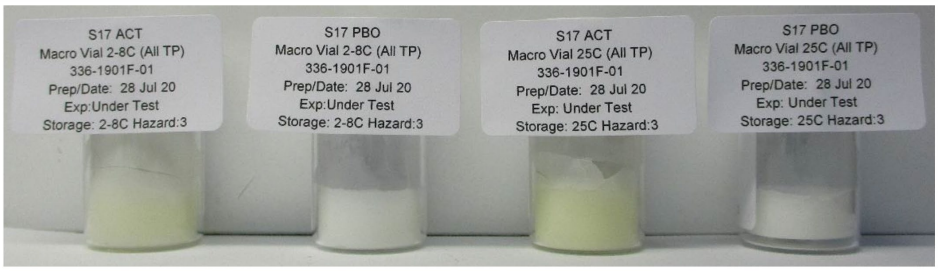
System	Macroscopic images
S12	
S17	

Table 20. Macroscopic images of the developed formulations following t=12 weeks.

System	Macroscopic images
S06	
S07	
S08	
S12	
S17	

(j) Microscopic appearance during short-term stability

The microscopic appearance of the formulations was assessed at t=0 and following t=12 weeks at 2-8 and 25 °C, and the results have been presented in Table 21 and Table 22, respectively. As with the observations during formulation development, it was challenging to identify drug particulates in the microscopy due to the large number of excipient particulates present in the formulations.

Table 21. Microscopic appearance of the developed formulations at t=0.

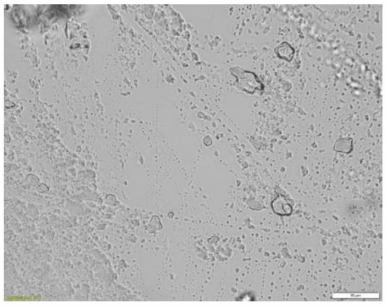
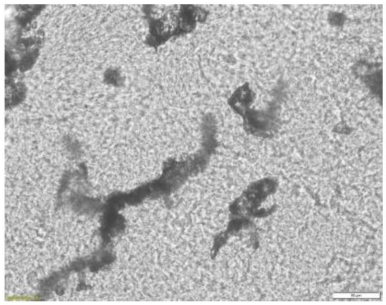
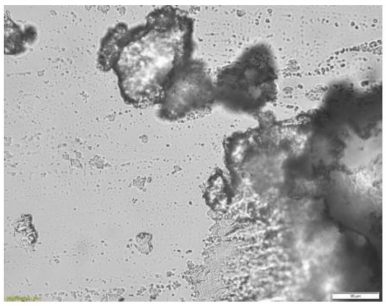
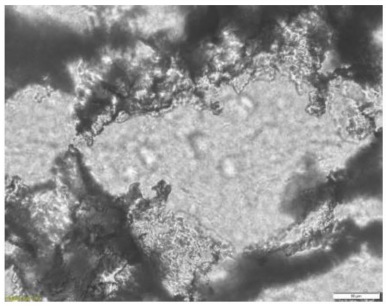
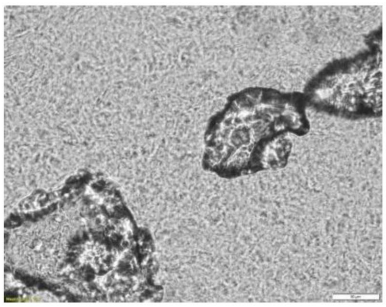
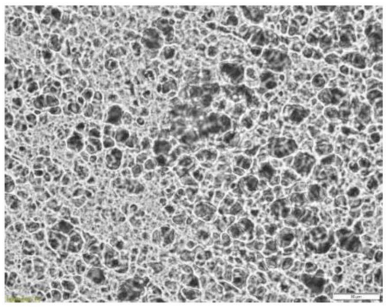
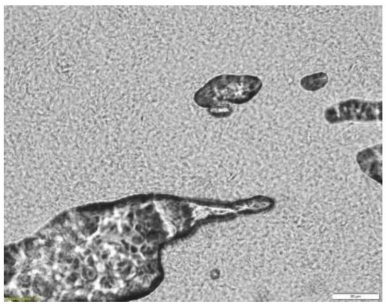
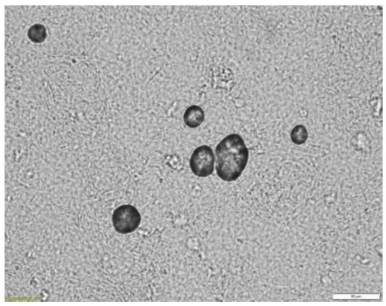
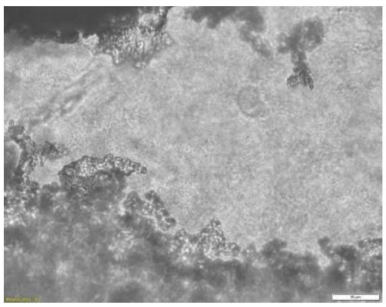
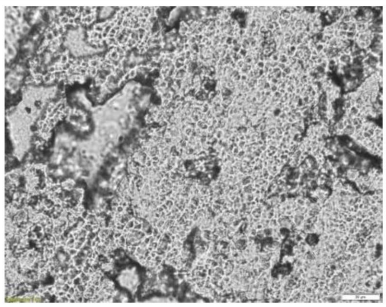
System	Microscopic appearance at t=0	
	Vehicle	Active
S06		
S07		
S08		
S12		
S17		

Table 22. Microscopic appearance of the developed formulations following t=12 weeks at 2-8 and 25 °C.

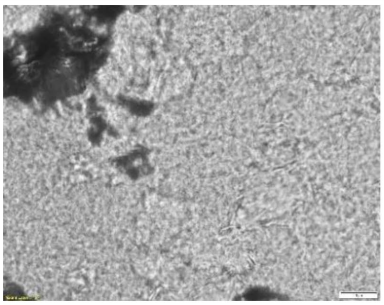
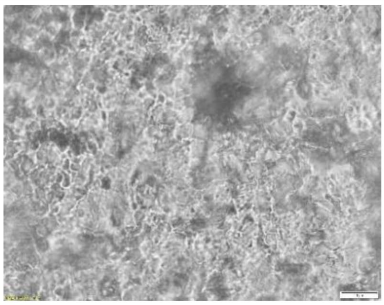
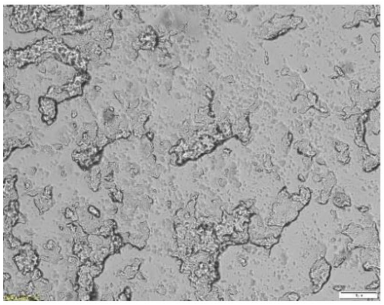
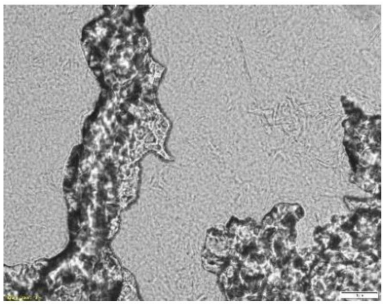
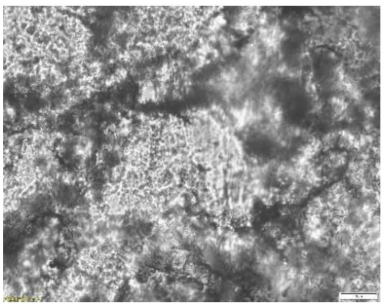
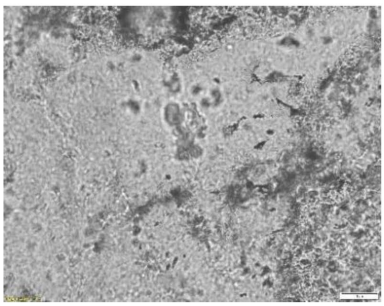
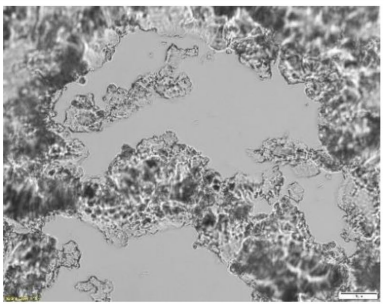
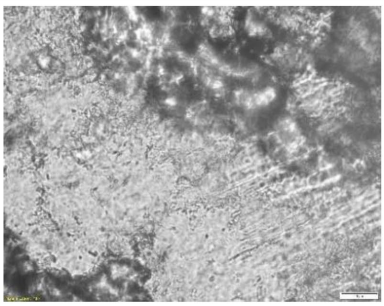
System		Microscopic appearance at t=12 weeks	
		2-8 °C	25 °C
S06	Active		
	Vehicle		
S07	Active		
	Vehicle		

Table 22 (continued). Microscopic appearance of the developed formulations following t=12 weeks at 2-8 and 25 °C.

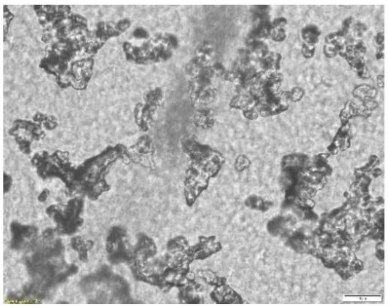
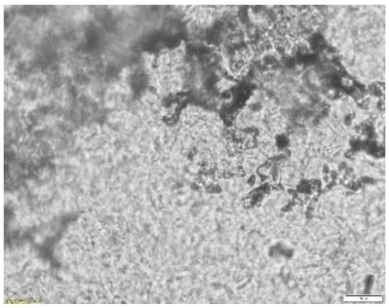
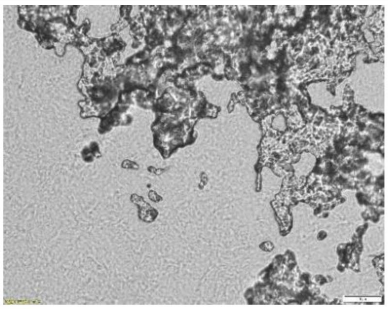
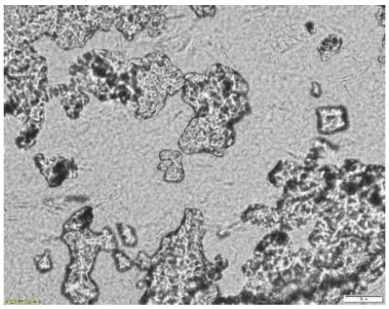
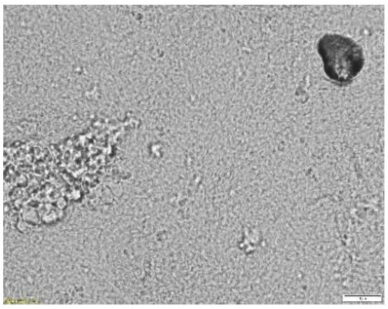
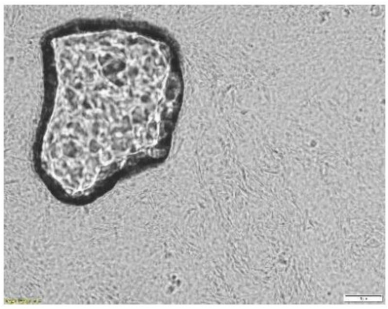
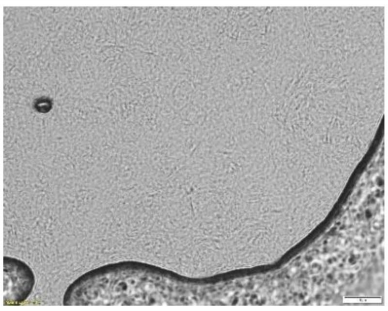
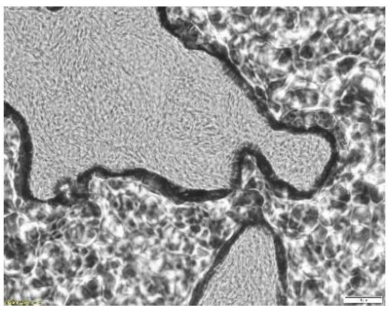
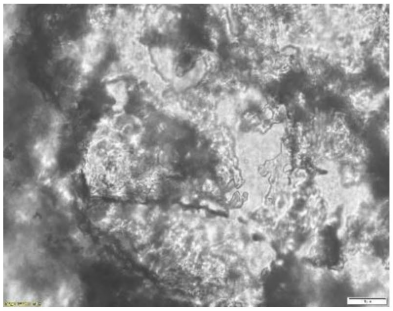
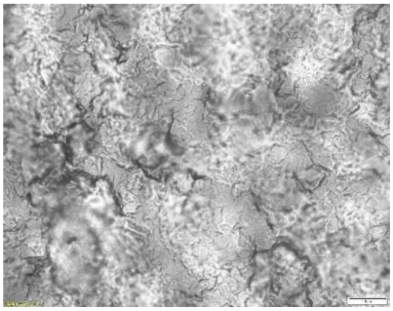
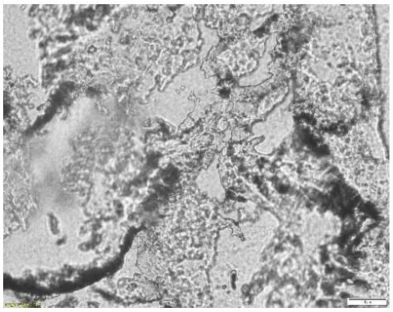
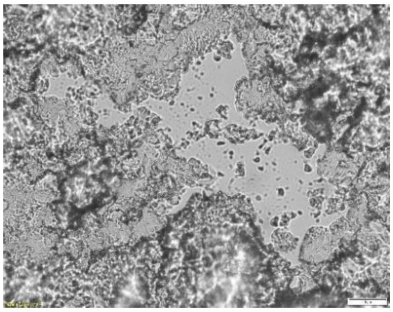
System		Microscopic appearance at t=12 weeks	
		2-8 °C	25 °C
S08	Active		
	Vehicle		
S12	Active		
	Vehicle		

Table 22 (continued). Microscopic appearance of the developed formulations following t=12 weeks at 2-8 and 25 °C.

System		Microscopic appearance at t=12 weeks	
		2-8 °C	25 °C
S17	Active		
	Vehicle		

(k) Selection of Formulations for Future Assessment

Based on the results of the stability testing, a decision matrix was prepared to aid the selection of formulation(s) for future assessment (Table 24). The parameters and criteria assessed are presented in Table 23. MedPharm recommended the selection of S06 (colored in green) due to its superior chemical stability at 2-8 °C.

Table 23. Parameters and criteria assessed in the decision matrix.

Parameter	Criteria	
Purity (% peak area) of NVN1000 following t=12 weeks at 2-8 °C	1	>90%
	2	80 – 90%
	3	<80%
Melting time following t=12 weeks at 2-8 °C	1	<20 mins
	2	20 – 30 mins
	3	>30 mins
Macroscopic appearance (actives)	1	No change from t=0
	2	Colour change from t=0
	3	Formulation separation observed at 25 °C

Table 24. Decision matrix to aid in the selection of formulations for future assessment.

Parameter	S06	S07	S08	S12	S17
Purity (% peak area) of NVN1000 following t=12 weeks at 2-8 °C	1	3	3	3	3
Melting time following t=12 weeks at 2-8 °C	2	3	2	1	2
Macroscopic appearance (actives)	3	1	2	1	1
Recommended for progression	Y	N	N	N	N

Y: Yes; N: No

Following t=12 weeks, S06 appears to be the most promising formulation candidate based on NVN1000 purity at 2-8 °C, indicating that NVN1000 may be more stable in PEG 1000 than the hard-fat excipients. All formulations were unstable at 25 °C and as such it is likely that refrigeration of the final product will be required. No material difference in the melt profile of any formulation was observed up to t=12 weeks at 2-8 or 25 °C, except for S07, where highly variable melt times were observed. In terms of visual appearance, no changes from t=0 were observed, except for the active samples for S06 and S08 which had become faint yellow at 25 °C (and t=8 weeks 2-8 °C for S06), with a stronger yellow discoloration in the top of the S06 samples potentially indicating separation of the excipients or heterogeneously distributed NVN1000. In the future, it may be possible to resolve these issues with S06 by improving the manufacturing process for the formulation.

Specific Aim 1.1, Deliverable 3: Development of a stability indicating chromatography method for the routine characterization of vaginal suppository at release and over time on stability.

Due to the variability of the initial NVN1000 excipient compatibility data (see Specific Aim 1.1, Deliverable 1), further analytical method development was performed by MedPharm on the extraction method. To further improve the extraction of NVN100 from excipients during analysis, a range of organic solvents potentially suitable for the liquid-liquid extraction of NVN1000 from oleaginous excipients was performed. These solvents and their justification for selection are presented in Table 25 below.

Table *25*. Organic solvents assessed initially, along with the justification for their selection

Organic solvent	**Justification for selection**
Chloroform	Used in the previous extraction procedure. Included as a reference solvent, and to determine if this component of the previous extractions caused the variable results.
Hexane	Aprotic hydrocarbon that MedPharm has experience using in liquid-liquid extractions in other products of this type.
Dichloromethane (DCM)	Alternative organochlorine compound to chloroform. Polar, but immiscible with water.
Diethyl ether	Aprotic, nonpolar solvent commonly used in liquid-liquid extractions

The recovery of NVNV1000 was measured using the MAP3-NONOate component as a marker, under the assumption that the other components (TEOS and MAP3), silane impurities (nitroso-MAP3 and nitro-MAP3) and non-silane impurities (nitrite and nitrate) have similar behavior. A series of experiments designed to assess the suitability of the extraction solvents is detailed below.

(a) Stability of NVN1000 (hydrolyzed) in organic solvents

The stability of hydrolyzed NVN1000 (i.e., broken down into its hydrolyzed monomers, as is typical for chromatographic evaluation) was evaluated in the organic solvents to ensure that nitric oxide from MAP3-NONOate does not decompose during extraction. A standard solution of NVN1000 (200 µg/mL MAP3-NONOate in 30 mM potassium hydroxide; 1.5 mL) was stirred in the organic solvents (2 mL) for 1, 3 and 5 hours to determine if they impacted the stability of the MAP3-NONOate. The data are shown in Table 26. Stir time did not appear to have a substantial effect on the stability of NVN1000 in any of the solvents, however lower percentage peak purities were generated from the diethyl ether sample. Thus, diethyl ether was not carried forward for further testing due to a lower observed peak purity compared to the other solvents, indicating instability of NVN1000 in that solvent.

Table *26*. Percentage peak purity of MAP3-NONOate following 1, 3 and 5 hrs of a 200 µg/mL MAP3-NONOate standard (in 30 mM potassium hydroxide) stirring in a selection of organic solvents.

Organic solvent	Timepoint	Percentage peak purity (% area)
QC2 (200 µg/mL MAP3-NONOate)	N/A	92.29
Chloroform	1 hr	92.45
	3 hr	92.38
	5 hr	92.40
Hexane	1 hr	92.35
	3 hr	92.42
	5 hr	92.38
DCM	1 hr	92.34
	3 hr	92.37
	5 hr	92.35
Diethyl Ether	1 hr	88.27
	3 hr	88.18
	5 hr	87.75

Example chromatograms of a standard solution of NVN1000 (200 µg/mL MAP3-NONOate in 30 mM potassium hydroxide (Figure 1) and the NVN1000 standard solution stirred in DCM (Figure 2) and diethyl ether (Figure *3*) for 5 hours are shown below.

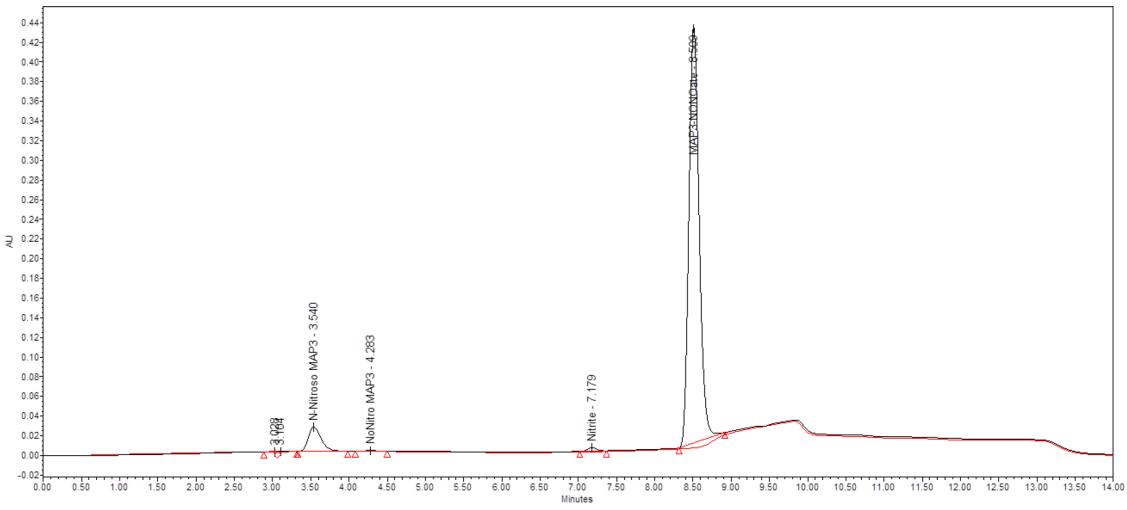


Figure 1. Example chromatogram of an NVN1000 standard (200 µg/mL MAP3-NONOate; black) overlaid with a blank (sample diluent; red).

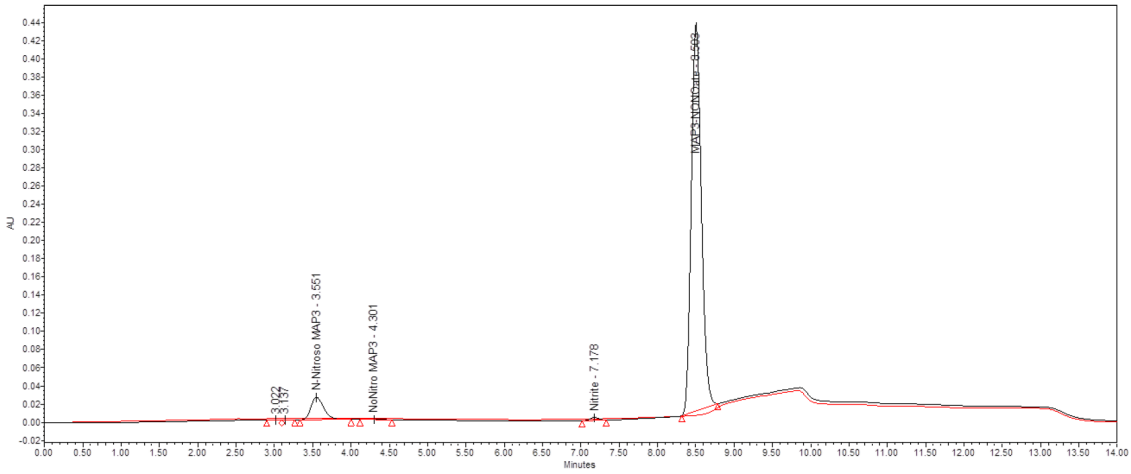


Figure 2. Example chromatogram of DCM following t=5 hours (black), overlaid with a blank (sample diluent; red).

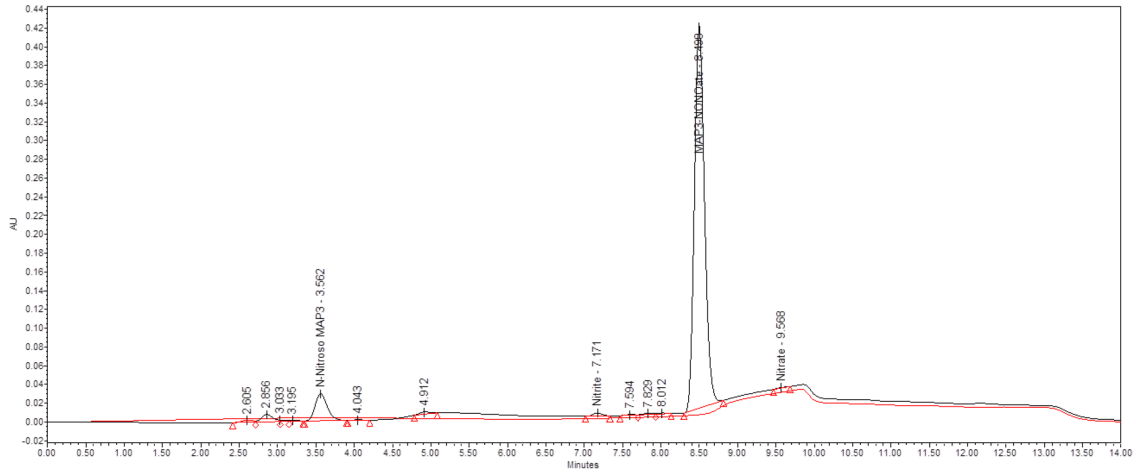


Figure 3. Example chromatogram of diethyl ether following t=5 hr (black) overlaid with a blank (sample diluent; red).

(b) Stability of NVN1000 (un-hydrolyzed) in organic solvents

The stability of un-hydrolyzed (i.e., intact) NVN1000 was evaluated in the organic solvents to ensure that nitric oxide from NVN1000 is not released during extraction. Suspensions of NVN1000 in the organic solvents were set up and stirred for 3 hours prior to extraction. The data are shown in Table 27. Slight downward trends in percentage peak purity were noted in all organic solvents over time. Additionally, NVN1000 was slightly less stable in hexane and most stable in DCM and chloroform. Based on these results, all organic solvents were progressed, and stir times were looked to be minimized where possible.

Table *27*. Percentage peak purity of MAP3-NONOate following 1, 3 and 5 hours of stirring in a selection of organic solvents.

Organic solvent	Timepoint	Percentage peak purity (% area)
Chloroform	1 hr	92.81 (92.77 – 92.84)
	3 hr	92.40 (92.34 – 92.46)
	5 hr	92.25 (92.05 – 92.37)
Hexane	1 hr	92.78 (92.72 – 92.86
	3 hr	92.14 (91.93 – 92.46)
	5 hr	91.96 (91.78 – 92.24)
DCM	1 hr	93.03 (93.01 – 93.04)
	3 hr	92.32 (92.28 – 92.39)
	5 hr	92.26 (92.22 – 92.33)

(c) Dissolution of excipients in organic solvents

The ability of each of the organic solvents to dissolve Suppocire DM, a representative excipient, was evaluated as follows. Suppocire DM pellets (10 g) were melted and allowed to solidify. Various volumes of the organic solvents were added, and the dissolution time of the excipient assessed. Data are shown in Table 28. Hexane (25 mL) was unable to fully dissolve Suppocire DM (10 g) following 1 hour of stirring and therefore this solvent was eliminated from testing at this stage. It was determined that a minimum ratio of 1:1 w/v Suppocire DM: Organic solvent was required to suitably dissolve the excipient. Additionally, approximate stir times for chloroform and DCM were established for testing in further experiments.

Table *28*. Results of the dissolution of Suppocire DM (10 g) in organic solvents.

Volume of organic solvent	Parameter	Organic solvent		
		Chloroform	DCM	Hexane
25 mL	Vortex time to free up stirrer bar	Approx. 45 s		
	Stirring time until full dissolution	4.5 min	4.5 min	>1 hour
15 mL	Vortex time to free up stirrer bar	3 min	2 min	Not tested
	Stirring time until full dissolution	14 min	8 min	
10 mL	Vortex time to free up stirrer bar	4 min	2 min	
	Stirring time until full dissolution	19 min	26 min	
5 mL	Vortex time to free up stirrer bar	12. 5 min	6 min	
	Stirring time until full dissolution	Mixture too thick	Mixture too thick	

(d) Stability of NVN1000 in various concentrations of potassium hydroxide (KOH)

To optimize the concentration of KOH used during the extraction, the stability of NVN1000 as a function of KOH concentration was evaluated. NVN1000 (ca. 400 µg/mL) was stirred in various concentrations of potassium hydroxide at ambient laboratory temperature and analyzed. Data are shown in Table 29. Poor stability of NVN1000 was noted in the lower concentration of (15 mM) KOH, while slightly higher percentage peak purities were observed in the higher concentration (50 mM) of KOH. Specifically, 15 mM KOH was not selected for further investigation due to lower observed peak purity at 24 hr, indicating instability of NVN1000 in the least alkaline concentration, as expected based on the pH-dependent decomposition of NVN1000 to release NO.

Table *29*. Percentage peak purity (% area) following various stir times in 15, 30, and 50 mM KOH.

Concentration of KOH	Timepoint	Percentage peak purity (% area)
15 mM KOH	1 hr	92.26
	24 hr	87.94
30 mM KOH	1 hr	92.56
	3 hr	92.49
	5 hr	92.48
	24 hr	90.57
50 mM KOH	1 hr	92.57
	3 hr	92.55
	5 hr	92.63
	24 hr	91.31

(e) Evaluation of potassium hydroxide concentration and stir time

The extraction parameters were further optimized in a trial extraction by varying the organic solvent, KOH concentration, and stir time. NVN1000 (0.1% w/w) was dispersed in mineral oil before dissolution in chloroform and DCM, 30 or 50 mM KOH was added and stirred for 1, 3 or 5 hours (Table 30). Lower recoveries of NVN1000 were noted when using 30 mM KOH with both organic solvents. For chloroform, minimal correlation of stir time of 50 mM KOH with recovery was observed. It was noted that 1 or 3 hr stir with 50 mM KOH offered the best recoveries from DCM, thus 50 mM KOH was selected for further progression.

Table *30*. Percentage recovery (%) of NVN1000 from chloroform and DCM following 1, 3 and 5 hr of stir with 30 or 50 mM KOH.

Organic solvent	Sample diluent	Percentage recovery from theoretical concentration (%)		
		1 hr	3 hr	5 hr
Chloroform	30 mM KOH	86.04 (75.05 - 93.54)	-	89.55 (83.37 - 96.36)
	50 mM KOH	98.21 (96.80 - 100.33)	99.89 (91.06 - 108.82)	101.08 (97.34 - 105.17)
DCM	30 mM KOH	92.43 (88.54 - 94.97)	-	92.67 (92.57 - 92.73)
	50 mM KOH	104.41 (97.93 - 108.51)	97.59 (84.53 - 104.84)	88.19 (86.30 - 91.59)

In order to further support the selection of DCM, and inform the selection of a KOH stir time, NVN1000 (0.1% w/w) was dispersed in Suppocire DM and the same experiment performed, but with only 50 mM KOH (Table 31). It can be seen from the %CV that the most consistent recoveries were observed when Suppocire DM was dissolved in DCM.

Based on the data, DCM and a 1 hour stir with 50 mM KOH were selected as optimal; however, a 3 hour stir time is also a viable option but did not offer a benefit in extraction performance based on the experiments conducted.

Table *31*. Model extractions at a target concentration of 200 µg/mL MAP3-NONOate from Suppocire DM (ca. 0.1% w/w NVN1000) with Chloroform and DCM and various stir times of 50 mM KOH.

Organic solvent	Stir time in 50 mM KOH	Percentage peak purity (% area)	Percentage recovery from theoretical concentration (%)	
			Mean	% CV
Chloroform	1 hr	92.89 (92.88 – 92.92)	100.32 (93.59 - 106.45)	6.43
	3 hr	92.60 (92.52 – 92.65)	100.62 (96.10 - 106.07)	5.02
	5 hr	92.60 (92.54 – 92.66)	96.82 (92.22 - 100.75)	4.44
DCM	1 hr	92.81 (92.80 – 92.83)	103.78 (101.61 - 105.67)	1.97
	3 hr	92.86 (92.83 – 92.89)	99.77 (98.11 - 102.88)	2.70
	5 hr	92.64 (92.63 – 92.66)	98.56 (94.87 - 100.86)	3.27

(f) Confirmatory extractions

Using the optimized extraction parameters (DCM, 1 hr stir time with 50 mM KOH), MedPharm performed confirmatory extractions (n=3) from each excipient. The higher target extraction concentration (650 µg/mL MAP3-NONOate) was selected as it is a more challenging extraction concentration to achieve. NVN1000 drug loadings of 0.1 and 1.0% w/w were assessed. The percentage peak purity and percentage recovery of NVN1000 are presented in Table 32.

Consistent percentage peak purities were observed, indicating minimal degradation of NVN1000 during sample set up and extraction. The low percentage peak purity observed from Ovucire 3460 0.1% w/w NVN1000 was caused by a growth in an impurity peak at RRT 0.82.

Mean recoveries within 100 ± 10% were generated from all excipients, with the highest % CV being from Suppocire DM 1.0% w/w NVN1000 (6.48% CV). While these results were outside of the success criteria defined at the start of the development (>95% percentage peak purity), the extraction procedure was deemed suitable for screening during excipient compatibility and formulation stability experiments.

Table 32. Model extractions (at a target concentration 650 µg/mL MAP3-NONOate) from the three excipients loaded at 0.1 or 1.0% w/w NVN1000. Data is presented as the mean of n=3, with the range in brackets.

Excipient	NVN1000 loading (% w/w)	Percentage peak purity (% area)	Percentage recovery from theoretical concentration (%)	% CV
Suppocire DM	0.1	92.38 (92.36 – 92.39)	96.32 (90.97 - 99.95)	4.91
	1.0	92.47 (92.42 – 92.50)	97.44 (92.36 - 104.51)	6.48
Mineral oil	0.1	92.57 (92.54 – 92.61)	93.49 (88.59 - 96.26)	4.55
	1.0	92.50 (92.46 – 92.53)	97.70 (96.97 - 99.06)	1.21
Ovucire 3460	0.1	91.56 (91.46 – 91.65)	92.38 (89.86 - 96.60)	3.98
	1.0	92.55 (92.49 – 92.61)	94.92 (92.81 - 98.35)	3.15

(g) LOQ evaluation

A batch of MAP3-NONOate reference standard (MP20200087) was used by MedPharm to assess the LOQ of the optimized method. MedPharm's criteria for the limit of quantification of a method is that a signal/ noise ratio (s/n) of >10 be measured when a standard of that concentration is analyzed on the method. The results are presented in Table 33.

An LOQ of 0.3 µg/mL NVN1000 (based on NVN1000 being 54.7% MAP3-NONOate) was selected since the s/n for the 0.3 µg/mL MAP3-NONOate standard was 13.95. Based on ICH Q3B (R2), which states impurities of 0.05% a/a should be reported for drug products with a maximum daily dose equivalent to >1 g of drug substance, a target extraction concentration of 1000 µg/mL NVN1000 was selected.

Table 33: Signal/ noise ratio calculated following the analysis of several standard solutions generated with the new MAP3-NONOate reference material. Concentrations have been presented in terms of MAP3-NONOate and NVN1000 (converted based on a MAP3-NONOate content of 54.7% in NVN1000) in 50 mM KOH.

Solution concentration (µg/mL)		s/n	
as MAP3-NONOate	as NVN1000	Shimadzu system	Waters system
0.1	0.18	6.69	3.88
0.2	0.37	20.46	8.19
0.3	0.55	38.09	13.95
0.325	0.59	29.37	9.03
0.4	0.73	31.85	19.29
0.5	0.91	61.76	23.74
0.6	1.10	50.00	3.88

Based on the totality of the analytical method development data above, Novan and MedPharm agreed that the analytical method was suitable for evaluating lead candidates.

Specific Aim 1.1; Deliverable 4 (execution of stability testing program at 3 recommended ICH climate conditions for up to 6 months in duration targeting at least one formulation having the minimum acceptable stability for clinical use.), Specific Aim 2.1 (in vitro dissolution testing of vaginal suppository), and Specific Aim 3.1 (additional performance testing).

In July 2022, Novan met with SO/GOR Emilee Senkevitch and received permission to stop any further work on Specific Aim 1 (). Based on the data from the prototype formulations and the funds remaining at the time, we determined that we were unlikely to meet the Go/No-go goal of 12 weeks of room temperature stability for the prototype formulation. Formulation S06 remains to be the most promising formulation that was developed, albeit

requiring refrigerated conditions to assure stability through 12 weeks. The funds originally dedicated to the abandoned deliverables sub-aims of Specific Aim 1 were rebudgeted to Specific Aim 2.

Specific Aim 2: Evaluate the effect of varying concentrations and treatment durations of NVN1000 in HPV-18 infected human raft cell cultures.

Specific Aim 2.1: Evaluation of NVN1000's antiviral activity across a higher range of concentrations in vitro.

Specific Aim 2.1, Deliverable 1: Regulatory Review and approval by the USAMRMC Human Research Protection Office.

Regulatory review and approval by the USAMRMC Human Research Protection Office (HRPO) was completed on November 7, 2019.

Specific Aim 2.1, Deliverable 2: Established minimally effective dose response of NVN1000 required to inhibit viral replication >90% qPCR.

The effects of varying doses of NVN1000 on uninfected and HPV-18 infected raft cultures (RCs) of primary human keratinocytes (PHK) were evaluated at the University of Alabama at Birmingham (UAB). Uninfected and infected PHK raft cell cultures were grown for 6 days and then treated with 0, 1, 2, 3, 4, and 5 mg/mL NVN1000 in 45 mM HEPES for 1 hour daily on days 7-12. Cultures were harvested either on day 13, or after a 4-day drug-free chase on day 16. Harvested cultures were analyzed by histology, fluorescence in situ hybridization of HPV DNA, in situ immunofluorescence microscopy of host DNA replication, and of viral major capsid protein synthesis in tissue sections. Apoptosis was probed by the TUNEL assay. In addition, total DNA from the infected raft cultures were extracted and subject to qPCR analysis to determine the relative HPV DNA copy number / cell.

(a) Histology

Although not unexpected based on previous evaluations of NVN1000, there was an visible reduction in growth and differentiation of uninfected RCs exposed to 1 and 2 mg/mL NVN1000 vs vehicle with thinning of tissues, fewer healthy cells, decreased granulation, incomplete terminal differentiation with residual nuclei in the squam (Figure 4). Higher concentrations (3-5 mg/mL) produced higher toxicity to uninfected RCs, confirming a previously observed dose-response of NVN1000 to RC toxicity.

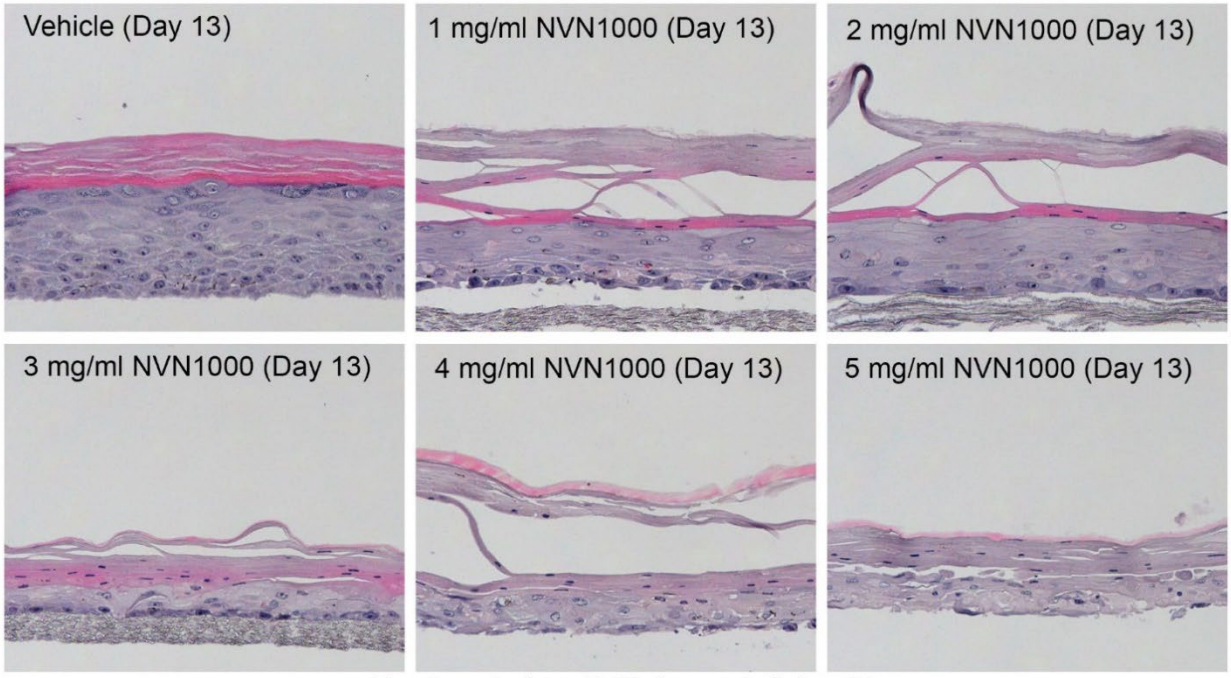


Figure 4. Histology of uninfected RCs after daily (days 7-12) 1 hr exposure to 0 (vehicle) or 1-5 mg/mL NVN1000 with harvesting on day 13.

Following a 4 day drug-free chase, the inhibitory or toxic effect of NVN1000 on growth and differentiation was persistent, but did not worsen. (Figure 5).

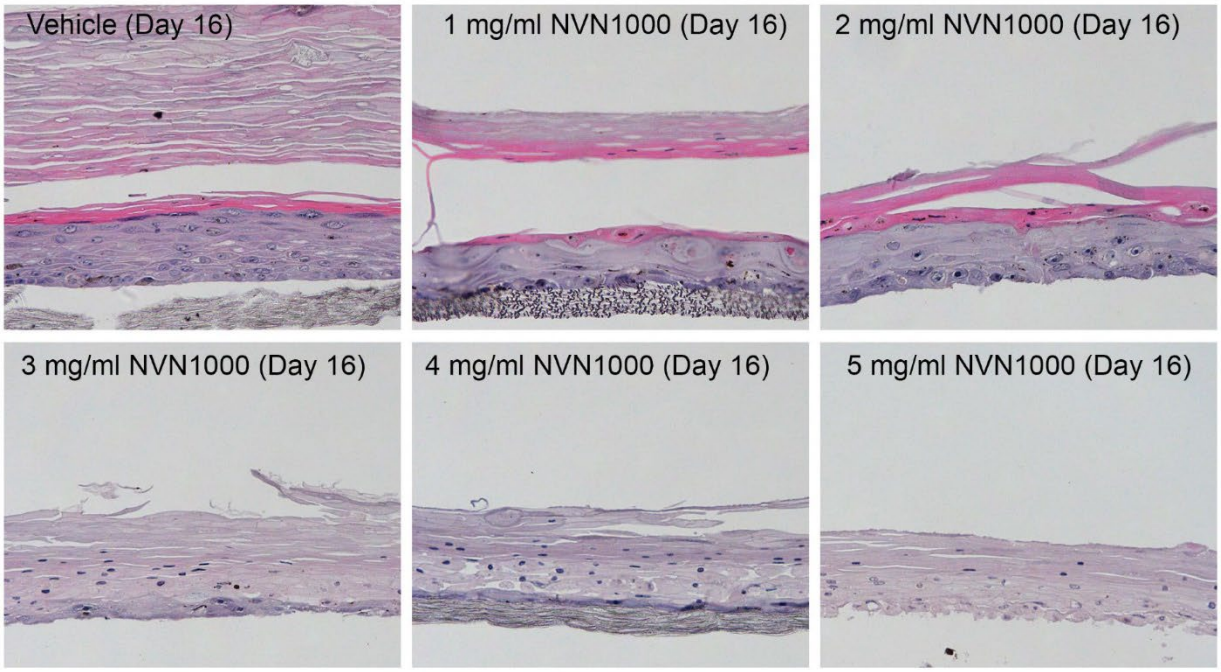


Figure 5. Histology of uninfected RCs after daily (days 7-12) 1 hr exposure to 0 (vehicle) or 1-5 mg/mL NVN1000 with harvesting after a 4-day drug-free chase.

Histology of NVN1000-treated HPV-18 infected RCs showed markedly less treatment-related toxicity than was observed in uninfected RCs. Cell density and granulocyte differentiation of RCs harvested on day 13 were reduced in a dose-dependent manner between 1-3 mg/mL compared to vehicle (Figure 6). Visible cytotoxic effects (pinkish cytoplasm, incomplete terminal differentiation (with residual nuclei in the squam) began to appear at 4 mg/mL and were exacerbated at 5 mg/mL. However, in contrast to uninfected RCs, even at higher concentrations, toxicity was not evident in the lower strata of HPV-18 infected PHK RCs. Additionally, in contrast with observations of uninfected RCs, the 4-day drug free chase partially restored stratification and granulocyte differentiation, but somewhat less so in RCs treated with 4 and 5 mg/mL NVN1000 (Figure 7). As expected, the residual nuclei remained in the squam in RCs treated with 4-5 mg/mL NVN1000; there are fewer cells in the stratified epithelium indicating basal cell proliferation was not completely restored. The reason for the difference in toxicity of NVN1000 to uninfected vs. HPV-18 infected cells is not well understood at this time, but a preliminary hypothesis is that the thicker HVP-18 RCs resulted in less nitric oxide diffused through the raft into the basal layer.

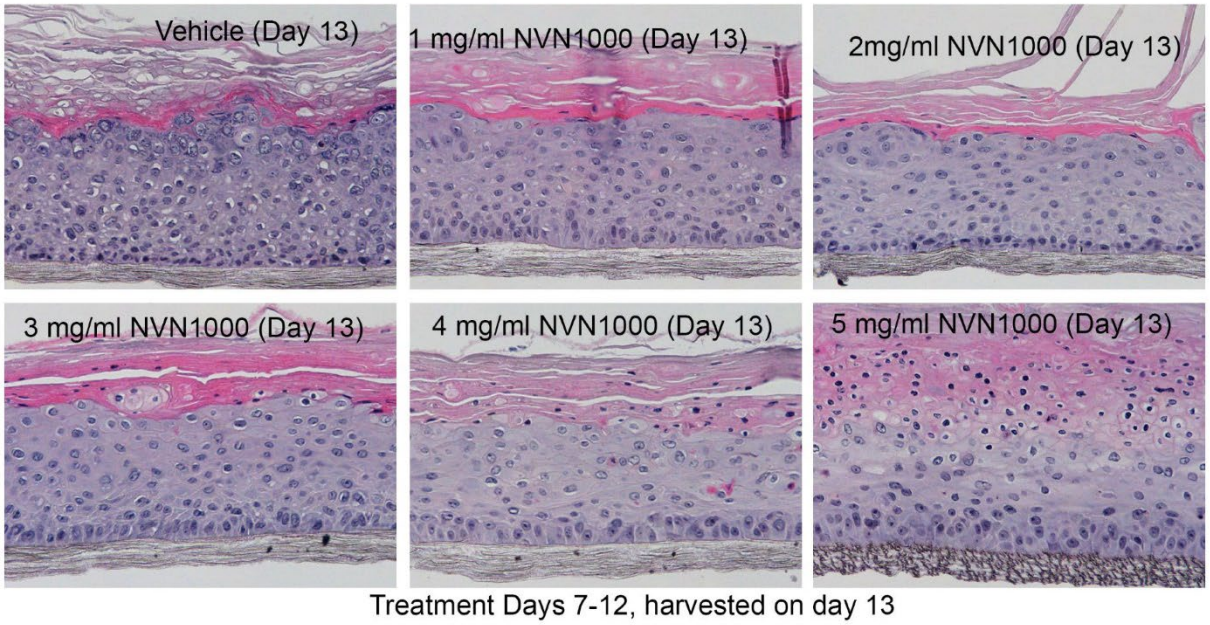


Figure 6. Histology of HPV-18 infected RCs after daily (days 7-12) 1 hr exposure to 0 (vehicle) or 1-5 mg/mL NVN1000 with harvesting on day 13.

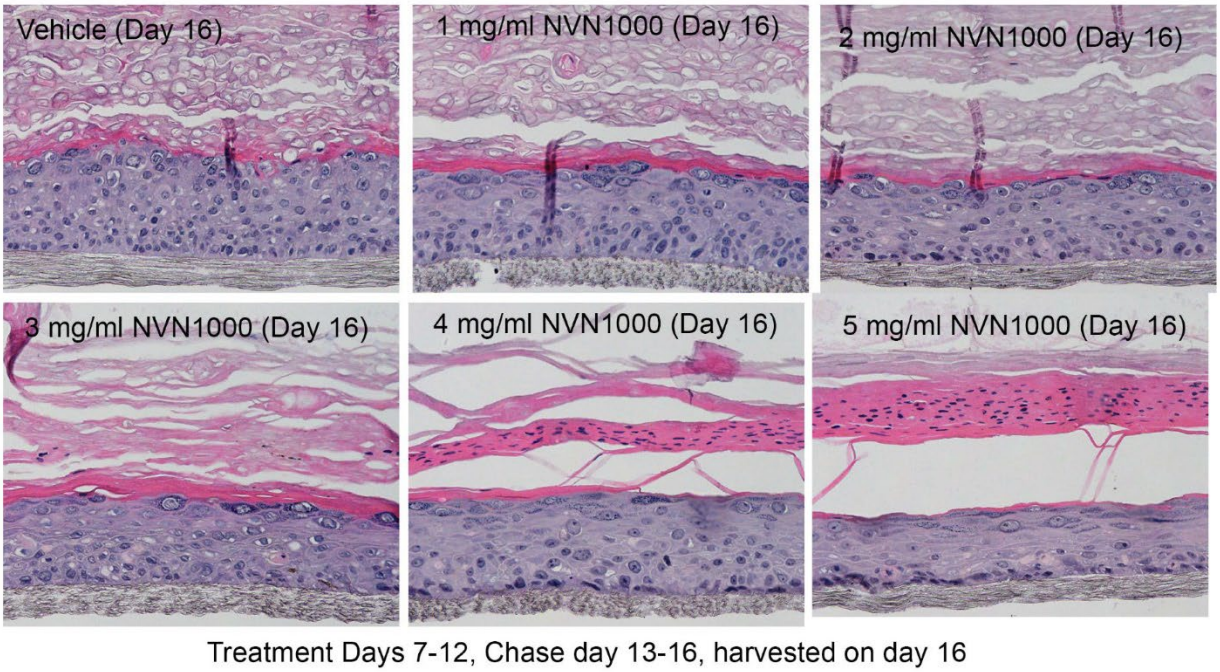


Figure 7. Histology of HPV-18 infected RCs after daily (days 7-12) 1 hr exposure to 0 (vehicle) or 1-5 mg/mL NVN1000 with harvesting after a 4-day drug-free chase.

(b) Fluorescence in situ hybridization of HPV DNA and Indirect immunofluorescence for BrdU incorporation.

Fluorescence in situ hybridization (FISH) of HPV DNA and in situ immunofluorescence microscopy of host DNA replication in vehicle- or NVN1000-treated HPV-18 infected raft culture sections showed that NVN1000 effectively inhibited HPV-18 DNA amplification (red signals) at all concentrations when harvested on day 13 (Figure 8). BrdU incorporation (green), which is indicative of host DNA replication promoted by the viral E7 oncoprotein was abrogated in the differentiated strata by NVN1000 treatment. The latter observation is consistent with the loss or significant reduction of viral DNA in these cells. Very low or dull red signals seen in 4 and 5 mg/mL NVN1000 treated cultures are likely nonspecific signals coming from residual nuclei from incompletely differentiated cells.

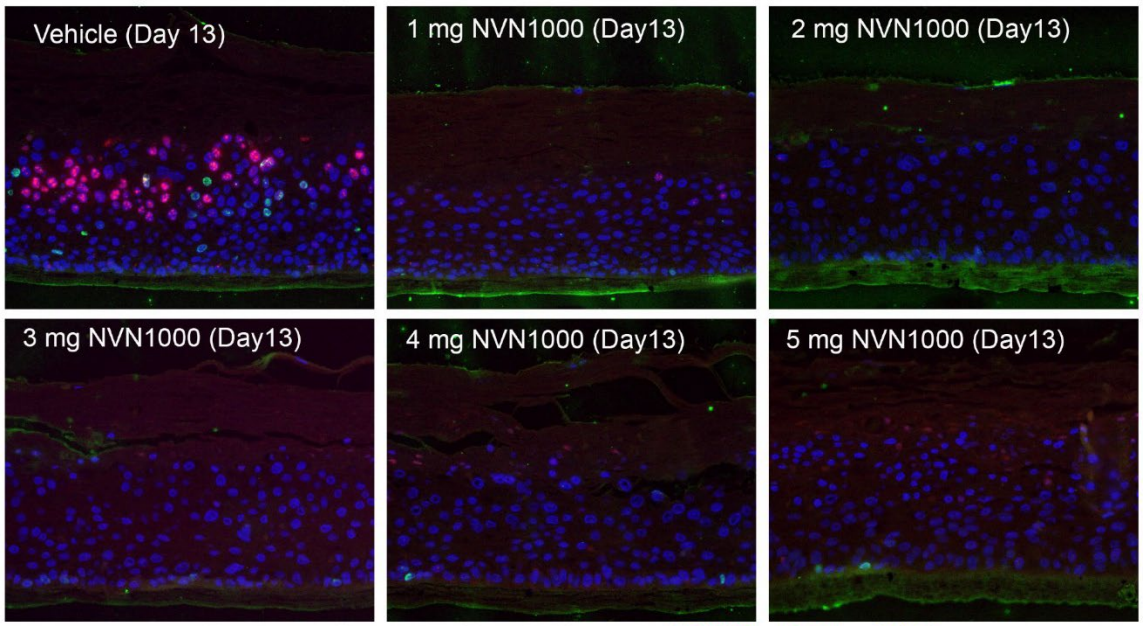


Figure 8. Fluorescence in situ hybridization and in situ immunofluorescence of HPV-18 infected raft cell cultures after daily (days 7-12) 1 hr exposure to 0 (vehicle) or 1-5 mg/mL NVN1000 with harvesting on day 13.

After a four-day drug-free chase, only occasional nuclei were positive for Brdu incorporation or viral DNA amplification at all NVN1000 concentrations, indicating a durable antiviral effect (Figure 9). The 5 mg/mL treatment appeared to have the highest antiviral activity. The BrdU incorporation was observed only in a few basal cells indicative of viable cells.

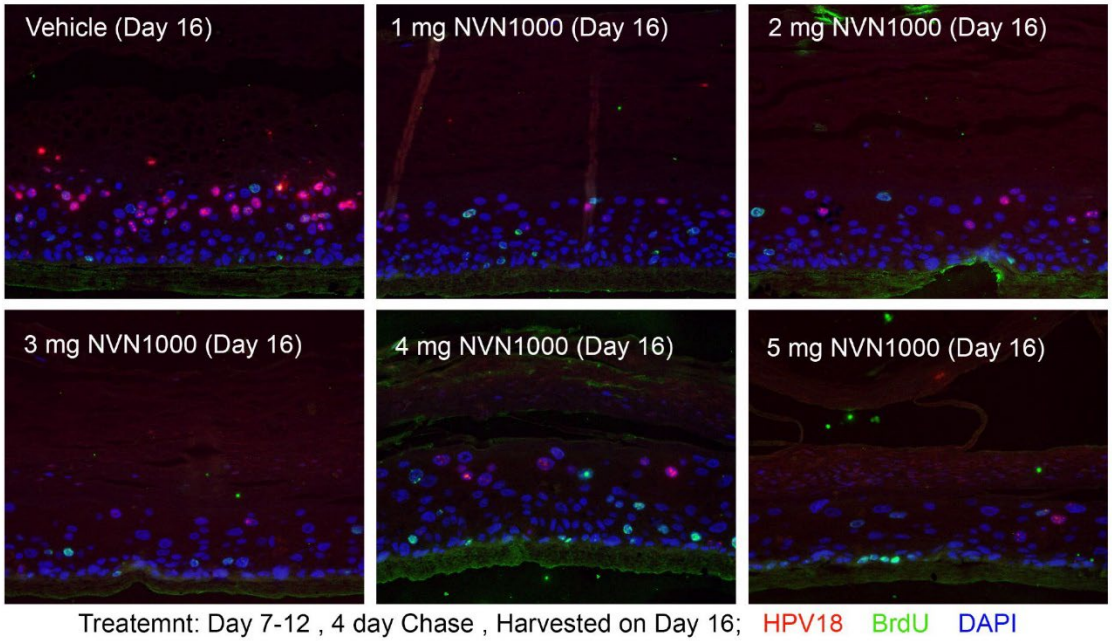


Figure 9. Fluorescence in situ hybridization and in situ immunofluorescence of HPV-18 infected raft cell cultures after daily (days 7-12) 1 hr exposure to 0 (vehicle) or 1-5 mg/mL NVN1000 followed by a 4 day drug-free chase with harvesting on day 16.

(c) HPV-18 DNA copy number detected by Quantitative Real Time PCR.

Total DNA was isolated from one RC of each treatment, and analyzed by real time quantitative PCR following the standard protocol used in earlier Novan projects (Banerjee et al, 2019) (Figure 10).

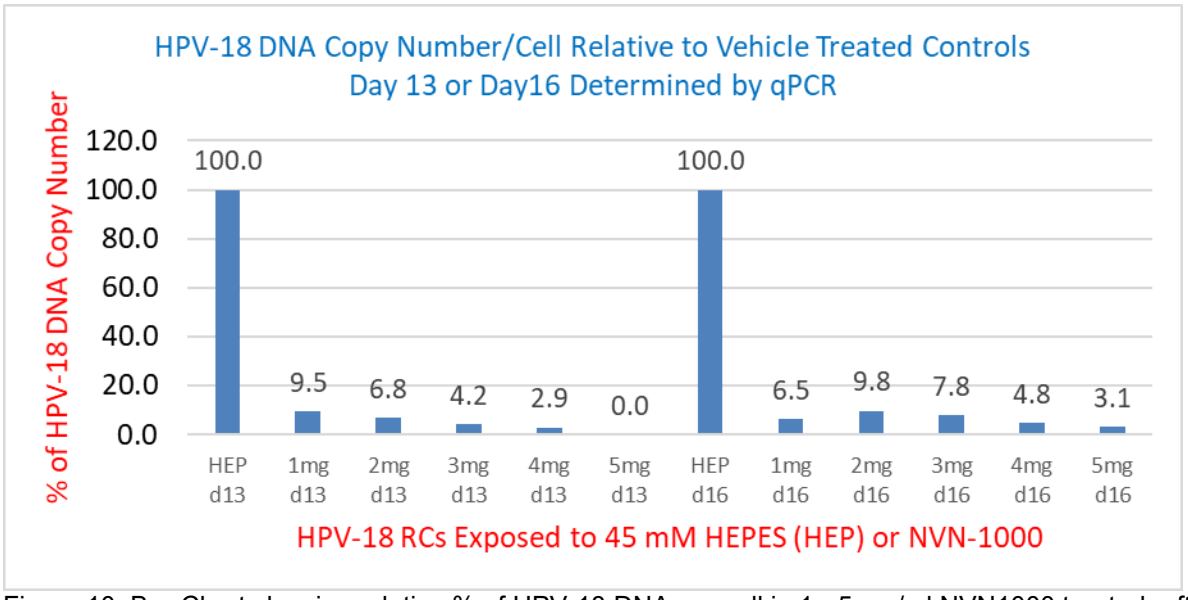


Figure 10. Bar-Chart showing relative % of HPV-18 DNA per cell in 1 - 5 mg/ml NVN1000 treated raft cultures (day 7-12) and harvested on Day 13 or Day 16 relative to those exposed to vehicle 45 mM HEPES buffer (HEPES, pH 7.1).

All concentrations of NVN1000 used were able to reduce HPV-18 copy number by >90%, with increasing efficiency at higher concentrations of the agent. Even after a drug free chase for 4 days, HPV-18 DNA copy number/cell remained below 90% of the respective vehicle treated control.

(d) L1 expression to assess effect of NVN1000 on Progeny Virus Production.

HPV-18 RCs were probed for progeny virus expression via L1 staining after harvesting on Day 13 and Day 16 (red in Fig. 11 and 12). L1 is the major capsid protein of HPV (against which the HPV vaccine is directed), while L2 is the minor capsid

protein. In this experiment, the L1 signal first emerged in the vehicle treated HPV-18 infected raft harvested on day 13, but the strongest expression was reached in day 16 untreated culture (Figs. 11, 12). L1 was not detected in any of the NVN1000 treated cultures harvested on day 13. Few nuclei in the stratum corneum were L1 positive in the day 16 cultures exposed to 1, 2, 3. 4, or 5 mg/ml NVN1000 (days 7-12). These data indicate that few cells resumed HPV-18 DNA amplification or virus production upon removal of the agent.

The same RCs were also probed for apoptosis by TUNEL assay (green in Figure 11 and Figure 12). In the day 13 harvested RCs, weak TUNEL signals were detected in stratum corneum of the vehicle treated rafts. In contrast, intense but sparse TUNEL positive signals were present in several cells of the live epithelium and stratum corneum of NVN1000 treated RCs (Figure 11). In day 16 cultures, the signals did not change in untreated cultures and in cultures treated with 1-4 mg/ml NVN1000. However, in the 5 mg/ml NVN1000 treated cultures, strong TUNEL signals were throughout the epithelium and strata corneum (Figure 12) indicating high cytotoxicity at that concentration and after the drug-free chase.

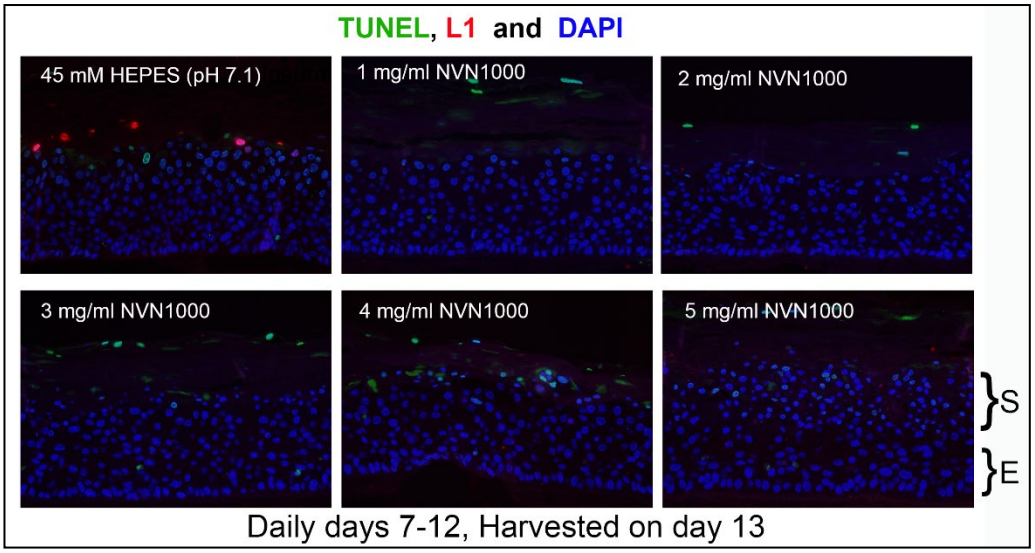


Figure 11. L1 protein expression in raft cultures exposed to vehicle (HEPES) or NVN1000 from days 7-12 and harvested on day 13.

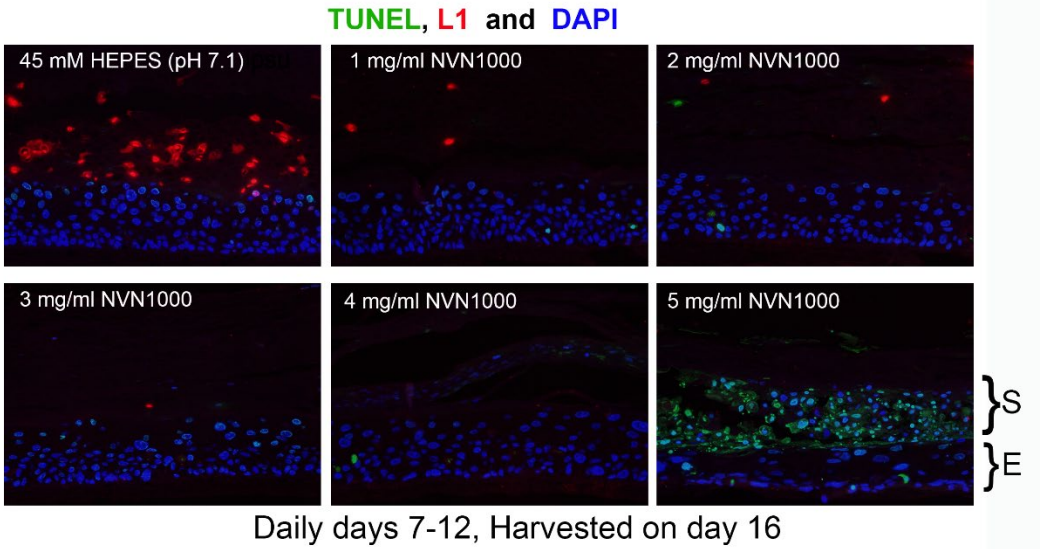


Figure 12. L1 protein expression in raft cultures exposed to vehicle (HEPES) or NVN1000 from days 7-12 and harvested on day 16.

Specific Aim 2.2: Optimization of NVN1000 frequency of administration to HPV-18 infected cultures

Specific Aim 2.2, Deliverables 1 and 2: Data for Application once every other day and Data for application once every three days.

Based on the dose-dependent efficacy and cytotoxicity data from Specific Aim 2.1 above, 2-4 mg/ml was determined to be the most optimal concentration range for daily application over 6 days to significantly reduce viral DNA amplification in a dose dependent manner. However, at the higher concentrations of NVN1000, cytotoxicity was observed in the uninfected PHK RCs. To reduce the toxicity during treatment, UAB evaluated application intervals of every 2nd, 3rd, and 4th day using 4, 5 or 6 mg/ml NVN1000 on HPV-18 RCs. The goal was to find minimal application schedules capable of blocking viral DNA amplification while having reduced toxicity to uninfected RC.

(a) Histology.

Hematoxylin and eosin staining of the infected raft cultures revealed that exposure to NVN1000 at 4, 5, or 6 mg/ml daily, or every 2nd, -3rd, or -4th day reduced live epithelial layers and induced cytotoxicity in a dose- and application frequency-dependent manner (Figure 13). Incomplete terminal differentiation and apoptosis, marked by condensed nuclei in the stratum corneum, was detected in the daily treatment with all three concentrations, every 2nd and every 3rd day treatment with 5 and 6 mg/ml, and in every 4th day treatment with 6 mg/ml NVN1000. Live cells were reduced to 1-3 in the cultures treated with higher concentrations and application frequency, relative to the typical 8-10 layers of cells in the vehicle treated infected cultures.

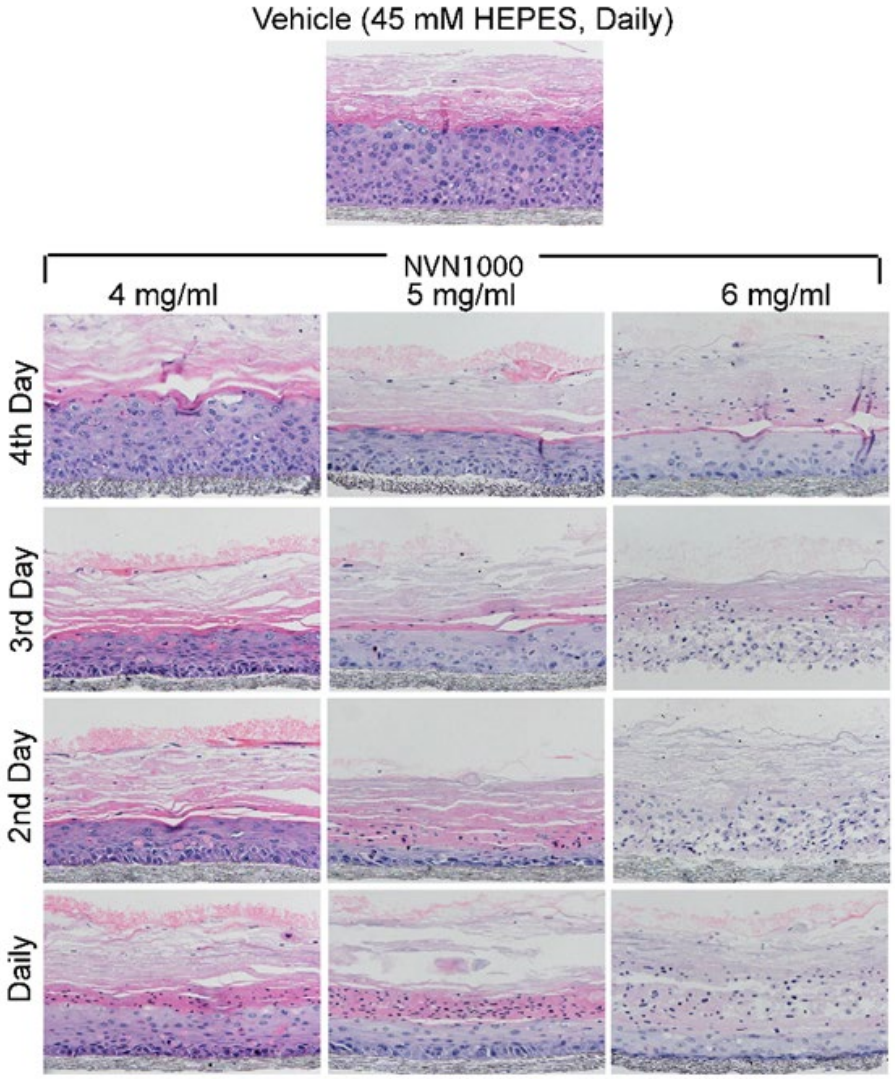


Figure 13. Histology of HPV-18 infected raft cultures revealed by hematoxylin and eosin staining. HPV-18 RCs were exposed to vehicle or to 4, 5 or 6 mg/ml NVN1000 from day 7 to 13, everyday (daily), every 2nd day (days 7, 9, 11, 13), -every 3rd day (days 7, 10, 13), or every 4th day (days 7, 11) and harvested on day 14. 20x Magnification.

(b) E7 Activity (BrdU incorporation).

Tissue sections were probed for BrdU incorporation as a marker for S-phase induction (Figure 14). As described before, cells in the differentiated strata initiate host DNA replication (S-phase re-entry) due to HPV-18 E7 mediated degradation of p130. The uninfected RCs exhibit host cell DNA replication and BrdU incorporation only in the basal layer of cells. As revealed by indirect immunofluorescence assay (green signal), stochastic incorporation of BrdU was detected abundantly in the

differentiated strata of the HPV-18 infected RC exposed to vehicle (daily 1 hour exposures from day 7 to day 13 and harvested on day 14).

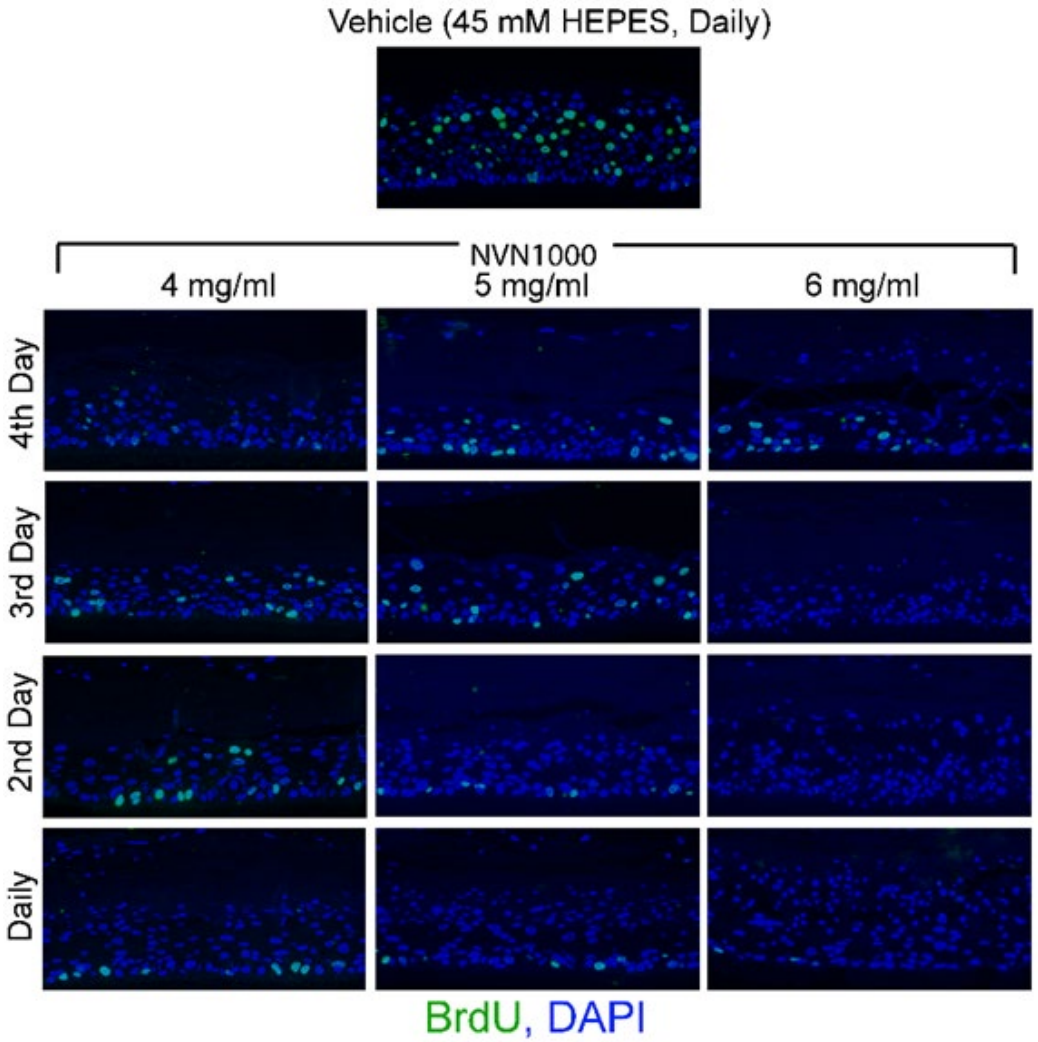


Figure 14. Indirect immunofluorescence detection of BrdU (green) as marker for S-phase entry. Nuclei were stained with DAPI. HPV-18 infected raft cultures were exposed to vehicle (45 mM HEPES, pH7.1), or NVN1000 (4, 5, or 6 mg/ml) from day 7 to 13 at indicated frequency (daily, every 2nd, 3rd, or 4th day) and all were harvested on day 14 after last 6 hours exposure to BrdU. 20x magnification.

Generally, basal and suprabasal BrdU incorporation was reduced with increasing NVN1000 concentration and application frequency. At 4 mg/ml NVN1000, applications every 4th day (2 total applications) significantly reduced the suprabasal S-phase reentry and daily application of 4 mg/mL completely abrogated suprabasal BrdU, but not basal Brdu. Complete abrogation of suprabasal BrdU was also observed at 5 mg/ml, with both daily and 2nd day application. However, only few cells in the basal layer were BrdU positive in the RCs exposed daily to 5 mg/mL. At 6 mg/ml, the live tissue was limited to 1-2 layers of cells even in the RCs exposed every 3rd day, 2nd day or daily, with almost no host DNA replication in the basal layer.

In conclusion, minimal exposures to NVN1000 (with regards to concentration and dosing frequency) as infrequent as to daily application of 4 mg/ml, every 2nd day application of 5 mg/ml, or every 3rd day application of 6 mg/ml virtually abrogated the HPV-18 E7 induced suprabasal S-phase reentry with acceptable cytotoxicity. Less frequent applications of these doses were not sufficiently effective. Daily dosing was only feasible for 4 mg/ml since higher concentrations were too toxic under the daily treatment regimen.

(c) HPV-18 DNA amplification.

Fluorescence in situ hybridization (FISH) showed abrogation of HPV-18 DNA amplification by ≥4 mg/ml at all dosing schedules (Figure 15). While a slight red hue was detectable in RCs exposed to daily or every 2nd or 3rd day applications of the 5 mg/ml or 6 mg/ml NVN1000, no HPV-18 DNA was detected in the HPV-18 infected RCs by qPCR. Moreover, these cultures experienced high cytotoxicity as revealed by histology and TUNEL assay (see below). Additionally, there was no host DNA replication in the differentiated strata of these RCs, which precedes HPV-18 DNA replication. Thus, these dispersed nuclear or cytoplasmic signals are likely due to non-specific background association of cy3-conjugated tyramide to the dying cells.

The above observations were confirmed by comparison of the HPV-18 DNA copy number per cell (determined by qPCR) in parallel raft cultures after each treatment (Figure 16).

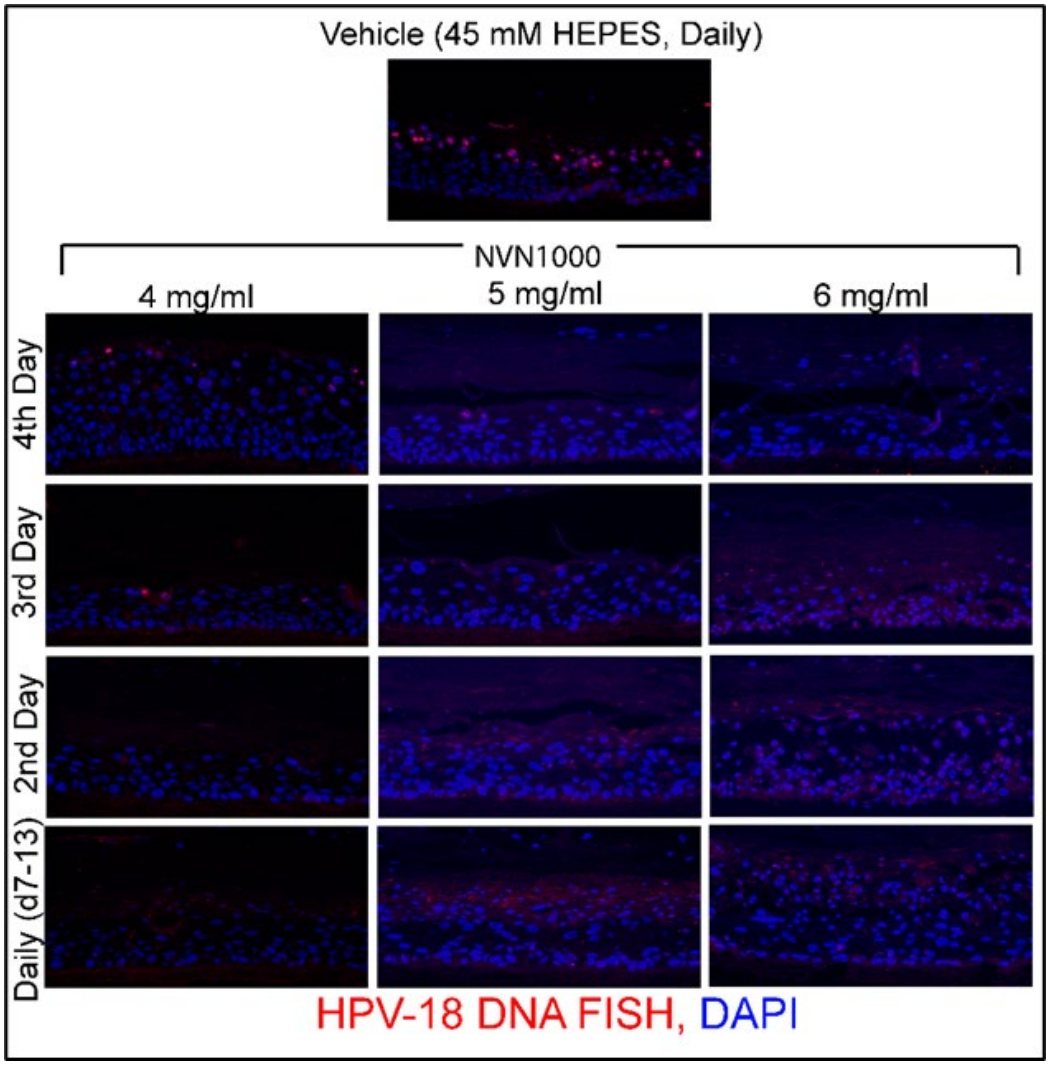


Figure 15. Fluorescence in situ hybridization to detect HPV-18 DNA amplification (red) in HPV-18 raft cultures. Nuclei were stained with DAPI (blue). Raft cultures were exposed to vehicle (45 mM HEPES, pH7.1), or NVN1000 (4, 5, or 6 mg/ml) from day 7 to 13 at the indicated frequencies (daily, every 2nd, 3rd, or 4th day), and all were harvested on day 14 after exposure to BrdU over the final 6 hours. 20x magnification.

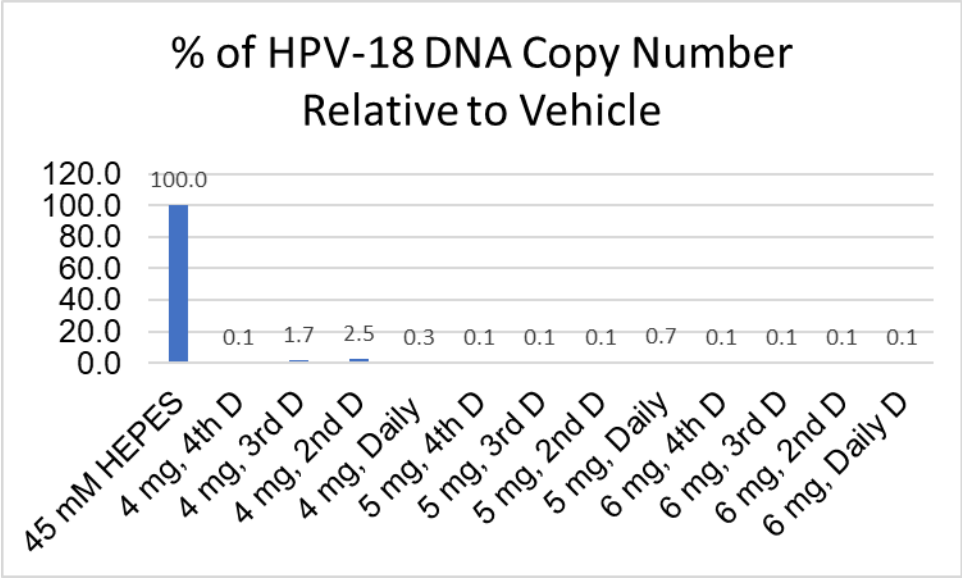


Figure 16. Relative HPV-18 DNA copy number per cell comparing to the control (vehicle). HPV-18 DNA copy number was determined by qPCR of total DNA isolated from the HPV-18 infected raft cultures, generated in parallel to those described in Figure 16.

(d)TUNEL assay and gamma-H2AX.

Cytotoxicity of NVN1000 was further evaluated by probing for γ-H2AX induction (measure of DNA damage) and TUNEL assay (cell apoptosis). Our results show that detectable γ-H2AX (red) and TUNEL (green) signals increased at higher concentrations and more frequent exposure to NVN1000 (Figure 17A and B).

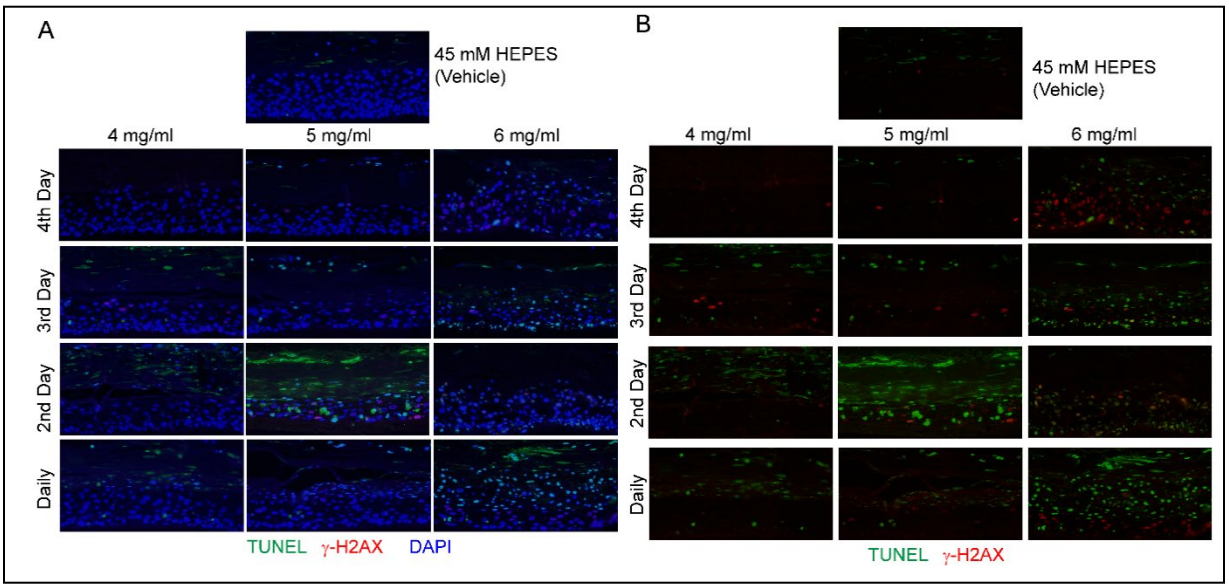


Figure 17. (A) Indirect immunofluorescence detection of nuclei by DAPI (blue), DNA damage by γ-H2AX (red) and TUNEL assay to detect apoptotic nuclei (green). (B) Same panels showing γ-H2AX and TUNEL assay without DAPI. Raft cultures were exposed to vehicle (45 mM HEPES, pH 7.1), or NVN1000 (4, 5, or 6 mg/ml) from day 7 to 13 at indicated frequencies (daily, every 2nd, 3rd, or 4th day) and all were harvested on day 14 after exposure to BrdU over the final 6 hours. 20x magnification.

TUNEL+ signals began to appear in the uppermost stratum corneum in 2nd and 3rd day application of 4 mg/ml of NVN1000 exposure. Apoptotic cells in the epithelial spinous layers were detected only at the daily application schedule of this concentration. The signal increased with increasing concentrations of NVN1000 and especially with more frequent applications at 6 mg/ml. Only 4 mg/mL applied every 4 days did not show any cytotoxicity.

In conclusion, the 6 mg/ml applications produced the highest cytotoxicity. These results are consistent with the histology of the cultures.

Based on the above data, the following frequency and concentration combinations might be suitable to prevent viral DNA replication, abrogation of HPV oncogene activity while demonstrating acceptable (i.e., minimal) cytotoxicity: daily or every 2nd day application of 4 mg/ml, every 2nd day application of 5 mg/ml or every 3rd day application of 6 mg/ml NVN1000. However, the every 2nd day application of 4 mg/mL was selected as the minimal effective dose to progress for further evaluation.

Specific Aim 2.2, Deliverable 4: Establish recovery and rebound of HPV DNA replication once NVN1000 challenge is removed. Data on drug-free chase experiments.

Treatment every 2nd day with 4 mg/ml NVN1000 was examined to establish recovery and rebound of HPV DNA once the NVN1000 challenge was removed (i.e., after a drug free chase period). In one experiment, raft cultures were exposed to topical application of NVN1000 daily or on alternate days between day-7 to day-13 and harvested on day-14 or after a 7-day drug-free chase on day-21. The total DNA was isolated, and HPV-18 DNA copy number was estimated by qPCR. Viral DNA amplification

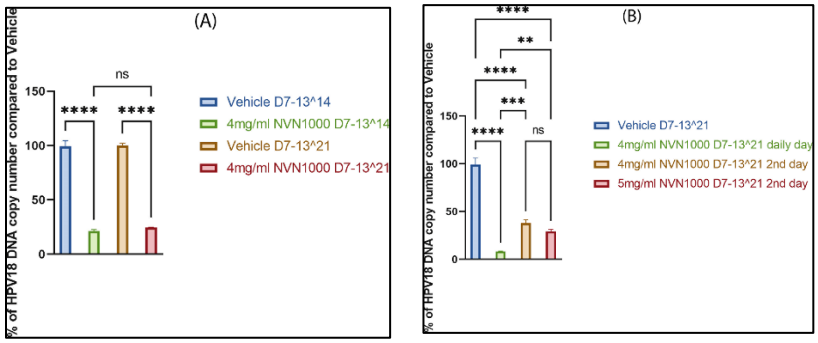


Figure 18: (A) Alternate day exposure to 4 mg/ml NVN1000 reduced HPV-18 DNA copy number per cell by ~80%. No significant recovery of HPV-18 DNA amplification was detected after a 7-day drug free chase. (B) Daily exposure to 4 mg/ml reduced HPV-18 DNA amplification by 90% and 4 or 5 mg/ml alternate day exposure maintained sustained inhibition of viral DNA amplification to about 37.8 and 29.3% of vehicle treated HPV-18 raft cultures.

was reduced to 21.4% of the control (vehicle treated), when assayed on day-14 after alternate day administration of 4 mg/ml NVN1000, and did not appreciably rebound (i.e., 24.6% of control) after a 7-day drug-free chase (Fig. 18A). Next, HPV-18 raft cultures were exposed to daily application of 4 mg/ml (green print) or alternate day exposure ("2nd day") to 4 or 5 mg/ml NVN1000 from day-7 to day-13 (brown and red). All were harvested on day 21 after a 7-day drug free chase to confirm efficiency of inhibition and sustainability. The daily exposure to 4 mg/ml NVN1000 sustainably reduced the viral DNA amplification to 8.3% of the control, but for alternate day exposure the reduction was 37.8% and 29.3% of control for the 4 and 5 mg/ml treatments, respectively (Fig. 18B). There was no significant difference between 4 mg/ml and 5 mg/ml treatments when delivered every alternate day. Because of the high cytotoxicity in the daily 4 mg/ml treated cultures, we performed protein analyses (see below) only with 4 mg/ml alternate-day treated cultures.

Specific Aim 2.3. Further the Understanding of nitric oxide's mechanism of action against E6 and E7 oncoproteins.

All the planned raft cultures were established, treated according to the optimized regimens for vehicle controls and NVN1000 exposures, and harvested. One raft culture per treatment was formalin-fixed and embedded in paraffin. Parallel raft culture samples were taken for RNA and DNA isolation and protein isolation. RNA-sequencing was completed and bulk data analysis performed, as described in Deliverable 3. Histological evaluation of the raft cultures was completed. HPV-18 DNA amplification, expression of E6 and E7 proteins, and other biomarkers were determined by immunoblot analysis. Possible rebound of HPV DNA replication and key protein biomarkers after removal of treatment were also evaluated. Details follow below.

Specific Aim 2.3; Deliverable 1: Data from in-situ assays.

(a) Histology.

HPV-18 raft cultures were exposed to vehicle or 4 mg/ml NVN1000 every alternate day from day-7 to day-13 and harvested on day-14 or harvested after a 7-day drug free chase on day-21. Histology was assessed by hematoxylin and eosin staining of the HPV-18 raft cultures (Fig. 19 A, B). A and B represent images from repeat experiments. Histology demonstrated highly proliferative and dysplastic histology of HPV-18 raft cultures exposed to vehicle on day-14, as expected. The day-21 raft culture had lower thickness due to reduced proliferation after 2-weeks of culture, which is expected based on previous studies. The 4 mg/ml NVN1000 reduced cell numbers and reduced cell layer thickness. This treatment's cytotoxic effects manifested as (1) enlarged cells, (2) gaps between the adjacent cells, and (3) apoptosis (condensed dark colored nuclei and eosinophilic (red) cytoplasm). The apoptotic effects are more acute when raft cultures were harvested one day after the last treatment (day-14 harvest, Fig. 19A). Though, in another experimental set apoptotic effects were less robust, nevertheless cell numbers and cell-cell attachment were still reduced (Fig. 19B). After 7-days of a drug free chase, basal cells resembled those of the vehicle treated cultures but some of the cytotoxic effects persisted.

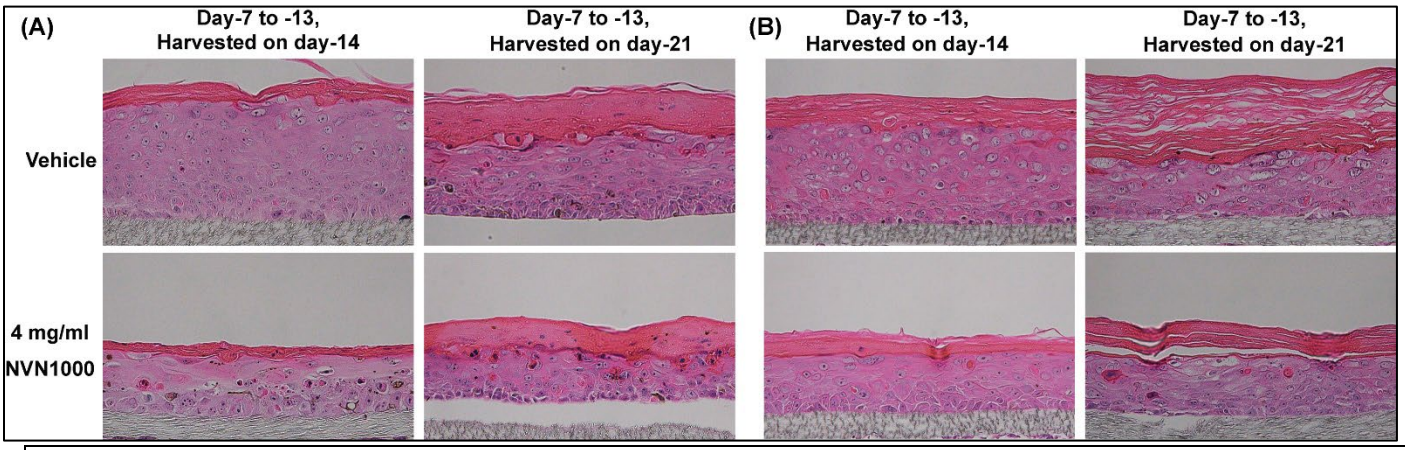


Figure 19. Histology of HPV-18 raft cultures exposed to 4 mg/ml NVN1000 or vehicle from day-7 to day 13 and harvested on day-14 or day-21. A and B are two replicates. These and other images were captured at 20X magnification.

(b) Data from the in-situ tests of raft culture tissues.

BrdU was detected as a marker for host cell DNA replication) and fluorescent in situ hybridization (FISH) was done to detect HPV-18 DNA amplification. FISH indicated about 80-90% reduction of HPV-18 DNA amplification after 4 mg/ml topical NVN1000 application to the raft cultures every other day (i.e., day 7, day 9, day 11 and day 13) (Fig. 20, red signal for HPV-18 DNA probe). DAPI (blue) is marker for nuclei. However, BrdU (green) was detected mostly in the upper differentiated layers and rarely in the basal layer of the vehicle treated cultures. In contrast, most BrdU positive cells are present in the basal and parabasal layer and only few in the upper layers of

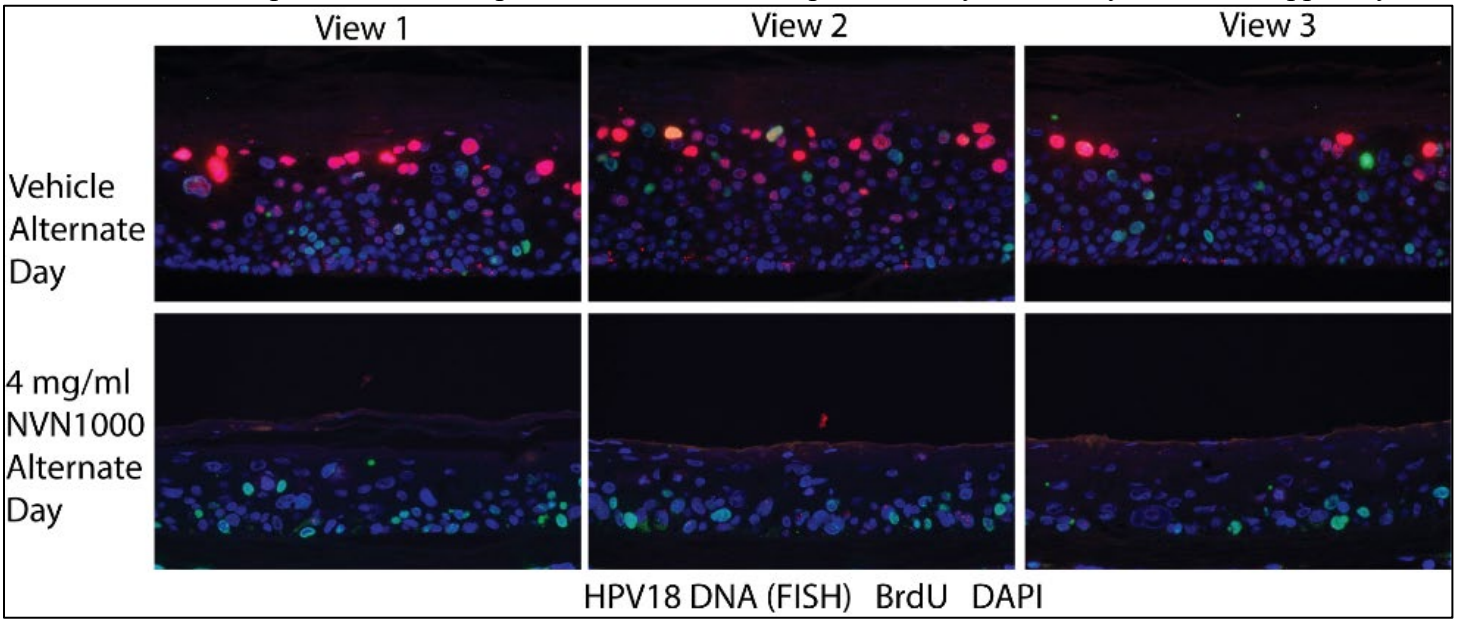


Figure 20. HPV18 DNA amplification (red) and BrdU incorporation (green) was detected by Fluorescent In situ Hybridization (FISH) and IHC.

the HPV-18 infected cultures. This phenotype indicated that E7 induced S-phase reentry induction in the upper differentiated layers was reduced. However, some DNA replication activity remained in the basal and parabasal layers. However, raft cultures showed fewer cell layers and cell numbers and irregular or collapsed nuclear morphology suggesting cytotoxicity in the NVN1000 treated cultures, consistent with histology (Fig.19). Nuclei, detected with DAPI (blue staining) in the upper differentiated layers of NVN1000 treated HPV-18 raft cultures were fewer and flatter compared to those in vehicle treated cultures.

Specific Aim 2.3; Deliverable 2: Data from biochemical assays.

(a) Immunoprecipitation experiments.

(1) The E6 oncoprotein mediates degradation of the tumor suppressor protein p53. By immunoblot assay, we confirmed that alternate day treatments with 4 mg/ml NVN1000 from day-7 to day-13 elevated p53 protein in day-14 harvest of HPV-18 raft cultures (Fig. 21A), supporting an effective reduction in E6 levels or activity. The co-immunoprecipitation of p53 bound protein complex in NVN1000-treated and vehicle-treated HPV-18 raft culture lysates did not reveal the presence of E6 or E6AP in two replicate experiments (Fig. 21A, Experiment sets 1-2 and 3-4). Hence, these experiments did not distinguish whether NVN1000 treatment interfered with E6/E6AP/p53 interaction. We interpret the finding that the complex could not be observed

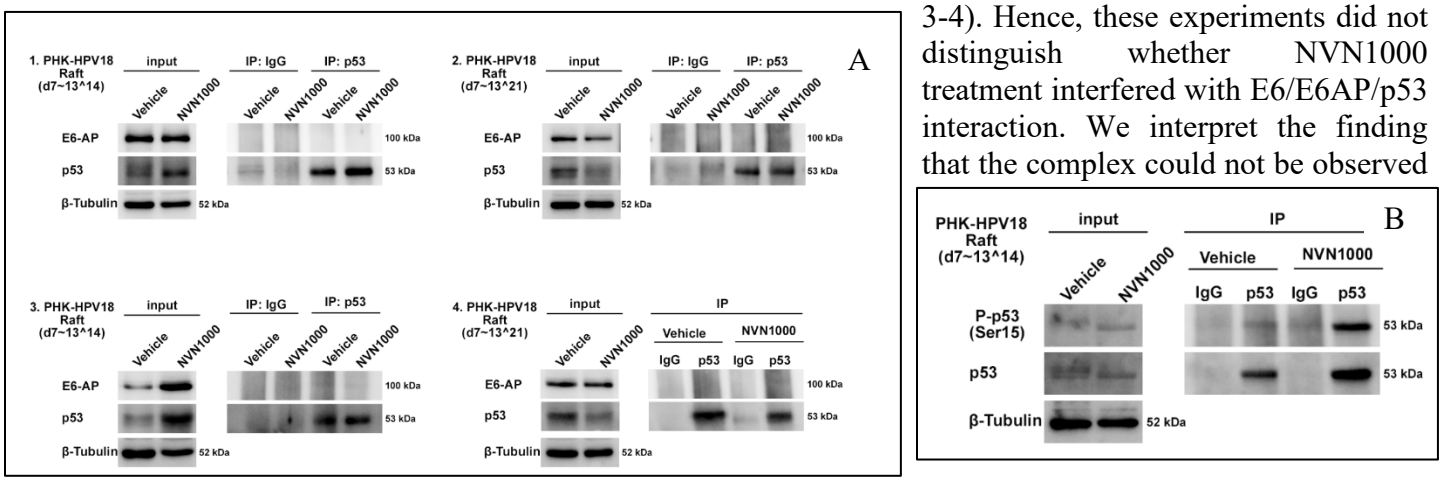


Figure 21: (A) Co-IP with p53 antibody did not precipitate bound E6AP from vehicle or NVN1000-treated HPV-18 raft cultures. (B) NVN1000 elevated phosphorylated p53 (S15).

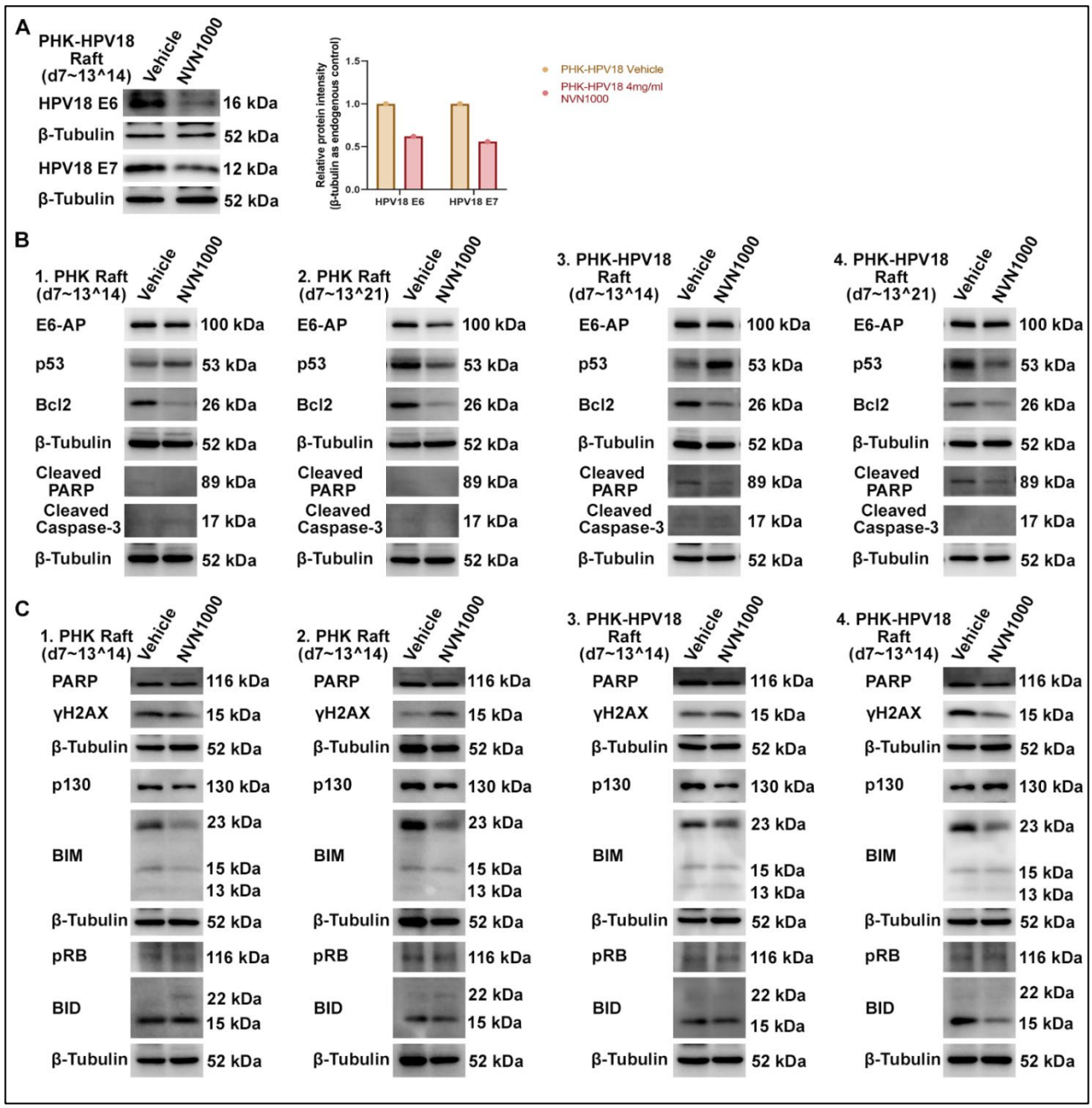
in vehicle-treatment to suggest that the E6/E6AP/p53 complex has a short half-life for detection by co-IP from raft cultures.

(2) We then probed for p53 phosphorylation in the immunoprecipitated protein, as a possible mechanism for stabilization against E6-induced degradation. Indeed, the immunoprecipitated p53 protein from NVN1000-treated HPV-18 raft culture lysate had higher phosphorylated $^{\text{Serine15}}$-p53 relative to vehicle treated control (Fig. 21B).
(3) Similarly to the effect of E6 on p53 protein, the E7 oncoprotein destabilizes pRB/p130 and thereby dysregulates the pathway controlled by these tumor suppressors. However, we could not perform co-IP for E7-pRB or E7-p130 to examine the effect of NVN1000-treatment on these proteins because protein lysates were insufficient. Additional raft cultures beyond what were budgeted in the grant would be necessary for this experiment.

(b) Immunoblot analysis.
We probed for the following proteins which are (a) HPV-18 E6 and E7, primary targets of E6 and E7 (p53, pRB and p130); (b) DNA damage induced gamma-H2AX, (c) pro-apoptotic proteins (cleaved caspase 3, cleaved PARP, BIM and BID), and (d) anti-apoptotic proteins (Bcl2).

(1) 1-hour topical application of 4 mg/ml NVN1000 every alternate day (from day-7 to day-13) reduced HPV-18 E6 and E7 proteins to 62 and 55% of the vehicle treated control, respectively (Fig 22A). There was insufficient protein to do the same assay for the day-21 harvested cultures.

(2) Alternate day topical application of 4 mg/ml NVN1000 reproducibly increased total p53 (Fig. 22B),



Figures 22. (A) Immunoblot analysis of E6 and E7 expression in day 14 HPV18 raft cultures. Bar chart depicts densitometric comparison of E6 and E7 protein bands. **(B)** Analysis of E6AP, p53, Bcl2, cleaved PARP and cleaved caspase 3 in uninfected and HPV18 infected raft cultures harvested on day 14 or after 7-day chase on day 21. **(C)** Immunoblot analysis of total PARP, gamaa-H2AX, p130, pRB, BIM and BID in uninfected and HPV18 infected raft cultures harvested on day 14. All raft cultures were treated every alternate day for 1 h exposure of from day-7 to day-13 and harvested on day-14 or day-21. Beta-tubulin was used as loading control.

corroborating our previous finding. We interpret that nitric oxide exposure induced p53 phosphorylation, which caused its stabilization. Loss of E6 protein by NVN1000 might have also contributed to elevated p53 protein levels. We did not detect a similar increase of p53 protein in uninfected PHK raft cultures by similar exposure to NVN1000.

However, after 7 days of drug free chase, the p53 protein level decreased in NVN1000 treated HPV-18 infected raft cultures relative to vehicle treated HPV-18 infected raft cultures. Since these day-21 raft cultures from the NVN1000 treated group display features of persistent cytotoxicity, we interpret that a loss of overall physiological activity in these raft cultures reduced the p53 level, but not because of rebound E6 activity.

(2) We did not detect any effect of NVN1000 on total pRB and p130 protein levels in HPV-18 raft cultures, relative to vehicle treated control (Fig. 22C). Hence, we cannot correlate reduced HPV-18 DNA amplification to loss of HPV-18 E7 activity, which normally degrades pRB and p130. The residual E7 protein in NVN1000 treated raft cultures might still be active and able to keep pRB and p130 low. This is consistent with IHC data (Fig 2), which did not show any significant reduction of BrdU incorporation (a surrogate marker of E7 activity) by alternate day application of NVN1000 in the HPV-18 raft cultures.

(3) DNA damage: We did not detect any robust increase of gamma-H2AX protein, a marker of double stranded DNA breaks in HPV-18 infected raft cultures (Fig. 22C). In previously published research (Banerjee et al, 2019), when NVN1000 was added daily (2 mg/ml) elevated expression of gamma-H2AX protein in HPV-18 raft cultures was detected, because reduced E6 and elevated p53, interrupted host DNA replication. Alternate day application of 4 mg/ml also reduced E6, increased p53 and induced p53 phosphorylation. Because of this, we expected NVN1000 would induce DNA damage and that this would be detectable as elevated gamma H2AX. However, we did not detect elevated level of gamma-H2AX protein in our assay. Possibly the 48-hour gap between two successive applications allowed repair of DNA damage by residual effect of E7. HPV E7 is known to induce DNA damage repair pathway in HPV-18 raft cultures. Alternatively, cells with excessive DNA damage could have died and degraded, which is consistent with reduced number of cells in NVN1000 treated HPV-18 raft cultures (Fig.18, 19).

(4) Pro-apoptotic proteins: We expected that elevated p53 might cause apoptosis and we would detect elevated levels of pro-apoptotic proteins. However, total PARP protein did not change, but cleaved PARP appeared to be decreased by NVN1000 treatment in uninfected and HPV-18 raft cultures. Similarly, there was no effect of NVN1000 alternate day applications on other pro-apoptotic proteins to include cleaved caspase 3, BIM or BID in uninfected and HPV-18 infected raft cultures. Cleaved PARP is indicator of early stage of apoptosis and cleaved caspase 3 is a marker of late stage of apoptosis. But these proteins are lost when cells are dead or have completed apoptosis. Thus, results from this part of immunoblot assay suggests that either apoptosis did not occur because of 48-hour gap between the two applications, or the dead cells were degraded. Both may be possible, because cell layers and numbers were reduced in 14-day NVN1000 treated cultures, as discussed above.

(5) Anti-apoptotic proteins: Bcl2 protein opposes apoptosis by sequestering pro-apoptotic protein Bax in the cytosol through direct binding. The four alternate day applications of NVN1000 reduced the level of anti-apoptotic Bcl2 in both uninfected and HPV-18 infected raft cultures (Fig. 22B). This reduced presence of Bcl2 might have contributed to some apoptosis in NVN1000 treated raft cultures.

The above results suggest that much of the effect from alternate day application of 4 mg/ml NVN1000 is through stabilization of active p53 in HPV-18 raft cultures. Phosphorylated p53 activity would inhibit HPV-18 DNA amplification, which is consistent with our earlier report that E6 mediated p53 degradation is necessary for efficient viral DNA amplification (Kho et al. 2013). The stabilized p53 is phosphorylated and hence should increase p53 regulated proteins in apoptotic, growth inhibitory or both kinds of pathways.

Specific Aim 2.3; Deliverable 3: Data from RNA sequencing assays.

(a) RNA samples.

Uninfected and HPV-18 infected PHK raft cultures were prepared and from day-7 to day-13 were exposed to vehicle or 4 mg/ml NVN1000 every other day and harvested on day-14, followed by RNA extraction. Total RNA was extracted from (A) four replicates of vehicle treated (SB9-12) or NVN1000 (SB13-16) treated uninfected PHK raft cultures harvested on day-14 ; (B) three replicates of raft cultures of vehicle treated (SB1-3) or NVN1000 (SB5-7) treated HPV-18 infected PHKs, harvested on day-14; (C) one each of vehicle (SB4) or NVN1000 (SB8) treated (days 7-14) HPV-18 infected raft cultures, harvested after 7-day drug-free chase on day 21. A total of 16 RNA samples were deposited for sequencing in the UAB Heflin Genomics Core Facility.

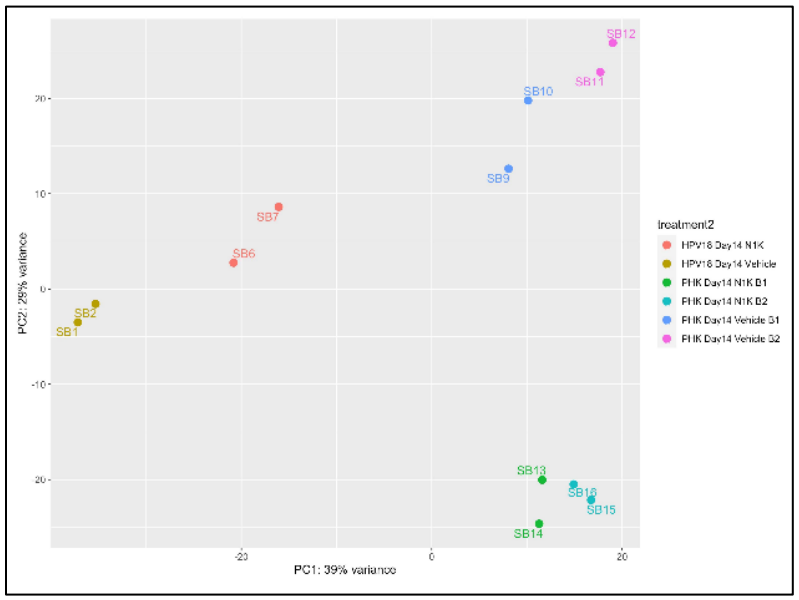


Figure 23. PCA analysis showing two each of vehicle treated and 4 mg/ml NVN1000 treated HPV-18 raft cultures clustered as separate groups. Similarly, 4 each of vehicle and NVN1000 treated raft cultures grouped separately. SB3 and SB5 were excluded for not clustering to respective groups. Day 21 cultures of HPV-18 (SB4 and SB8) were not included.

(b) RNA sequencing.

RNA processing, cDNA library preparation and sequencing were performed at the Heflin Genomics Core by Dr. Michel Crowley following standard protocols. RNA quality was checked by analyzing RIN value. Kits from NEB were used for ribosomal RNA reduction. NEB Next Ultra II Directional kit was used to prepare the sequencing library. Sequencing was performed at 100 million paired end reads per sample on the NovaSeq6000 using standard protocols.

(c) RNA sequence data analysis.

Data normalization, processing, and analysis were done by Dr. Michael Crossman from the Heflin Genomics Core under a fee for service arrangement. He reported as follows:

"*Raw sequence reads were first trimmed to remove primer adapters using Trim Galore (version 0.6.10; parameters used: --trim-n --paired --nextseq 20). The trimmed sequences were then aligned to Gencode GRCh38 p13 Release43 reference genome using STAR (version 2.7.11a; parameters used: --outReadsUnmapped Fastx --outSAMtype BAM SortedByCoordinate --outSAMattributes All) (1). Over 80% of the sequence reads aligned uniquely to the reference genome. Following alignment, transcript abundances were calculated using HTSeq-count (version 2.0.2; parameters used: -m union -r pos -t exon -i gene_id -a 10 -s no -f bam) (2). Normalization and differential expression were then applied to the count files using DESeq2 (3) following their default parameters in their vignette*."

(1) DATA quality: Sequenced data matched well to human genome data based on STAR alignment stats. The number of input reads varied between 116,729,412 to 244,188,515 for HPV-18 samples and between 93,526,658 to 128, 510,665 for uninfected PHK raft cultures. The average input read length was 199 bases and average mapped length was 196. Following are the main findings.

(2) Principal Co-ordinated Analysis (PCA) analysis for making clusters for comparison (Figure 23): Sequences from one of the vehicle treated and one of the NVN1000 treated HPV-18 raft culture did not cluster to their respective group, hence were excluded from the pathway analyses.

(3) HPV-18 transcripts: Analysis of the RNA sequencing data revealed that NVN1000 reduced transcription of HPV E6 containing mRNAs in HPV-18 raft cultures. But we did not detect any increase of p53 transcription. Moreover, transcription of hTERT, which is another protein elevated by high-risk HPV E6, was reduced by >log2 fold. Thus, increased p53 protein and reduced hTERT mRNA testify for reduced E6 protein or its activity upon NVN1000 treatment in HPV-18 raft cultures.

(4) Volcano-plots: Volcano-plots of the differentially expressed genes identified many genes were up- or down-regulated by NVN1000 (4 mg/ml) in HPV-18 infected (Fig. 24) or uninfected (Fig. 25) PHK raft cultures. Although the HPV-18 infected and uninfected raft cultures share a list of transcripts whose level were altered by NVN1000, they were not changed in the same ways. For example, genes upregulated by NVN1000 in HPV-18-uninfected raft cultures sometimes down regulated in uninfected PHK RCs. More importantly, NVN1000 altered unique sets of genes in HPV-18 infected and uninfected raft cultures.

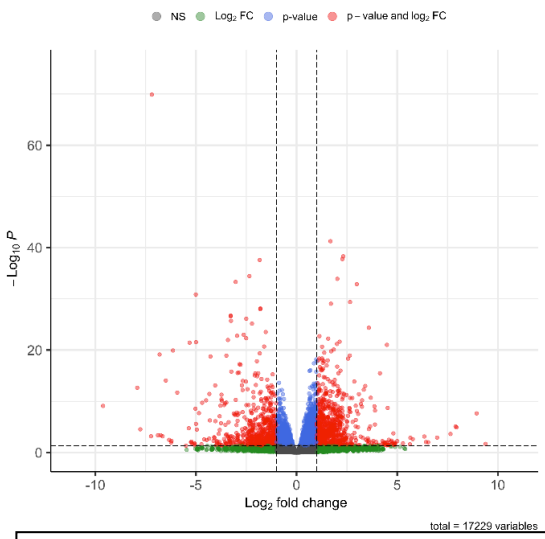


Figure 24: Volcano plot depicts down and up regulated genes in HPV-18 infected raft cultures after alternate day treatment with 4 mg/ml NVN1000 (day7-14) and harvested on day-14.

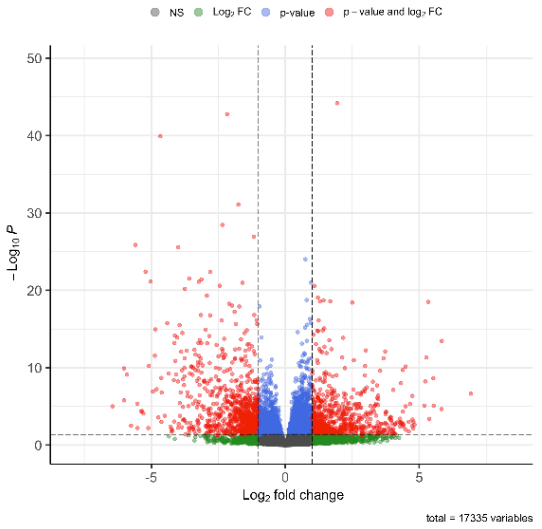


Figure 25: Volcano plot depicts down and up regulated genes in uninfected PHK raft cultures after alternate day treatment with 4mg/ml NVN1000 (day7-14) and harvested on day-14.

(5) Top 10 uniquely altered genes in HPV-18 raft cultures: The top 10 uniquely down-regulated genes in HPV-18 raft cultures with >50 FPKM (fragments per kilobase of transcript per million fragments) value are, SOCS7 (673-folds), HLA-DQB1(358-folds), DDR1 (137-folds), AOC1 (11-folds), CRISP3 (71-folds), PLAAT4 (61-folds), KCNQ1 (41- folds), PADI2 (40-folds), LETR1 (34-folds), and CD7 (31-folds). Of these 10 genes, AOC1 is known to directly regulate proliferation by activating AKT1 pathway, while DDR1 participates in the DNA damage repair pathway. PADI2 participates in activation of MEK, wnt and notch pathways in mammalian cells. In addition, this study discovered many more genes uniquely but significantly downregulated by NVN1000 in HPV-18 raft cultures. These downregulated genes have documented functions in important pathways such as cell proliferation, oncogenesis, invasion, and inflammation. A separate study would be necessary to explore their role in NVN1000 response on the HPV infection program.

The top 10 uniquely up-regulated genes are GNL1 (246-folds), TRIM26 (239-folds), NPAP1L (227-folds), TCF19 (223-folds), XIRP2 (201-folds), MDC1 (93-folds), MAGEA3 (86-folds), ZFP57 (81.5-folds), TUBB (57.4-folds), ZNF66 (30-folds). However, based on the available literature, their potential implication in the HPV life cycle is not clear. It is important to note that upregulated transcripts had much less reading depth relative to the

down-regulated transcripts. Importantly, abundance of several of these transcripts were reverted after the drug free chase, suggesting direct effects of NVN1000 on these genes.

(6) Top 10 altered genes both in PHK and HPV-18 raft cultures:

The downregulated genes are AQP5 (-10.5 folds), MAL (-10.2 folds), CYP4P8 (-7 folds), CLIC6 (-6.4 folds), CCL2 (-6 folds), FAM3D (-6 folds), FMO2 (-5.6 folds), IRAG2 (-5.6 folds), KRT13 (-4.6 folds), ACE2 (-4.6 folds).

The upregulated genes are LGR5 (23-folds), KRT9 (10.6 folds), SHE (6.3 folds), BAALC (7.3 folds), PCDH19 (6.9 folds), BPIFC (6.3 folds), GLB1L3 (6.1 folds), PNPLA5 (5.2 folds), SERPINE1 (4.9 folds). Importantly, while many of them were upregulated in HPV-18 rafts, they were reduced in uninfected PHK RC.

Again, the potential roles of these transcripts on HPV life cycle are not clear and will require more detailed literature search and investigation. There are many more genes significantly altered in both uninfected and HPV-18 infected PHK raft cultures. Importantly, abundance of several of these transcripts were reverted after the drug free chase, suggesting direct effect of NVN1000 on these genes.

(7) The p53 regulated pathways: There was no change of p53 mRNA or transcripts of classical proapoptotic or growth suppressive genes comprising p53 pathways. Expression of 144 genes in the expansive and complex p53 pathways were altered. However, amongst the known p53 transactivated genes, transcription of some were upregulated (p53AIP1, DNAI3, SERPINE1), but mRNA expression of others was decreased (BIRC3, CSF1 etc.) or did not change (Bax, PUMA, Noxa, Apaf, p21Cip1, etc). PLAAT4 and PADI2 mRNA were reduced by about 6 and 4 log2 folds respectively despite their promoters being transactivation targets of p53. Much deeper combing of this data might well provide a more comprehensive understanding.

(8) Gene Ontology: Gene ontology-based cluster profiling revealed that 4 mg/ml NVN1000 (alternate day, day-7-14) significantly altered expression levels of thousands of genes belonging to several biological processes (BP), Cellular components (CC) and Molecular Functions (MF) in both uninfected and HPV-18 infected PHK raft cultures (Figures 26-32). The top identified BP, CC and MFs in HPV-18 RCs are depicted below. Please note in the plots that “count” indicates number of genes in the related pathway.

Figure 26. Biological Processes (BP) down-regulated by NVN1000 in HPV-18 RCs.

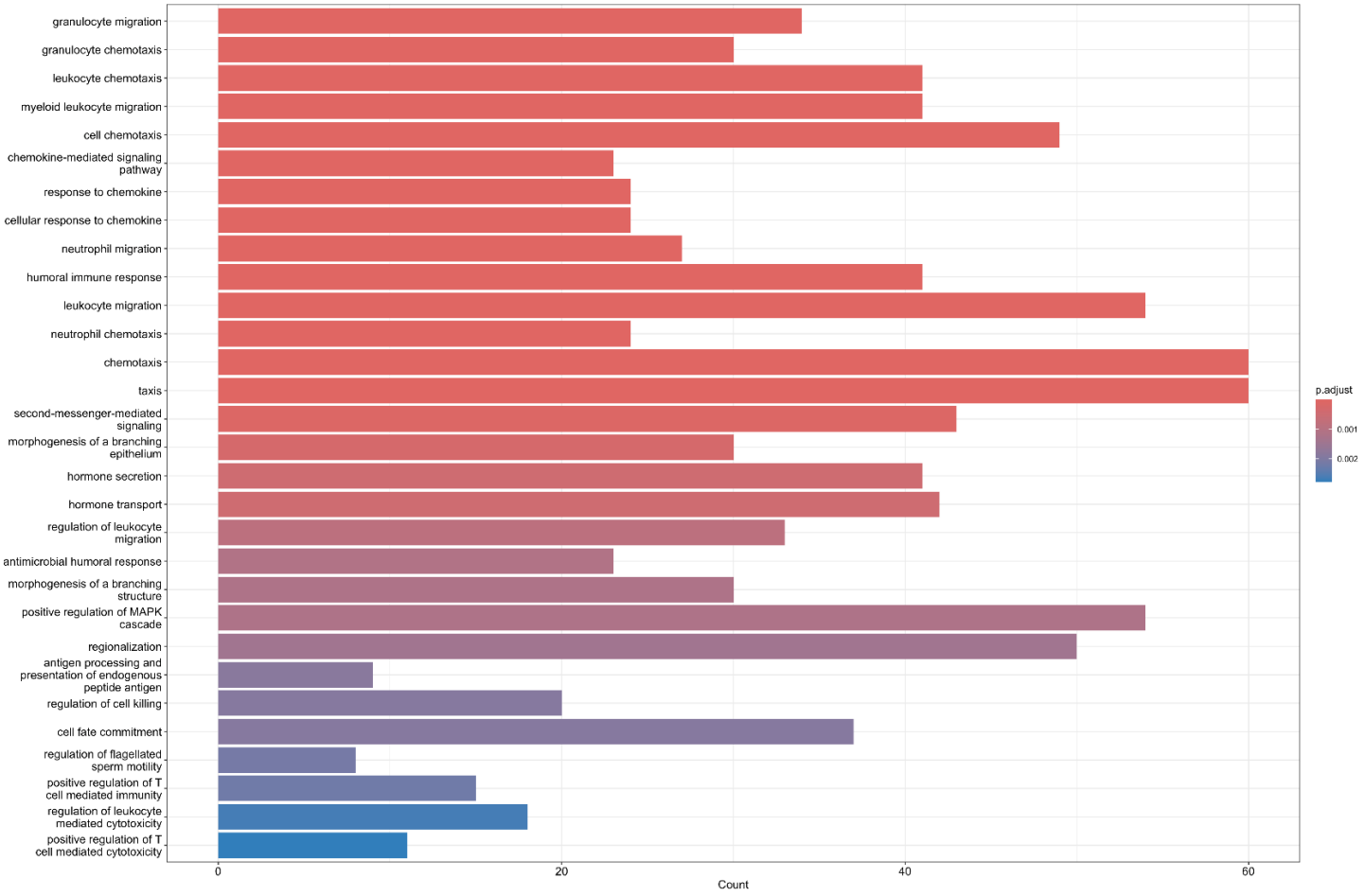


Figure 27. Cellular Components (CC) down-regulated by NVN1000 in HPV-18 RCs.

External side of plasma membrane

Collagen-containing Extra cellular matrix

MHC-protein complex

Cytoplasmic vesicle lumen

Vesicle lumen

Luminal side of endoplasmic reticulam membrane

Specific granule lumen

Secretory granule lumen

Lumenal side of membrane

Specific granules

Apical plasma membrane

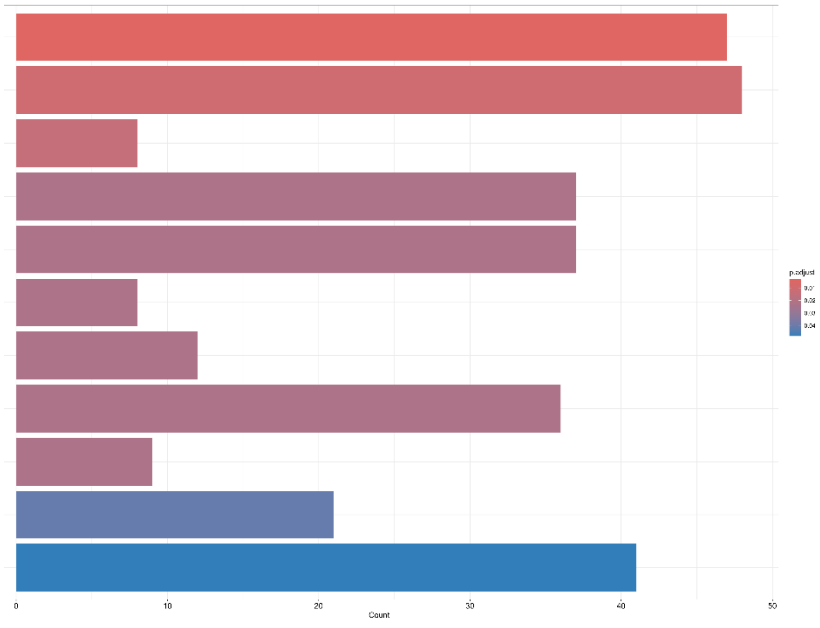


Figure 28. Molecular Functions down-regulated by NVN1000 in HPV-18 RCs.

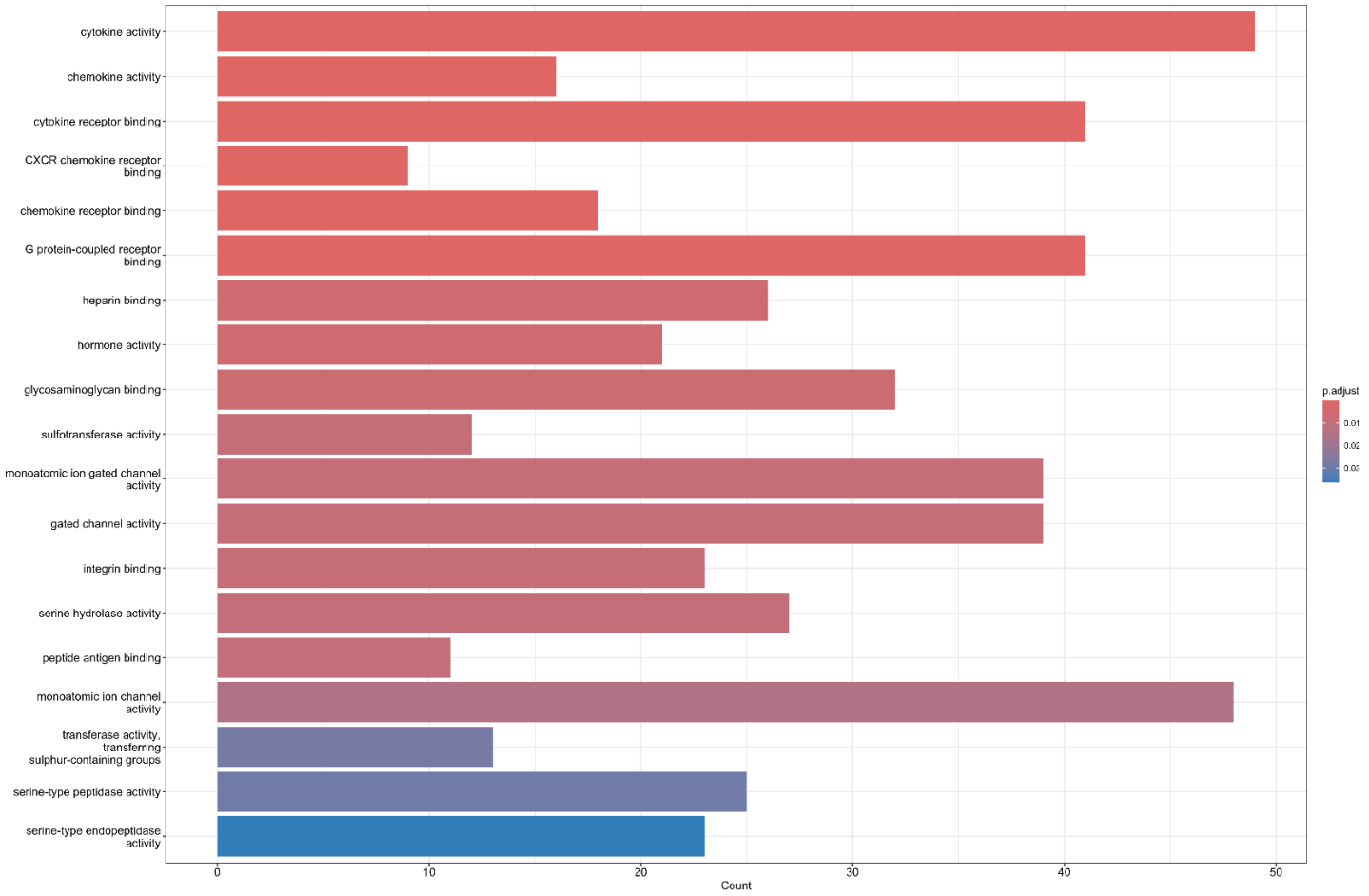


Figure 29. Biological Processes (BP) up-regulated by NVN1000 in HPV-18 RCs.

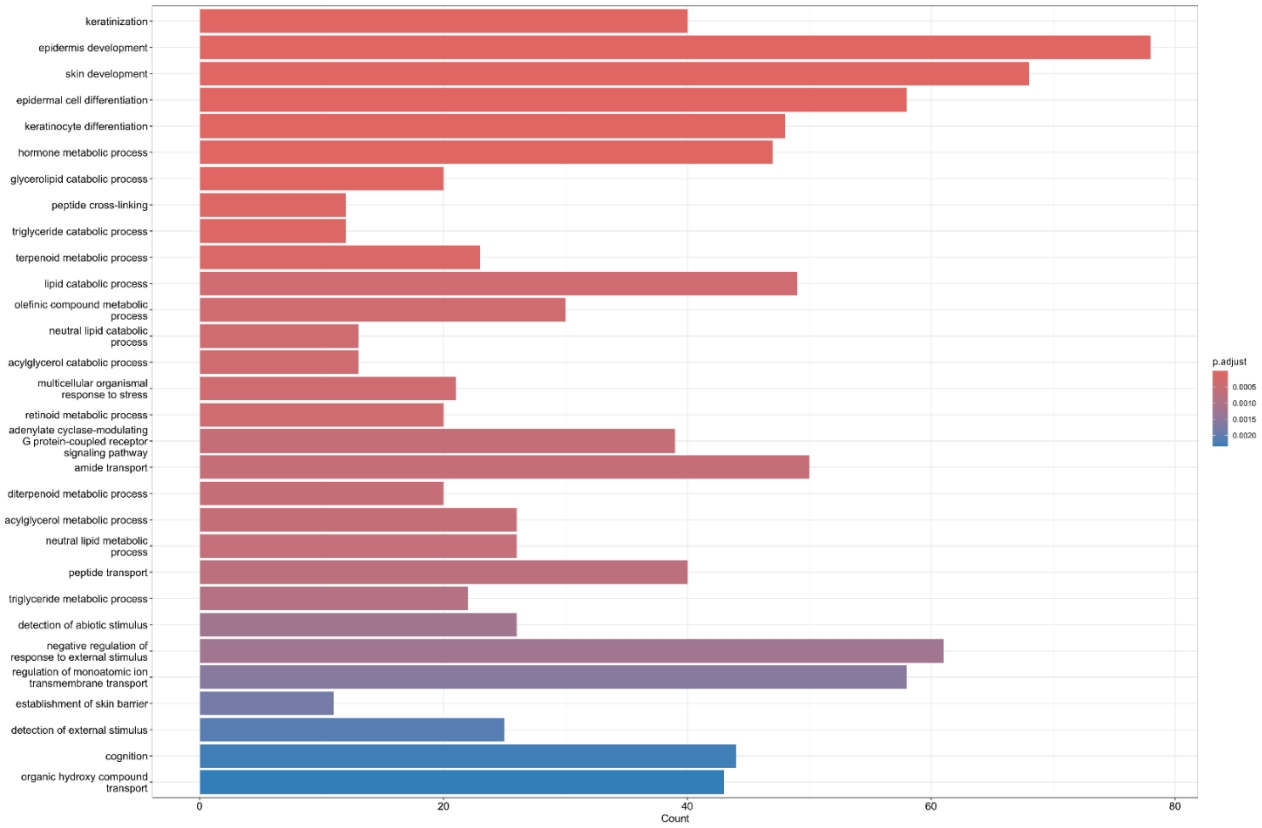


Figure 30. Cellular Components (CC) up-regulated by NVN1000 in HPV-18 RCs.

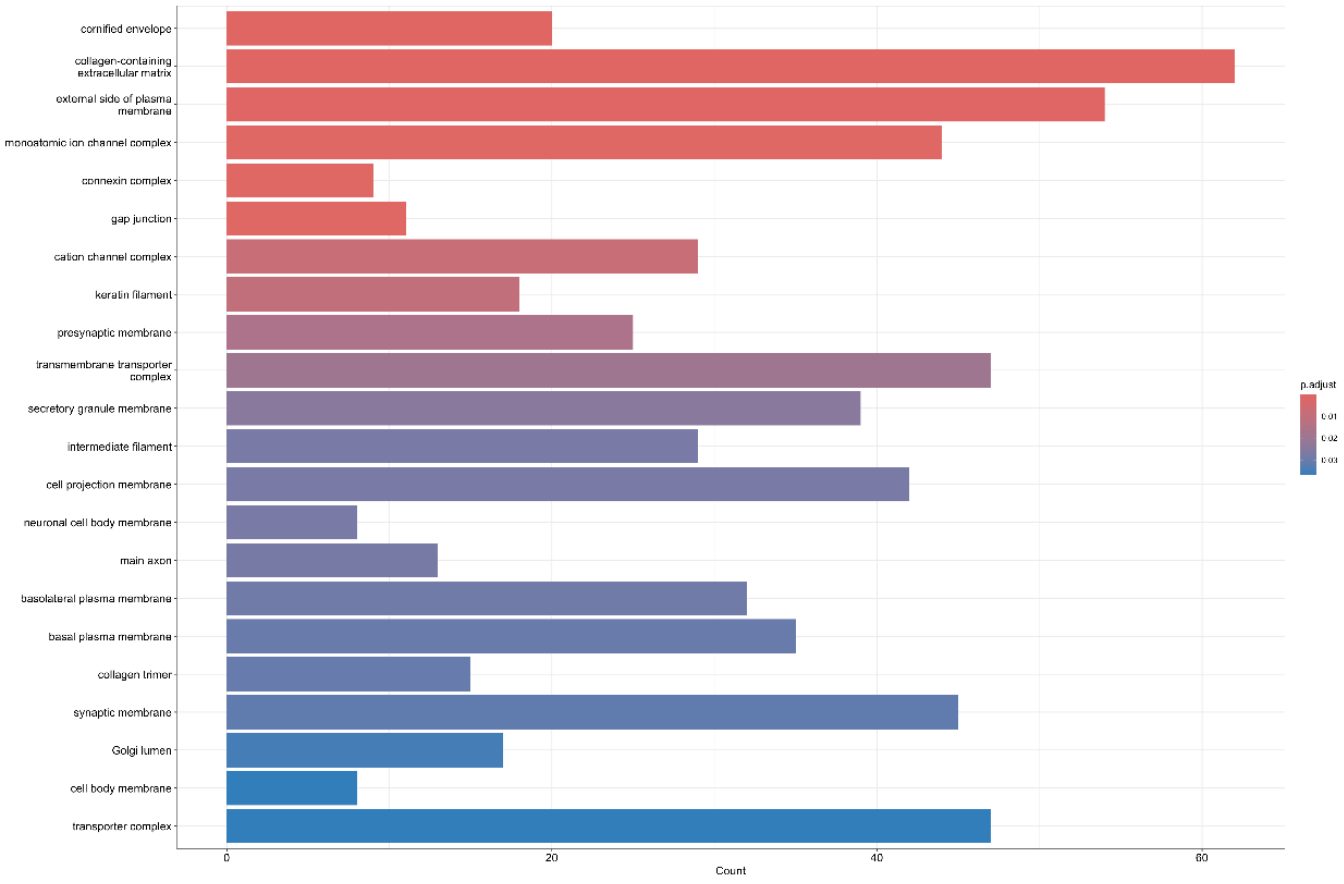


Figure 31. Molecular Functions (MF) up-regulated by NVN1000 in HPV-18 RCs.

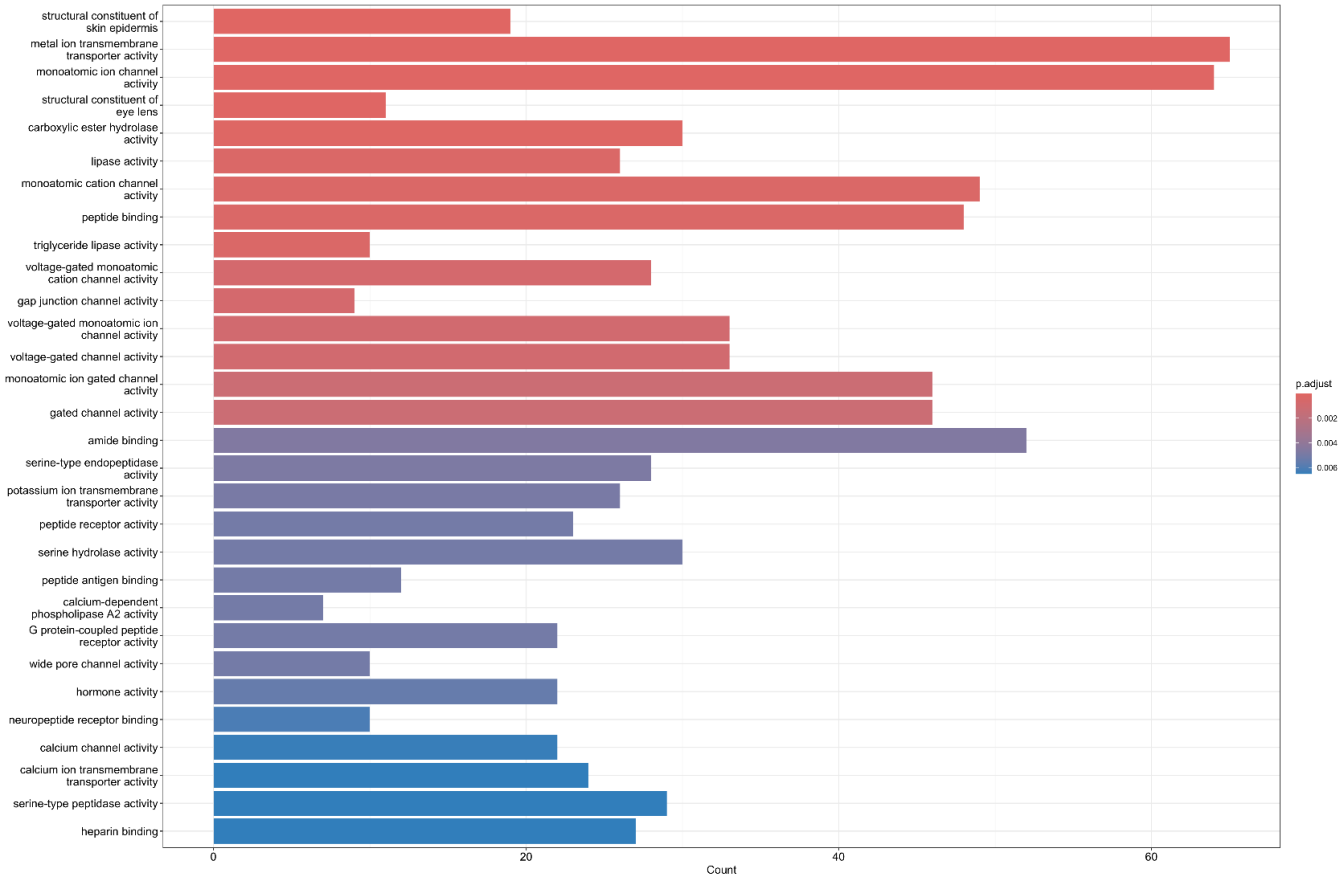
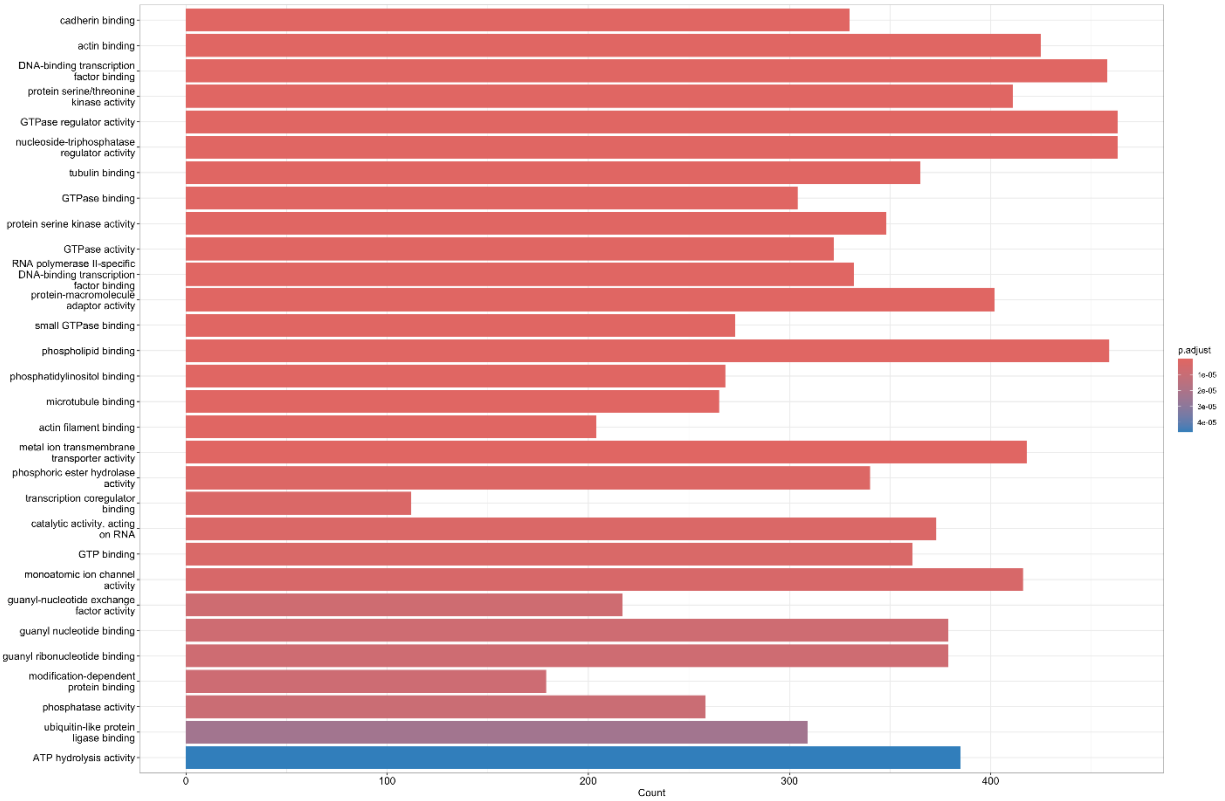


Figure 32. Molecular Functions altered (i.e., up- or down-regulated) by NVN1000 in HPV-18 RCs:



Similarly, Ingenuity pathway analysis identified the following interesting canonical pathways relevant to HPV induced pathology, which were either up-regulated or down-regulated by NVN1000:

1. Glucocorticoid Receptor signaling
2. Wound Healing
3. Oxidative Phosphorylation
4. Mitochondrial Dysfunction
5. Molecular Mechanisms of Cancer
6. Granzyme A signaling.
7. Tumor Microenvironment Pathway
8. ERK/MAPK signaling.
9. Protein Citrullation
10. Regulation of Cellular Mechanics by calpain

Specific Aim 2.3; Deliverable 4: Final Report and Manuscript Preparation.

The work detailed in this report has been drafted as a manuscript and will be submitted for consideration of publication with 1-2 months. Target journals for submission that are being considered include "Viruses" and "Antiviral Research."

In summary, 4 mg/ml NVN1000, upon daily 1 hour application from day-7 to day-13 and a 7-day drug free chase produced 90% reduction of viral DNA amplification relative to the control. But tissues had significant cytopathic effect. Alternate day application of 4 or 5 mg/ml NVN1000, every 48 h between day-7 to day-13 (a total of 4 applications) reduced HPV-18 DNA amplification by ~80%. A 7-day drug free chase of the alternate day treated culture reported small rebound of HPV-18 DNA amplification. There was no robust benefit with the 5 mg/ml NVN1000 dose compared to the 4 mg/ml dose under this dosing schedule. An ~80% reduction from alternate day treatment with 4 mg/ml concentration and ~70% reduction even after a 7-day drug free chase suggests sustainable efficacy of NVN1000 against HPV-18 DNA amplification in raft cultures. Histology clearly demonstrated that NVN1000—treated HPV-18 raft cultures did not fully recover after the 7-day drug free chase, in terms of cell

numbers, cell layers and cell-cell adhesion. Based on these data we envisage that repeat cycles of 4 mg/ml alternate day treatment in preclinical and clinical settings would eliminate most of the HPV-18 infected cells.

The most effective dose and schedule of NVN1000 would be 4 mg/ml daily application, which destroys most of the affected or treated cells, HPV18-infected or -uninfected in our in vitro raft culture experiments. Because the nitric oxide release from NVN1000 is transient, the 4 mg/ml daily application could also be a feasible alternative. Under preclinical and clinical settings, after the infected tissue is destroyed the surrounding cells of healthy epithelium may grow back and heal the wound.

However, because daily application is extremely cytotoxic under the conditions of these raft culture experiments and thus severely reduces the amount of available tissue for analysis, we applied 4 mg/ml on alternate days for biochemical studies to understand the mechanism of action of NVN1000.

Immunoblot analysis revealed elevated expression of p53 and its phosphorylation (S-15) upon four alternate day applications of 4 mg/ml NVN1000. Anti-apoptotic Bcl2 protein was also reduced. But we did not detect any increase of DNA damage marker, gamma-H2AX, or apoptotic proteins, cleaved PARP or cleaved caspase 3 in HPV-18 raft cultures. We interpret that the 48-hour gap between successive treatments, allowed time to repair any damaged DNA or reduction of apoptotic proteins. However, our co-immunoprecipitation could not detect any effect of NVN1000 on interaction between E6 and p53 in raft culture experiment. It is possible that any bound complex between these proteins is quickly destabilized as the complex was not observed in vehicle-treated conditions either.

Surprisingly, even though the steady state level E7 protein decreased (immunoblot, Fig 22A), level of its target proteins pRB and p130 did not increase in 4 mg/ml NVN1000 treated HPV-18 raft cultures (immunoblot, Fig. 22C). But because S-phase re-entry was reduced by NVN1000 applications (indirect immunofluorescence, Fig. 20), we interpret that reduced E7 protein might have caused increased pRB/p130 activity in the differentiated layers of treated HPV-18 raft cultures. Taken together, increase of p53 and inhibition of host DNA replication are manifestation of reduced E6 and E7 protein and their activity by NVN1000.

For further assessment of mechanism of action of NVN1000, bulk RNA sequencing was performed with RNA extracted from uninfected and HPV-18 infected PHK raft cultures. The HPV-18 E6E7 transcripts were reduced significantly in NVN1000 treated cultures. The results then revealed that NVN1000 exposure significantly altered multiple host pathways through up-regulation or down-regulation of their mRNA expressions. A reduction of hTERT mRNA (for telomerase) testified for reduced HPV-18 E6 protein level and activity. Transcription of 144 p53 regulated genes were altered, and thus p53 appears to be one of the major regulators of anti-HPV activity of NVN1000. However, we did not detect elevated expression of several p53 downstream effector proteins in NVN1000 treated cultures. For example, mRNAs of proapoptotic p53AIP was slightly elevated by 2.44-fold, but transcription of classical p53 regulated proapoptotic proteins, Bax, PUMA, NOXA etc. or mRNA of growth suppressor protein p21CIP1 did not increase. Effects on multiple pathways, biological processes, molecular functions and cellular components suggest broad pleiotropic effects of NVN1000 on HPV-18 raft culture. Thus, a thorough investigation is necessary to pinpoint which NVN1000 regulated genes had decisive effects on HPV-18 DNA amplification.

Future research should focus on the top genes whose transcription altered (up or down regulated) in HPV18 raft cultures but not in uninfected PHK raft culture. The rational for this approach is that those genes should be regulated by HPV infection, and their alteration by NVN1000 may reveal an inhibitor- specific effect on the HPV life cycle. The top genes selected should be based on expression amount (>50 FPKM) either in control or treated, those altered by lowest p-adjusted value (false discovery rate) and those which have literature-based relevance to physiological aspects important to HPV life cycle. Additionally, the top 10 up or down regulated genes from the p53 pathway should be specifically looked at. A deeper bioinformatic analysis could be performed to determine the list of candidate genes as mediators of NVN1000 activity. A potential approach could be (1) confirmation of the effect of NVN1000 on HPV-18 transcription and alternative splicing by RT-PCR and quantitative PCR. (2) confirmation of RNA seq data by RT-PCR/qPCR analysis (3) confirmation translation of differentially expressed genes by immunoblot and IHC analysis, if antibodies are available (4) analysis of the relevance of these differentially expressed genes with respect to E6 and E7 expression and activity and in the HPV18 DNA amplification. (5) Finally, a study on the mechanism of action of NVN1000 on genes which could have the best inhibitory influence on HPV-18 DNA replication or HPV E6/E7 expression. Additionally, a single cell mRNA

sequencing of HPV-18 raft cultures could determine differentially expressed genes in cells which retained HPV-18 E7 activity and those which did not.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report as this is the final report for this award.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

The work conducted under the entirety of this award furthered the understanding of the antiviral activity of NVN1000, a nitric oxide-releasing drug, specifically against HPV-18. Knowledge on the mechanism of action for how the drug may combat against HPV viral infection can inform such aspects that would be studied under clinical development, including dose response and dosing frequency. Further, the formulation development conducted under this award can inform future formulations for NVN1000 or for other vaginal suppositories with a potentially different active pharmaceutical ingredient.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

The aim of the work under this award was to progress development of a novel treatment for HPV infection and CIN. Ultimately, work focused on healthcare topics involving a novel treatment could inform public health policy and ensuing regulatory practices. However, the stage of development for this therapy is not expected to have any impact at this point in development.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

In July 2022, Novan met with SO/GOR Emilee Senkevitch and received permission to stop any further work on Specific Aim 1 (as detailed above). Based on the data from the prototype formulations and the funds remaining, it was determined that the Go/No-go goal of 12 weeks of room temperature stability for the prototype formulation was unlikely to be met as the developed prototypes were found to require refrigerated conditions (i.e., 2-8 °C) to maintain stability. This resulted in abandoning the in vitro dissolution testing of the vaginal suppository prototypes as well as the additional performance testing (e.g., condom compatibility, mucoadhesion testing) that was planned. Remaining funds were rebudgeted to Aim 2 as agreed, and the project was granted a 1 year no-cost extension to further mechanistic work against HPV that was not reliant on need for a formulation as this in vitro work required the active pharmaceutical ingredient instead.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report as this is the final report for this award.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable, so nothing to report.

Significant changes in use or care of vertebrate animals

Not applicable, so nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report currently, but a manuscript on the in vitro mechanistic work against HPV is drafted and planned for submission. The final authors are still pending, but will include N. Sanjib Banerjee and Thomas R. Broker.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report. Previous patents existed prior to this award. The research conducted under this award has not resulted in any inventions or patent applications or licenses.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

From project initiation through the date of this final report, none of our personnel have worked more than one-person month on the project. These lower personnel hours are expected because our personnel have operated in supporting and oversight roles while our collaborator, MedPharm, performed against the key deliverables under Aim 1.1 of the project. Since the majority of work under Specific Aim 1 was abandoned as discussed above due to technical challenges regarding stability, the work that was planned to increase the Novan personnel hours (e.g., in vitro dissolution testing of vaginal suppository) never occurred.

While none of our personnel have worked more than one-person month to date, we deemed it appropriate to list those key personnel who have contributed more than one-person month during the total project duration or whom were active employees involved at close of this award.

Name	**Carri Geer, Ph.D.**
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	Year 1 – 1.8 calendar months, Year 2 – 0.9 calendar months, Year 3 – 0.1 calendar months; Year 4 – 0.1 calendar months; Year 5 – 0.0 calendar months
Contribution to Project:	Led the work performed by consultants and subcontractors and worked closely with Novan's Pharmaceutical Development team and MedPharm to formulate a nitric oxide-releasing vaginal suppository and with collaborators at the University of Alabama – Birmingham.
Name	**Hussaini Qhattal, Ph.D.**
Project Role:	(former employee) Associate Director of Product Development
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	Year 1 – 2.8 calendar months, Year 2 – 1.8 calendar months, Year 3 – 0.1 calendar months
Contribution to Project:	Lead the formulation development with MedPharm
Name	**Shashank Jain, Ph.D. (Replaced Hussaini Qhattal in Q2 2021)**
Project Role:	Director of Product Development
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	Year 3 – 0.0 calendar months, Year 4 – 0.1 calendar months, Year 5 – 0.0 calendar months
Contribution to Project:	Lead the formulation development with MedPharm

Name	**Benjamin "BJ" Privett, Ph.D.**
Project Role:	(former employee) Associate Director, Drug Substance Development
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	Year 1 – 4.5 calendar months, Year 2 – 1.8 calendar months, Year 3 – 0.1 calendar months, Year 4 – 0.1 calendar months, Year 5 – 0.0 calendar months
Contribution to Project:	Worked closely with MedPharm to guide experiments, analyze data, and summarize major conclusions. Served as the grant manager and assisting in vendor oversight.

Name	**Shaylyn Walter**
Project Role:	(former employee) Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	Year 1 – 2.5 calendar months, Year 2 – 0.6 calendar months
Contribution to Project:	Providing technical support and guidance during formulation development.

Name	**Daniel Riccio, Ph. D**
Project Role:	Senior Director, Discovery and Translation
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	Year 5- 0.0. calendar months
Contribution to Project:	Served as technical grant manager for this final submission.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES:

AWARD CHART

CA180741: Topical Nitric Oxide Therapy to Treat Cervical Neoplasias and Prevent HPV-Associated Cancers

PI: Carri Geer, Ph.D., Novan, NC **Budget:** $1,112,679.00

Topic Area: CDMRP **Mechanism:** W81XWH-18-PRCRP-IPA

Research Area(s): 0104, 0800, 0801, 0803 **Award Status:** 30-SEP-2019 TO 29-SEP-2023

Study Goals:

The goal of this study was to develop a nitric oxide-releasing vaginal suppository that can be self-administered by female patients as a treatment for cervical neoplasias to eradicate latent HPV infection and inhibit disease progression to cancer.

Specific Aims:

The aims were to 1) formulate a stable vaginal suppository with well-characterized physical chemical properties suitable for intravaginal administration and 2) evaluate the effect of varying concentrations and treatment durations of NVN1000 against HPV-18 in human raft cell culture in vitro studies.

Key Accomplishments and Outcomes:

1. Established a range of potential suppository excipients that are compatible with NVN1000
2. Developed lead prototype formulations and methods of manufacture
3. Evaluated stability of lead prototypes over 8 weeks and established that refrigeration is required and that additional development may be needed to achieve room temperature stability
4. Identified lead prototype with sufficient compatibility with NVN1000
5. Confirmed the antiviral efficacy of NVN1000 against HPV-18 and E6/E7 in a human raft cell culture model
6. Established the minimally effective dose of 4 mg/mL NVN1000 treated every 2nd day is capable of sustaining an antiviral response through a drug-free period
7. Screened numerous genes that may be impacted by NVN1000 treatment and antiviral efficacy

Publications: none to date

Patents: none

Funding Obtained: none

Award Expiration Transition Plan

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.
This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** ☑ or **No** ☐
These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

The data support that NVN1000 can be formulated into a vaginal suppository to yield a potential nitric oxide-releasing therapy; however, the data support that refrigeration is required to maintain stability over a period of 12 weeks. Room temperature stability may be able to be attained but would require further formulation development and potential improvements to any manufacturing process. The final data obtained under this award also support that NVN1000 has potent antiviral efficacy against HPV-18, specifically impacting E6 and E7 levels and/or activity. Furthermore, a dosing schedule of 4 mg/mL NVN1000 applied every other day is the minimally effective concentration to have potent antiviral activity, while mitigating unwanted cytotoxicity. The anti-HPV-18 activity was maintained even after a period of drug free exposure, demonstrating a sustainable and desired impact against HPV-18 viral replication. Finally, the RNA sequencing data support that NVN1000 can impact multiple pathways in its fight against HPV infection and these pathways can be the focus of future research.

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award.*

No, we have not.

4. What will be **the next step(s)** for this project?

There are no current plans for this project, but future directions for such research should revisit the formulation development of the suppository and conduct those aims/deliverables that were not completed during the course of this study (e.g., additional performance testing and in vitro dissolution testing). Finally, the mechanistic work for NVN1000 against HPV warrants additional focus to deconvolute any key pathways that may be critical in the antiviral response against HPV-18 that NVN1000 evokes. This may help inform inprovements to the intended therapy and a deeper understanding of its mechanism of action against this virus.

5. How would you classify your **lead candidate product**? a
(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Small Molecule
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify:

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

The relevance of this candidate product is to both the general population and the military. As a high risk strain of the human papillomavirus, HPV-18 is associated with cerivical intraepithelial neoplasia (CIN) and cervical cancers, as well as many other cancers of the vagina, vulva, rectum, and penis. In female service members, HPV prevalence is reported to be higher than in the general population, but in both cases the incidence rates are staggeringly high. This candidate product was aimed at being a self-administered treatment for many stages of CIN and to ultimately reduce progression of the disease to cancer. An easily administered, safe, and efficacious product would allow for a flexible treatment option for both service women, as well as women in the general population.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

As the research (i.e., formulation development) conducted under specific aim 1 of this award did not reach the initial go/no-go milestone of achieving room temperature stability, this is an area that should be revisited. In parallel, the current lead formulation can be progressed through preclinical stages if the need for refrigeration of the final product was not viewed as a nonstarter. NVN1000 is the intellectual property of Novan and LNHC, Inc and would require that entities' involvement in progressing this therapy. Any additional IP or patent opportunities should be examined after formulation development. Regardless, once formulation development is complete, preclinical safety of the formulation needs to be assessed so that the appropriate IND-enabling studies can be used to support opening an IND with the FDA in an attempt to clinically develop such a formulation.
The in vitro studies conducted by UAB would support the pharmacology of any potential candidate, but additional in vivo papillomavirus models could also be examined.