Published in final edited form as:

Antiviral Res. 2018 February ; 150: 164–173. doi:10.1016/j.antiviral.2017.12.013.

Evaluation of ODE-Bn-PMEG, an Acyclic Nucleoside Phosphonate Prodrug, as an Antiviral Against Productive HPV Infection in 3D Organotypic Epithelial Cultures

N. Sanjib Banerjee^{1,*}, Hsu-Kun Wang^{1,†}, James R. Beadle², Karl Y. Hostetler², and Louise T. Chow¹

¹Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294-0005

²Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0676

1. Introduction

Human papillomaviruses (HPV) are double-stranded DNA viruses that infect mucosal or cutaneous epithelial tissues (Bravo and Felez-Sanchez, 2015). The mucosotropic low-risk types cause benign anogenital warts and recurrent respiratory papillomatosis, whereas persistent infections by high-risk HPVs can progress to cancers. Over 95% of cervical cancers (Walboomers et al., 1999), 70% of oropharyngeal cancers and about 5–30% of other head and neck carcinomas are caused by high-risk HPVs (Prigge et al., 2017). In 2012, 266,000 deaths worldwide resulted from 530,000 cases of cervical carcinomas (Ferlay et al., 2015). Prophylactic vaccines (Gardasil, Gardasil-9 and Cervarix) are highly effective, but they are type-restricted and do not impact pre-existing infections (Schiller and Muller, 2015). Moreover, HPV vaccination programs around the world have been met with varied acceptability (Bruni et al., 2016). Importantly, there are no pharmaceutical agents that reliably and efficaciously treat HPV infections by specifically targeting viral DNA amplification and persistence.

All HPV genotypes establish infection in basal keratinocytes of a squamous epithelium, where extrachromosomal viral genomes are maintained as low copy number plasmids with minimal early gene expression in order to dodge immune surveillance. In active infections,

*Corresponding author: N. Sanjib Banerjee, Ph.D., Telephone: +1 205-975-8304, banerjee@uab.edu. current address: MilliporeSigma, Temecula, CA 92590

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest

The authors declare the following competing financial interests: K.Y.H. is a consultant to and equity holder in Antiva Biosciences, Inc. J.R.B. is an equity holder in Antiva Biosciences, Inc. N.S.B., H-K.W and L.T.C. have no competing interest to declare.

Supplemental Materials:

Following supplementary information is submitted.

1. Appendix A: Detailed Experimental Procedures.
2. Appendix B: Supplementary Figure Legends.
3. Four Supplementary Figures.

elevated viral RNA transcription and DNA amplification occur in the differentiating epithelium (Chow et al., 2010; Doorbar et al., 2015; Stoler et al., 1989). Three dimensional (3D) organotypic (raft) cultures of PHKs recapitulate the proliferation and differentiation of a squamous epithelium and support virus production in explanted warty lesions (Dollard et al., 1992; Wilson et al., 1992). By utilizing the Cre-LoxP recombination, high titers of infectious HPV-18 virus are produced in raft cultures of PHKs transfected with recombinant HPV-18 plasmids. In situ analyses of these cultures have produced molecular details of the viral productive program (Wang et al., 2009). The HPV-16 and HPV-31 life cycles have also been studied in raft cultures of immortalized NIKS cell lines or in human foreskin keratinocytes after prolonged passaging (Lambert et al., 2005; Wilson et al., 2005), but viral DNA amplification was not as robust.

Papillomaviral DNA replication requires the viral E2 and E1 proteins. E2 binds to the replication origin and helps recruit the replicative DNA helicase E1. The host supplies all other replication proteins and substrates (Kuo et al., 1994). To enable viral DNA amplification in differentiated cells, HPV oncoproteins E6 and E7 destabilize the major tumor suppressors, p53 and the pRB family proteins, respectively (Chow and Broker, 2013; Galloway and Laimins, 2015). E7 protein reactivates the cell cycle genes and S-phase reentry (Cheng et al., 1995; Genovese et al., 2008) along with the DNA damage response (DDR) (Banerjee et al., 2011; Hong and Laimins, 2013; Moody and Laimins, 2009). Subsequent to S-phase, the differentiated cells transition into a prolonged G2-phase, during which the viral genome amplifies (Banerjee et al., 2011; Wang et al., 2009). Prolonged G2 is evidenced by suprabasal accumulation of cytoplasmic cyclin B1. Kinases activated by DDR stabilize p53, which, in turn, is inactivated or destabilized by E6 in association with E6-AP. This E6 function is critical for efficient viral DNA amplification (Kho et al., 2013; Wang et al., 2009). Following viral DNA amplification, E7 activity wanes and becomes extinguished, as deduced from the cessation of cellular DNA replication and the loss of cyclin B1. Viral transcription then switches to late gene expression, producing capsid proteins for progeny virion assembly. Thus, biomarkers for HPV E7 activity include induction of suprabasal proliferating cell nuclear antigen (PCNA), suprabasal bromodeoxyuridine (BrdU) incorporation into replicating host DNA, and accumulation of suprabasal cytoplasmic cyclin B1, whereas p53 accumulation is a marker for reduced E6 function.

Acyclic nucleoside analogs have been developed to inhibit the replication of diverse virus families (De Clercq, 2003a; De Clercq and Li, 2016). For example, (S)-1-[(3-hydroxy-2-phosphonomethoxy) propyl]cytosine (HPMPC, i.e., Cidofovir (CDV)) has a 100–500 fold higher affinity for human cytomegalovirus (CMV) DNA polymerases than for cellular DNA polymerases (Hitchcock et al., 1996; Kramata and Downey, 1999) and is effective in treating CMV retinitis (De Clercq, 2003b). HPVs do not encode a DNA polymerase, but CDV has been used to treat preneoplastic HPV infections of mucosal epithelia with mixed results (De Clercq, 2003a, b; Stier et al., 2013; Van Pachterbeke et al., 2009). The bioavailability of CDV is low, and delivery of high concentrations over long durations may cause systemic toxicity and renal failure (Bienvenu et al., 2002). Importantly, the efficacy of CDV in blocking productive HPV DNA amplification or viral persistence has not been critically examined.

Lipid esters of acyclic nucleoside phosphonate antivirals show greatly increased cell uptake and antiviral activity (Hostetler, 2009). Octadecyloxyethyl benzyl 9-[2-phosphonomethoxyethyl]guanine (ODE-Bn-PMEG, i.e., ABI-1968) is a diester of the acyclic guanine-based phosphonate. Benzyl diesters exhibit very slow intracellular conversion to the active diphosphate metabolite, providing sustained effects (Andrei et al., 2015; Beadle et al., 2016). Because it lacks a 3' hydroxyl, DNA chain elongation ceases following PMEG incorporation. It effectively inhibits HPV origin-dependent plasmid amplification in transfected HEK-293 cells (Beadle et al., 2016). In the present study, we investigated ODE-Bn-PMEG as a prodrug inhibitor of HPV-18 genomic DNA amplification in organotypic epithelial cultures. This 3D raft culture is a highly informative model well suited to reveal the mechanisms of action and possible side effects in uninfected tissues. We demonstrate that ODE-Bn-PMEG at 1.5 μ M has superior antiviral effects relative to 15 μ M CDV. ODE-Bn-PMEG blocks HPV DNA amplification, virion production and possibly viral persistence, as well as causing apoptosis in suprabasal infected cells.

2. Materials and methods

PHKs were isolated from circumcised neonatal foreskins (Wilson et al., 1992) with approval by the UAB Institutional Review Board. Raft cultures of PHKs with or without HPV-18 genomic plasmid were prepared as described (Wang et al., 2009). ODE-Bn-PMEG (HPLC purity = 94.6%) was synthesized as described (Beadle et al., 2016). 10 mM stock solution of ODE-Bn-PMEG or CDV in DMSO was added to the cultures to final concentrations and for durations indicated for each experiment. Raft cultures were harvested, fixed in 10% buffered formalin and embedded in paraffin. Four micron sections were used for in situ assays. Unfixed tissues were used to determine viral DNA copy number per cell. Detailed protocols and reagents are provided in Supplementary Materials (Appendix A).

3. Results

3.1. ODE-Bn-PMEG inhibited HPV-18 DNA amplification and cellular DNA replication

HPV-18 DNA amplification takes place between day 10 and day 14 after the cultures are placed at the liquid medium-air interface (Wang et al., 2009). In uninfected cultures, S phase cells, as indicated by BrdU incorporation into replicating host cell DNA, were observed only in the basal stratum. Untreated HPV-18 raft cultures were hyperplastic relative to uninfected cultures (Fig. 1A), and stochastic suprabasal S-phase reentry was induced in a substantial fraction of the differentiated cells (Wang et al., 2009) (Fig. 1B). Based on previous results (Beadle et al., 2016) and the toxicity in submerged PHK cultures, a subset of the cultures was treated with ODE-Bn-PMEG at 0.16 μ M, 0.5 μ M or 1.5 μ M. Application started on day 6 (refreshed on days 8, 10 and 12) and cultures were harvested on day 13. Upon exposure to 0.5 μ M and 1.5 μ M, uninfected and HPV-infected cultures became thinner than the untreated cultures, with decreasing DAPI-positive nuclei (Fig. 1A, B). In uninfected raft cultures, BrdU-positive basal nuclei were virtually abolished, suggestive of a cytostatic effect. In the infected cultures, positive nuclei and the signal intensity were both greatly reduced (Fig. 1A, C, see also Fig. S1B). Real time qPCR in three independent experiments conducted with separate batches of PHKs revealed a concentration-dependent decrease in viral DNA copy

numbers per cell, down to about 20% of those in untreated control cultures (Fig. 1D). Fluorescence in situ hybridization to HPV DNA in tissue sections supported this conclusion (Fig. 1E).

3.2. The durable antiviral effect of ODE-Bn-PMEG

To test the durability of ODE-Bn-PMEG, we exposed normal and HPV-18 infected raft cultures to 1.5 μ M ODE-Bn-PMEG over days 6 to 10 (applied on days 6 and 8), days 6 to 12 (applied on days 6, 8, and 10), or days 6 to 14 (applied on day 6, 8, 10, and 12). One set of cultures was harvested on day 14 and another set on day 18. Compound-treated day 14 HPV-18 and normal cultures were thinner than untreated control cultures, and BrdU-positive cells were greatly reduced (Fig. 2, Fig. S1B), in agreement with the previous experiments (Fig. 1A). Importantly, neither culture recovered after additional days of culturing in compound-free media (Fig. 2, Fig. S1B). These results indicate a durable inhibition, consistent with the slow conversion of ODE-Bn-PMEG to a DNA replication substrate in human fibroblasts (Beadle et al. 1016). In addition, chain termination would preclude further elongation during the chase. In this series of experiments, we observed suprabasal condensed nuclei in the infected cultures, suggestive of apoptosis (Fig. 2A).

Real Time qPCR revealed that untreated HPV-18 infected cultures harbored 5,000 and 4,000 copies/cell on days 14 and 18, respectively. In samples exposed to ODE-Bn-PMEG for 4 days or longer, viral DNA was reduced by more than 90%. Viral DNA amplification did not rebound after a chase with compoundfree medium (Fig. 3A). Indeed, FISH detected sparingly few cells with amplified HPV DNA upon short or long exposure (Fig. 3B, C). The capsid L1 protein was not detected in treated day 18 cultures, consistent with the dependency of L1 expression on high viral DNA amplification (Kho et al., 2013; Wang et al., 2009) (Fig. 3D).

3.3. ODE-Bn-PMEG significantly reduced E7-mediated induction of PCNA and cyclin B1

The absence of suprabasal BrdU incorporation does not necessarily indicate the absence of the viral genome. We probed the tissues for the well-established E7-induced biomarkers PCNA and cyclin B1 for evidence of persisting viral activities. Indirect IF assays showed that ODE-Bn-PMEG reduced basal PCNA and cyclin B1 in infected and uninfected cultures (Fig. 4, Fig. S1A). In the infected cultures, a greater reduction of suprabasal signals was observed in cultures exposed over days 6–14 than those exposed over days 6–10. In day 14 cultures, suprabasal viral activity was still evident (Fig. S2, rows 4 and 5). However, in cultures harvested on day 18, only very few basal cells were weakly positive for these two biomarkers (Fig. 4, rows 4 and 5). The patterns and signal intensities resembled those in normal PHK raft cultures (Fig. 4, compare rows 1 to 5), suggesting that ODE-Bn-PMEG effectively abrogated E7 activities.

3.4. ODE-Bn-PMEG induced DNA damage in HPV-18 raft cultures

γ -H2AX is a marker for double-stranded DNA breaks (Rogakou et al., 1998). Indirect IF assays detected signals primarily in suprabasal cells of HPV-18 cultures treated with 1.5 μ M ODE-Bn-PMEG (Fig. 5A). The percentage of positive nuclei increased from 30% to over 50% of total nuclei, with increasing duration of exposures (Fig. 5B). This percentage did not

decrease following a 4-day chase in day 18 cultures. By comparison, nuclei positive for γ -H2AX were detected only in 1 or 2 per 100 cells in the untreated HPV-18 raft cultures. Untreated normal PHK cultures had no signal, but positive nuclei were detected in treated cultures (Fig. 5A). Thus replicating DNA is vulnerable to this inhibitor irrespective of infection, but infected cultures accumulate more γ -H2AX-positive nuclei due to the high numbers of suprabasal cells in S-phase.

3.5. ODE-Bn-PMEG induced apoptosis in HPV-18 raft cultures

The high percentage of γ -H2AX positive nuclei and incidence of condensed nuclei in HPV-18 raft cultures exposed to ODE-Bn-PMEG (Figs. 2, 5) are suggestive of apoptosis. Positive TUNEL signals indicative of extensive DNA fragmentation and cell death indeed detected in infected cultures harvested on day 14, but primarily in the differentiated strata. Positive nuclei increased with a higher concentration and longer duration of exposure (Fig. 6A and B, see also Fig. S3).

The cell number and hence thickness of uninfected raft cultures normally decrease with age as basal cell proliferation diminishes. Infected raft cultures also became thinner because of reduced basal cell proliferation and reduced HPV early gene expression following viral DNA amplification (Wang et al., 2009) (Fig. 6C, compare total nuclei in untreated HPV-18 cultures harvested on day 18 and day 14). In day 14 and day 18 infected cultures exposed to 1.5 μ M ODE-Bn-PMEG, DAPI-positive nuclei were reduced to about 20% – 30% of untreated cultures of the same age (Fig. 6C). These results are consistent with reduced basal cell proliferation, continued loss of apoptotic cells, or both.

In contrast, TUNEL-positive nuclei were rarely detected in control or PHK cultures exposed to 1.5 μ M ODE-Bn-PMEG for various durations (Fig. 6A, top row). Along with the loss of basal BrdU incorporation, these results suggest that 1.5 μ M ODE-Bn-PMEG is largely cytostatic to normal PHK raft cultures.

3.6. Cidofovir (CDV) reduced HPV DNA amplification but did not significantly affect viral activity

CDV at 6 μ M reduced the titer of camel pox virus (CML1) in PHK raft cultures by 99% (Duraffour et al., 2007). However, it had little effect on HPV ori-dependent plasmid amplification in transiently transfected HEK-293 cells (Beadle et al., 2016). In parallel to the time course experiments just described, we also examined normal and HPV-18 infected day 14 and day 18 raft cultures after an exposure to 15 μ M CDV over days 6–14. CDV reduced viral DNA amplification, but not as effectively as ODE-Bn-PMEG (Fig. 3A). L1 expression was abolished (Fig. 3D). The tissues were slightly thinner (Fig. 2A) and contained fewer DAPI-positive cells than controls, but twice as many as in ODE-Bn-PMEG exposed cultures (Fig. 6C). However, suprabasal BrdU-positive cell numbers did not appear to change significantly (Figs. 2 and S4). The suprabasal cells also remained positive for PCNA and cyclin B1 (Figs. 4 and S2). These observations demonstrate that, despite reduced HPV-18 plasmid amplification, transcriptionally competent viral genomes persisted. In contrast, in uninfected PHK cultures, Cidofovir inhibited BrdU incorporation and eliminated cyclin B1 signal in basal cells (Fig. S1B), indicative of a cytostatic effect in normal raft cultures.

In the infected cultures, CDV exposure induced γ -H2AX-positive nuclei, but the number was significantly lower than cultures treated with 1.5 μ M ODE-Bn-PMEG (Figs. 5A, B). The DNA damage appeared to be transient or not extensive, as the cultures exhibited no condensed nuclei characteristic of apoptosis (Fig. 2) and were virtually negative for TUNEL signals (Figs. 6A, B). Thus, CDV reduced the cell number to 48% of the untreated control (Fig. 6C), largely due to the inhibition of basal cell proliferation. The differential cell number relative to ODE-Bn-PMEG treated cultures could be attributed to the absence of TUNEL-positive apoptosis in CDV-treated cultures (Figs. 6A, B). In normal PHK raft cultures, CDV induced few if any γ -H2AX-positive or TUNEL-positive nuclei (Figs. 5, 6).

3.7. Cidofovir stabilized p53 to inhibit HPV-18 DNA amplification

Why did CDV exposure (days 6–14) reduce HPV-18 DNA amplification by 80% (Fig. 3A), while the E7 activity was hardly affected (Figs. 4 and S2)? We previously demonstrated that E6 mutants that are unable to destabilize p53 do not amplify viral DNA to high copy numbers (Kho et al., 2013; Wang et al., 2009). Double IF was performed in inhibitor-treated HPV-18 raft cultures. While control cultures had cyclin B1 signal but no p53 signal, both p53 and cyclin B1 were detected in CDV-treated (Fig. 7). In contrast, in ODE-Bn-PMEG treated cultures, neither p53 nor cyclin B1 was detected (Fig. 7, 3rd and 4th rows from top), in agreement with a loss of the E7 activity. Normally the expression of E7 in raft cultures causes p53 stabilization in the absence of E6 (Jian et al., 1998).

4. Discussion

In this study, we investigated the efficacy of ODE-Bn-PMEG as an anti-HPV agent in a 3D organotypic model system prepared from PHKs containing HPV-18 genomic plasmid. Our results reveal that the compound is effective and durable in abrogating viral DNA amplification and virus production. This antiviral effect is attributable to obligate chain termination during cellular and viral DNA replication. A high efficacy was reached at 1.5 μ M after an exposure for 8 days (days 6 to 14). Viral DNA copy numbers/cell were reduced by at least 90%. We further conclude that suprabasal viral DNA was rendered transcriptionally inactive, as the induction of PCNA, suprabasal BrdU incorporation, and cytoplasmic cyclin B1 accumulation, all biomarkers of E7 activity (Banerjee et al., 2011), were dramatically reduced (Figs. 4 and S2). These results are expected, as transcription takes place only on supercoiled templates. Replication intermediates trapped by chain termination or broken into fragments are not effective templates. Incomplete viral genomes are also subject to destruction by nucleases. In fact, in the day 18 cultures after a 8-day treatment and 4-day chase, the basal cells of infected cultures resembled those in uninfected cultures when probed for PCNA or cyclin B1 (Fig. 4). These results suggest the possibility that at least some of the basal cells had lost viral activity, if not viral DNA. Since all HPVs use the same mechanisms to amplify their DNA in the differentiated cells, these inhibitory effects are expected to extend to other HPV genotypes.

Our results agree with the evidence that ODE-Bn-PMEG and its active metabolite PMEG-pp have a long intracellular stability (14 days after a 48 hours exposure) in human fibroblasts (Beadle et al., 2016). Cellular DNA replication and viral DNA amplification did not rebound

after a chase of 4 - 8 days (day 14 or day 18 cultures after an exposure on days 6–10, 6–12 or 6–14), as judged from multiple biomarkers. These results imply that DNA damage was not repaired, consistent with a lack of reduction in γ -H2AX positive nuclei during the chase. Consequently, extensive apoptosis took place in the suprabasal differentiated strata. Chain termination after the incorporation of ODE-Bn-PMEG metabolite also interferes with basal cell DNA replication and cell division of infected or uninfected cultures. Despite the cytostatic effect in uninfected basal cells, 1.5 μ M ODE-Bn-PMEG rarely caused apoptosis. We suggest that, relative to basal cells, infected suprabasal cells are sensitized to cytotoxic effects of ODE-Bn-PMEG due to elevated viral E7 gene activities. For instance, S-phase genes, including PCNA (Fig. 4 and S2) and the p180 catalytic subunit of DNA polymerase α (Chien et al., 2000), are expressed to higher levels in the differentiated cells as compared to the basal cells.

We further treated raft cultures with 2.5 μ M of ODE-Bn-PMEG for various durations, with or without a chase. Viral DNA copy number per cell was further reduced (Fig. 3A). However, apoptosis was induced in both infected and uninfected raft cultures (Fig. S3A, B). Thus the selectivity of infected over uninfected cultures was diminished or lost at this high concentration.

We also examined the effects of CDV, which has been used for clinical treatment of infections with a variety of DNA viruses, including HPV-induced warts, condyloma and laryngeal papillomas. The results were mixed (Andrei et al., 2000; De Clercq, 2003a, b). In our model system, CDV at 15 μ M reduced viral DNA amplification and induced DNA damage. However, it induced very little or no apoptosis and did not reduce viral E7 activity. These observations are consistent with the notion that CDV is not an obligate chain terminator and is inefficiently incorporated into DNA by cellular DNA polymerases (Wolfgang et al., 2009). Interestingly, while E7 activity persisted, E6 activity with regard to p53 destabilization was somewhat compromised. Thus, the stabilized p53 could be responsible in part for the reduced HPV-18 DNA amplification. Previous reports indicated that CDV induced p53 stabilization in HPV-positive cervical cancer cell lines (Abdulkarim et al., 2002; Andrei et al., 2000; Sirianni et al., 2005), but the mechanism was unknown. Abdulkarim et al. (2002) also reported that CDV reduced E7 protein and increased pRb in these cells. Although we cannot rule out that E7 activity was also diminished in CDV-treated cultures, our probing for surrogate biomarkers of E7 activities such as S-phase re-entry (BrdU incorporation), PCNA induction, and cytoplasmic cyclin B1 accumulation strongly indicate that significant E7 activity persisted in CDV-treated raft cultures (Figs. 4, 7, S2 and S4).

CONCLUSIONS

ODE-Bn-PMEG (ABI-1968) is superior to Cidofovir as an inhibitor of pre-neoplastic HPV infection, abolishing viral DNA amplification and production of progeny virus. Furthermore, it causes apoptosis in suprabasal infected keratinocytes while sparing uninfected cultures. ODE-Bn-PMEG is a very promising antiviral candidate for additional preclinical development leading to clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank DeeAnne S. Jackson, M.D. and the UAB Hospital Newborn Nursery for providing freshly harvested neonatal foreskins for the isolation of PHKs and gratefully acknowledge Ms. Dianne W. Moore and Mr. Sourav Banerjee for technical assistance.

Funding

This research was supported by a contract from the USPHS NIAID Collaborative Antiviral Testing Group (HHSN272201100016 I) (TRB); USPHS 2R01 CA83679 (LTC); USPHS 5P30 CA013148 to the UAB Comprehensive Cancer Center (pilot award to NSB); the Anderson Family Endowed Chair to LTC; and research contracts from Antiva Biosciences (UAB and KYH).

ABBREVIATIONS

PMEG	9-[2-phosphonomethoxy ethyl]guanine
ODE-Bn-PMEG	Octadecycloxyethyl benzyl 9-[2-phosphonomethoxyethyl]guanine
HPMPC	(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, also known as Cidofovir (CDV)
HPV	human papillomavirus
HR	high-risk
LR	low-risk
ori	papillomavirus replication origin sequences
PHK	primary human keratinocyte
qPCR	quantitative real time polymerase chain reaction
3D	three dimensional
HEK-293	human embryonic kidney-293 cell line
Gardasil	4 valent Gardasil vaccine, targeting HPV types 6, 11, 16, 18
Gardasil-9	9 valent Gardasil vaccine, targeting HPV types 6, 11, 16, 18, 31, 33, 45, 52, 58)
DAPI	4',6-diamidino-2-phenylindole
BrdU	bromodeoxyuridine
FISH	fluorescence in situ hybridization
IF	immunofluorescence

p53	tumor suppressor protein p53
PCNA	proliferating cell nuclear antigen
DMSO	dimethyl sulfoxide
TUNEL	<u>Terminal deoxynucleotidyl transferase dUTP Nick End</u> <u>Labeling of DNA breaks</u>
H&E	hematoxylin and eosin histological stain
hCMV	human cytomegalovirus

References

- Abdulkarim B, Sabri S, Deutsch E, Chagraoui H, Maggiorella L, Thierry J, Eschwege F, Vainchenker W, Chouaib S, Bourhis J. Antiviral agent Cidofovir restores p53 function and enhances the radiosensitivity in HPV-associated cancers. *Oncogene*. 2002; 21:2334–2346. [PubMed: 11948417]
- Andrei G, Snoeck R, Schols D, De Clercq E. Induction of apoptosis by cidofovir in human papillomavirus (HPV)-positive cells. *Oncol Res*. 2000; 12:397–408. [PubMed: 11697818]
- Andrei G, Topalis D, De Schutter T, Snoeck R. Insights into the mechanism of action of cidofovir and other acyclic nucleoside phosphonates against polyoma- and papillomaviruses and nonviral induced neoplasia. *Antiviral Res*. 2015; 114:21–46. [PubMed: 25446403]
- Banerjee NS, Wang HK, Broker TR, Chow LT. Human papillomavirus (HPV) E7 induces prolonged G2 following S phase reentry in differentiated human keratinocytes. *J Biol Chem*. 2011; 286:15473–15482. [PubMed: 21321122]
- Beadle JR, Valiaeva N, Yang G, Yu JH, Broker TR, Aldern KA, Harden EA, Keith KA, Prichard MN, Hartman T, Buckheit RW Jr, Chow LT, Hostetler KY. Synthesis and Antiviral Evaluation of Octadecyloxyethyl Benzyl 9-[(2-Phosphonomethoxy)ethyl]guanine (ODE-Bn-PMEG), a Potent Inhibitor of Transient HPV DNA Amplification. *J Med Chem*. 2016; 59:10470–10478. [PubMed: 27933957]
- Bienvenu B, Martinez F, Devergie A, Rybojad M, Rivet J, Bellenger P, Morel P, Gluckman E, Lebbe C. Topical use of cidofovir induced acute renal failure. *Transplantation*. 2002; 73:661–662. [PubMed: 11889450]
- Bravo IG, Felez-Sanchez M. Papillomaviruses: Viral evolution, cancer and evolutionary medicine. *Evol Med Public Health*. 2015; 2015:32–51. [PubMed: 25634317]
- Bruni L, Diaz M, Barrionuevo-Rosas L, Herrero R, Bray F, Bosch FX, de Sanjose S, Castellsague X. Global estimates of human papillomavirus vaccination coverage by region and income level: a pooled analysis. *Lancet Glob Health*. 2016; 4:e453–463. [PubMed: 27340003]
- Cheng S, Schmidt-Grimminger DC, Murant T, Broker TR, Chow LT. Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. *Genes Dev*. 1995; 9:2335–2349. [PubMed: 7557386]
- Chien WM, Parker JN, Schmidt-Grimminger DC, Broker TR, Chow LT. Casein kinase II phosphorylation of the human papillomavirus-18 E7 protein is critical for promoting S-phase entry. *Cell Growth Differ*. 2000; 11:425–435. [PubMed: 10965847]
- Chow, LT., Broker, TR. Human papillomavirus infections: warts or cancer?. In: Bell, SD.Méchali, M., DePamphilis, ML., editors. *DNA Replication and Human Disease*. Cold Spring Harb. Perspect. Biol. 5. Cold Spring Harbor Lab Press; Cold Spring Harbor, NY: 2013. p. 469-485.
- Chow LT, Broker TR, Steinberg BM. The natural history of human papillomavirus infections of the mucosal epithelia. *APMIS*. 2010; 118:422–449. [PubMed: 20553526]
- De Clercq E. Clinical potential of the acyclic nucleoside phosphonates cidofovir, adefovir, and tenofovir in treatment of DNA virus and retrovirus infections. *Clin Microbiol Rev*. 2003a; 16:569–596. [PubMed: 14557287]

- De Clercq E. Potential of acyclic nucleoside phosphonates in the treatment of DNA virus and retrovirus infections. *Expert Rev Anti Infect Ther.* 2003b; 1:21–43. [PubMed: 15482100]
- De Clercq E, Li G. Approved Antiviral Drugs over the Past 50 Years. *Clin Microbiol Rev.* 2016; 29:695–747. [PubMed: 27281742]
- Dollard SC, Wilson JL, Demeter LM, Bonnez W, Reichman RC, Broker TR, Chow LT. Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. *Off Genes Dev.* 1992; 6:1131–1142. [PubMed: 1321068]
- Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. Human papillomavirus molecular biology and disease association. *Rev Med Virol.* 2015; 25(Suppl 1):2–23. [PubMed: 25752814]
- Duraffour S, Snoeck R, Krecmerova M, van Den Oord J, De Vos R, Holy A, Crance JM, Garin D, De Clercq E, Andrei G. Activities of several classes of acyclic nucleoside phosphonates against camelpox virus replication in different cell culture models. *Antimicrob Agents Chemother.* 2007; 51:4410–4419. [PubMed: 17893157]
- Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 2015; 136:E359–386. [PubMed: 25220842]
- Galloway DA, Laimins LA. Human papillomaviruses: shared and distinct pathways for pathogenesis. *Curr Opin Virol.* 2015; 14:87–92. [PubMed: 26398222]
- Genovese NJ, Banerjee NS, Broker TR, Chow LT. Casein kinase II motif-dependent phosphorylation of human papillomavirus E7 protein promotes p130 degradation and S-phase induction in differentiated human keratinocytes. *J Virol.* 2008; 82:4862–4873. [PubMed: 18321970]
- Hitchcock MJM, Jaffe HS, Martin JC, Stagg RJ. Cidofovir, a New Agent with Potent Anti-Herpesvirus Activity. *Antiviral Chemistry and Chemotherapy.* 1996; 7:115–127.
- Hong S, Laimins LA. Regulation of the life cycle of HPVs by differentiation and the DNA damage response. *Future Microbiol.* 2013; 8:1547–1557. [PubMed: 24266355]
- Hostetler KY. Alkoxyalkyl prodrugs of acyclic nucleoside phosphonates enhance oral antiviral activity and reduce toxicity: current state of the art. *Antiviral Res.* 2009; 82:A84–98. [PubMed: 19425198]
- Jian Y, Schmidt-Grimminger DC, Chien WM, Wu X, Broker TR, Chow LT. Posttranscriptional induction of p21cip1 protein by human papillomavirus E7 inhibits unscheduled DNA synthesis reactivated in differentiated keratinocytes. *Oncogene.* 1998; 17:2027–2038. [PubMed: 9798674]
- Kho EY, Wang HK, Banerjee NS, Broker TR, Chow LT. HPV-18 E6 mutants reveal p53 modulation of viral DNA amplification in organotypic cultures. *Proc Natl Acad Sci U S A.* 2013; 110:7542–7549. [PubMed: 23572574]
- Kramata P, Downey KM. 9-(2-phosphonylmethoxyethyl) derivatives of purine nucleotide analogs: A comparison of their metabolism and interaction with cellular DNA synthesis. *Mol Pharmacol.* 1999; 56:1262–1270. [PubMed: 10570054]
- Kuo SR, Liu JS, Broker TR, Chow LT. Cell-free replication of the human papillomavirus DNA with homologous viral E1 and E2 proteins and human cell extracts. *J Biol Chem.* 1994; 269:24058–24065. [PubMed: 7523366]
- Lambert PF, Ozbun MA, Collins A, Holmgren S, Lee D, Nakahara T. Using an immortalized cell line to study the HPV life cycle in organotypic “raft” cultures. *Methods Mol Med.* 2005; 119:141–155. [PubMed: 16353335]
- Moody CA, Laimins LA. Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. *PLoS Pathog.* 2009; 5:e1000605. [PubMed: 19798429]
- Prigge ES, von Knebel Doeberitz M, Reuschenbach M. Clinical relevance and implications of HPV-induced neoplasia in different anatomical locations. *Mutat Res.* 2017; 772:51–66.
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998; 273:5858–5868. [PubMed: 9488723]
- Schiller JT, Muller M. Next generation prophylactic human papillomavirus vaccines. *Lancet Oncol.* 2015; 16:e217–225. [PubMed: 25943066]
- Sirianni N, Wang J, Ferris RL. Antiviral activity of Cidofovir on a naturally human papillomavirus-16 infected squamous cell carcinoma of the head and neck (SCCHN) cell line improves radiation sensitivity. *Oral Oncol.* 2005; 41:423–428. [PubMed: 15792615]

- Stier EA, Goldstone SE, Einstein MH, Jay N, Berry JM, Wilkin T, Lee JY, Darragh TM, Da Costa M, Panther L, Aboulafia D, Palefsky JM. Safety and efficacy of topical cidofovir to treat high-grade perianal and vulvar intraepithelial neoplasia in HIV-positive men and women. *AIDS*. 2013; 27:545–551. [PubMed: 23032420]
- Stoler MH, Wolinsky SM, Whitbeck A, Broker TR, Chow LT. Differentiation-linked human papillomavirus types 6 and 11 transcription in genital condylomata revealed by in situ hybridization with message-specific RNA probes. *Virology*. 1989; 172:331–340. [PubMed: 2549716]
- Van Pachterbeke C, Bucella D, Rozenberg S, Manigart Y, Gilles C, Larsimont D, Vanden Houte K, Reynders M, Snoeck R, Bossens M. Topical treatment of CIN 2+ by cidofovir: results of a phase II, double-blind, prospective, placebo-controlled study. *Gynecol Oncol*. 2009; 115:69–74. [PubMed: 19647859]
- Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*. 1999; 189:12–19. [PubMed: 10451482]
- Wang HK, Duffy AA, Broker TR, Chow LT. Robust production and passaging of infectious HPV in squamous epithelium of primary human keratinocytes. *Genes Dev*. 2009; 23:181–194. [PubMed: 19131434]
- Wilson JL, Dollard SC, Chow LT, Broker TR. Epithelial-specific gene expression during differentiation of stratified primary human keratinocyte cultures. *Cell Growth Differ*. 1992; 3:471–483. [PubMed: 1390334]
- Wilson R, Fehrmann F, Laimins LA. Role of the E1–E4 protein in the differentiation-dependent life cycle of human papillomavirus type 31. *J Virol*. 2005; 79:6732–6740. [PubMed: 15890911]
- Wolfgang GH, Shibata R, Wang J, Ray AS, Wu S, Doerrfler E, Reiser H, Lee WA, Birkus G, Christensen ND, Andrei G, Snoeck R. GS-9191 is a novel topical prodrug of the nucleotide analog 9-(2-phosphonylmethoxyethyl)guanine with antiproliferative activity and possible utility in the treatment of human papillomavirus lesions. *Antimicrob Agents Chemother*. 2009; 53:2777–2784. [PubMed: 19398642]

HIGHLIGHTS

- ODE-Bn-PMEG abolishes human papillomavirus productive infection in 3D cultures of primary human keratinocytes (PHKs).
- Incorporation of activated ODE-Bn-PMEG into replicating DNA causes DNA breaks and abolishes viral activities.
- ODE-Bn-PMEG induces apoptosis selectively in HPV-infected, differentiated keratinocytes while sparing uninfected tissues.
- Cidofovir, which is used to treat human cytomegalovirus retinitis, neither induces apoptosis nor abolishes HPV activity.
- With its efficacy, selectivity and mechanism of action, ODE-Bn-PMEG strongly merits further development and clinical trials.

Figure 1.

Effects of ODE-Bn-PMEG on day 13 HPV-18 and PHK raft cultures. Cultures were exposed to ODE-Bn-PMEG (0, 0.16, 0.5 and 1.5 μM) from day 6 to day 13, with media refreshed every other day. (A) Histology of PHK (1st row) and HPV-18 (3rd row) raft cultures was revealed by H&E staining. BrdU incorporation (red) detected with indirect IF revealed S-phase nuclei in the PHK (2nd row) and HPV-18 (4th row) raft cultures. (B) Numbers of nuclei per microscopic field averaged from four non-overlapping regions of PHK and HPV-18 raft cultures. (C) Quantitative estimates of percentages of BrdU-positive nuclei relative to total nuclei in four images from each condition. (D) Relative copy numbers of HPV-18 plasmids per cell; data were compiled from 3 independent sets of raft cultures using different batches of PHKs and separate transfections. (E) FISH to detect HPV-18 DNA amplification in the presence of increasing concentrations of ODE-Bn-PMEG from one of

the experiments. Images were captured at 20X magnification. Error bars indicate standard deviation. ‘**’ and ‘***’ indicate p values <0.05 and <0.005 .

Figure 2.

Inhibitory effects of ODE-Bn-PMEG and Cidofovir on cellular DNA replication in HPV-18 raft cultures. (A) Histology (upper row) and IHC for BrdU incorporation (reddish-brown in the lower row) in HPV-18 raft cultures unexposed or exposed to 15 μ M CDV from day 6 to day 14 or to ODE-Bn-PMEG at 1.5 μ M from day 6 to day 10 or 14. (B) Histology (upper row) and IHC for BrdU incorporation (lower row) in uninfected PHK raft cultures unexposed or exposed to 15 μ M CDV from day 6 to day 14 or to 1.5 μ M ODE-Bn-PMEG from day 6 to day 10 or 14. All cultures were harvested on day 14. Images were captured at 20X magnification.

Figure 3.

Short exposure to ODE-Bn-PMEG induced durable inhibition of HPV-18 DNA amplification. (A) Relative HPV-18 DNA copy numbers per cell were determined by quantitative real time PCR in raft cultures exposed to CDV (day 6 to day 14) or to ODE-Bn-PMEG (from day 6 to day 10, 12 or 14). Cultures were harvested on day 14 or day 18. HPV-18 DNA was not detected in raft cultures exposed to the inhibitor from day 6 to 12 or day 6 to day 14 harvested on day 18. (B) HPV-18 plasmid amplification was detected by FISH in HPV-18 raft cultures unexposed (left panel) or exposed to ODE-Bn-PMEG from day 6 to day 10 (middle panel) or from day 6 to day 14 (right panel). All cultures were harvested on day 14. HPV DNA failed to recover and amplify during the 4 days of drug-free chase (middle panel). (C) Quantitative estimates of HPV-18 DNA positive nuclei averaged from four images in each treatment. Error bars indicate standard deviation and ‘***’ indicate

p value <0.005. (D) IHC detection of the L1 capsid protein (reddish-brown) in control or treated HPV-18 raft cultures on day 18. Images were captured at 20X magnification.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4.

In situ assays for biomarkers of HPV infection. PCNA (red, left panels) and cyclin B1 (green, middle panels), merged along with DAPI nuclear stain (blue, right panels) were detected by indirect IF microscopy in raft cultures harvested on day 18. Images are shown for untreated PHK culture (top row), untreated HPV-18 culture (row 2), and HPV-18 cultures exposed to 15 μ M CDV from day 6 to day 14 (row 3), or to 1.5 μ M ODE-Bn-PMEG from day 6 to day 10 (row 4) and from day 6 to day 14 (row 5). Images were captured at 20X magnification.

Figure 5.

Induction of γ -H2AX in uninfected and HPV-18 infected raft cultures. Cultures were untreated (control) or exposed to CDV or to ODE-Bn-PMEG and harvested on day 14 or day 18. The inhibitors, their concentrations and exposure durations are indicated in the panels.

(A) γ -H2AX (red) was detected by indirect IF staining. Nuclei were stained with DAPI (blue). (B) Percentages of γ -H2AX-positive nuclei among all DAPI-positive nuclei in HPV-18 cultures were determined by enumeration of positive nuclei from 4 non-overlapping microscopic fields from day 14 and day 18 raft cultures. Images were captured at 20X magnification. Error bars indicate standard deviation and ‘***’ indicate p value <0.005.

Figure 6.

TUNEL assay to detect apoptosis. (A) Apoptotic nuclei (green) were revealed by TUNEL assay in uninfected and HPV-18 infected raft cultures. Cultures were unexposed (control) or exposed to 1.5 μ M ODE-Bn-PMEG from day 6 to day 10 or 14, or to 15 μ M CDV from day 6 to 14 and harvested on day 14 or day 18. Nuclei were stained with DAPI. (B) Percentages of TUNEL-positive nuclei relative to total nuclei were determined using counts from 4 different microscopic fields for each treatment of the HPV-18 raft cultures. (C) Average numbers of DAPI stained nuclei per microscopic field were counted in HPV-18 raft cultures. Images were captured at 20X magnification. Error bar indicate standard deviation. ‘***’ and ‘****’ indicate p value <0.05 and <0.005.

Figure 7.

Fluorescence microscopy to detect p53 (red), cyclin B1 (green) and DAPI in HPV-18 raft cultures. Day 18 control, untreated cultures (top row), cultures exposed to 15 μ M CDV from day 6 to day 14 (row 2), or to 1.5 μ M ODE-Bn-PMEG from day 6 to day 10 (row 3) and from day 6 to day 14 (row 4). Digitally enlarged views of two areas (L and R) from the CDV-treated raft culture (row 2) reveal stabilization of p53 in the nuclei of cells with cytoplasmic cyclin B1. Images were captured at 20X magnification.