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Novel antivirals inhibit early steps in HPV infection

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ABSTRACT

The future incidence of cervical cancer is forecast to decline because of the remarkably effective prophylactic vaccines against human papillomaviruses. However, lack of access to these expensive vaccines in the developing countries where cervical cancer is most frequent, and the restricted genotypes these vaccines protect against, will limit their impact. Clearly, there is still a need for identifying other modalities for preventing HPV infections. Ready access to effective, inexpensive antivirals represents one potentially valuable approach to the prevention of genital HPV infections. We developed a well-validated high throughput screening (HTS) assay for identifying compounds that inhibit HPV infection and applied this assay to identify lead compounds that act by inhibiting an early step in infection. We screened over 40,000 small molecules that were available at the University of Wisconsin Small Molecule Screening Facility (UW-SMSF). The top 22 compounds were chosen for further analyses based upon the pharmacological property, scaffold diversity, strength of the inhibitory activity and lack of nonspecific cytotoxicity. Of these compounds, #13 and #14 had the most acceptable properties of low to submicromolar IC_{50}'s and low cytotoxicity. Optimal antiviral activities were elicited by exposure of cells to the #13 and #14 during the initial 12 h following infection. Twenty-nine #13-like and 15 #14-like analogs were identified *in silico* and tested for their antiviral activities corresponded to the altered structures comparing to #13 and #14, informing on the pharmacophore structure of each compound. Studies indicate that both compounds inhibit infection post-entry.

1. Introduction

Human papillomaviruses (HPVs) are the most common sexually transmitted pathogens known today, infecting approximately 75% of sexually active individuals (Koutsky, 1997). In the US over 20 million people are actively infected at any one time with over 5 million new infections arising each year (Cates, 1999). Cervical cancer, a leading cause of death by cancer among women worldwide, with approximately a half million new cases and nearly a quarter of a million deaths each year (Arbyn et al., 2011), is caused by a subset of "high-risk" HPVs, including HPV16, 18, 31, 33 and 45 that are classified as carcinogens by the World Health Organization (Cogliano et al., 2005). Among the "high risk" HPVs, HPV16 is most notable, whose DNA can be detected in over half of cervical cancers. These high-risk HPVs are also causally associated with other anogenital cancers of the vulva, vagina, penis, anus and perineal region, and approximately 20% of head and neck cancers, particularly of the tongue, tonsil and oropharynx (Zur Hausen, 2009).

Prevention of HPV infections remains the primary means by which we can reduce the incidence of HPV-associated cancers. Vaccines against human papillomaviruses, Cervarix and Gardasil, have made this goal achievable; they possess remarkable efficiency at inhibiting infection in vaccinated populations by those HPV genotypes they are designed to protect against (Frazer et al., 2011). These vaccines reduced by at least 90% the infection rate and early neoplasia by those genotypes targeted by the vaccines (HPVs 16 and 18 in the case of Cervarix, HPVs 6, 11, 16 and 18 in the case of Gardasil) (Garland et al., 2007; Paavonen et al., 2007; Villa et al., 2005). However, the long-term effectiveness of these prophylactic vaccines in those vaccinated remains unclear. The current vaccines, even if 100% effective will only prevent cervical cancers arising from infection by two of the high-risk HPV genotypes, HPV16 and HPV18. As cancers caused by other high-risk HPVs account for approximately 25–30% of all cervical cancers, second-generation vaccines with broader range effectiveness for more

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high-risk HPV genotypes or alternative means to prevent infection by these high-risk HPVs are needed. Another critical issue is accessibility of the vaccines. These vaccines are unlikely to be widely disseminated in most developing countries where cervical cancer is more frequent because of the high cost of whole scale vaccinations of young women in these populations. Clearly, there is need for identifying other less expensive modalities for preventing HPV infections.

Ready access to effective antivirals represents one potentially valuable approach to the prevention of sexually transmitted diseases (STDs) including genital HPV infections (Howett and Kuhl, 2005). To make an effective topical HPV antiviral commercially available, it must fulfill several criteria. It should efficiently target an early step in HPV infection, possess minimal side-effects/cytotoxicity, it should be easy to formulate and chemically stable in the formulation, and inexpensive to manufacture.

Recent advances now make it possible to pursue HPV antiviral development. Specifically, methodologies to efficiently produce HPV pseudoviruses carrying reporter genes (Buck et al., 2004) or infectious papillomavirus (Pyeon et al., 2005) at very high yields have been developed. These advances overcame the major limitation in the identification of HPV-antivirals, by providing access to useful amounts of virus particles for high throughput assays to identify small molecules that inhibit early steps in infection.

In this study, we identified small molecules available in the University of Wisconsin Small Molecule Screening Facility (UW-SMSF) that fulfill the above stated criteria for an effective HPV-antiviral using a well-validated cell-based high throughput screening (HTS) assay using HPV16 pseudoviruses expressing a reporter gene. Candidate lead compounds were screened in multiple secondary and tertiary screens using HPV11, 16 and 31 pseudoviruses containing alternative reporter genes as well as infection with bona fide HPV16 particles. Lead compounds were chosen for further analyses based upon the pharmacological property, scaffold diversity, strength of the inhibitory activity and low cytotoxicity. Analogs of the best two lead compounds were screened to inform on the pharmacophore for each compound. Initial studies indicate these compounds act at early steps of infection, in the first 12 h post-exposure of cells to virus like particles, but do not prevent internalization of the bulk of virus particles based upon in situ microscopy studies. Potential mechanisms of action of these compounds are discussed.

2. Materials and methods

2.1. Plasmids

pEF399, containing the complete W12E HPV16 genome (Flores et al., 1999), and all other plasmids used for virus packaging were previously described (Pyeon et al., 2005). pSEAP (pSEAP-control) for expression of secreted alkaline phosphatase (SEAP) was purchased from Clontech. pHPV16wpA-RL was cloned with HPV16 long control region (LCR, nucleotide position 7155-861), into pRL-null (Promega) to prepare a Renilla luciferase reporter system driven by native HPV16 promoter. pXuLL encodes HPV16 L1 and L2 was provided by Dr. John Schiller. p16SheLL, a 10.8 kilo base pair (kbp) plasmid (too large to be packaged in HPV VLPs) that expresses the HPV16 major capsid protein, L1, and minor capsid protein, L2. pLucf, encodes both firefly luciferase (Luc) and green fluorescent protein (GFP). Both p16SheLL and pLucf were gifts from Dr. Chris Buck at the National Cancer Institute.

2.2. Cell lines

Human embryonic kidney cell line 293T from ATCC was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). 293FT cell line expressing enhanced SV40 large T antigen purchased from Invitrogen was maintained in DMEM containing 10% FBS supplemented with 0.1 mM MEM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen) and 500 μg/ml Geneticin (Invitrogen). Immortalized human keratinocyte cell line, HaCaT, was maintained in F-media (Invitrogen, 3 parts F-12 and 1 part DMEM), supplemented with 10% FBS.

2.3. Production of virus particles

HPV virions and pseudovirions (HPV virus-like particles carrying encapsidated reporter plasmids) were prepared as described previously (Buck et al., 2004; Buck and Thompson, 2007; Pyeon et al., 2005). Briefly, HPV16 capsid proteins expression plasmid, pEF399 or pXuLL, as well as reporter plasmid pSEAP or pHPV16wpA-RL were cotransfected in 293FT cells to produce wild type HPV16 virions, hpv16SEAP and hpv16wpA-RL, respectively. Pseudovirions were harvested by detergent lysis of the cells 48 h after transfection. Particles were allowed to mature overnight (Buck et al., 2004, 2005; Buck and Thompson, 2007), and were purified by ultracentrifugation through Optiprep gradients. Viral genome equivalent were determined as previously reported (Pyeon et al., 2009). Alternatively, HPV16:LucF pseudoviruses are generated with p16SheLL and pLucf using methods described above. The titers of HPV16:LucF pseudoviruses were determined by flow cytometric analysis of 293FT cells treated with various dilutions of purified pseudovirion stock. HPV11:LucF and HPV31:LucF pseudoviruses were gifts from Dr. Michelle Ozbun in University of New Mexico.

2.4. Small molecule compounds and libraries

Known Bioactive Library (KBA01) consists of three commercially available collections totaling 4160 compounds. KBA01 consists of 880 high purity compounds of known safety and bioavailability in humans of which over 85% are marketed s from Prestwick Chemical. The Prestwick compounds cover several therapeutic areas including neuropsychiatry, cardiology, immunology, inflammation, analgesia, etc. Also included in the KBA01 library are 2000 diverse FDA-approved drugs and natural products from Spectrum Chemical Collection From Microscource Discovery Systems, Inc. and 1280 compounds from the LOPAC of Sigma representing marketed drugs, failed development candidates and "gold standards" that have well-characterized activities. These compounds are the results of lead optimization efforts and have been rationally designed by structure–activity relationship studies. The NCI03 library consists of 235 natural products that were selected from the NCI open repository on the basis of structural diversity and availability of compounds. The 16,000 compounds from the Chembridge DIVERSet are universally diverse, pre-designed drug-like small molecules. The compounds have been rationally selected based on 3D pharmacophore analysis to cover the broadest part of biologically relevant pharmacophore diversity space. The Maybridge HitFinder collection of screening compounds consists of 14,400 premier compounds representing the drug-like diversity of the Maybridge Screening Collection. All screening compounds fit Lipinski's guidelines for "drug-likeness".

2.5. High throughput screening

293TT cells in 384-well plates were treated with 3.3 μM of library compounds for 4 h prior to being inoculated with hpv16SEAP at 50 vge (viral genome equivalent)/cell for 48 h. Culture supernatant 2.5 μl was used for virus infectivity assay using a Phospha-Light a chemiluminescent alkaline phosphatase assay (Applied

Biosystems) and cell lysate was used for cell viability assay using CellTiter-Glo LuMinescent cell viability assay (Promega). Biomek FX (Beckman Coulter) was used for automated liquid handling and a Victor 3-V plate reader (Perkin Elmer) was used for measuring luminescence. Screening was performed in the Small Molecule Screening Facility at the University of Wisconsin Comprehensive Cancer Center.

2.6. Infectivity assay

The activity of pSEAP-control-encapsidated pseudovirions (hpv16SEAP) was measured using a Phospha-Light chemiluminescent alkaline phosphatase assay (Applied Biosystems) and the activity of pHPV16wpA-RL-encapsidating pseudovirions (hpv16wpA-RL) was assayed using Renilla Luciferase Assay System (Promega). Infectivity of wild type HPV16 virions was examined by quantitative reverse transcriptase PCR (qRT-PCR) by amplifying viral mRNA signals from HPV16-treated 293TT cells. 293TT cells were inoculated with 50–100 vge/cell of packaged virus and incubated for 48 h at 37 °C. Total RNA was isolated using the RNeasy total RNA purification kit (Qiagen), treated with RQ DNaseI (Promega) to remove possible DNA contaminants, purified again on RNeasy columns to remove DNaseI, and quantified by spectrophotometer. cDNA was synthesized from 20 μg of total RNA with oligo (dT) using a SuperScript cDNA synthesis kit (Invitrogen), and qPCR was performed with QuantiTect SYBR Green PCR Kit (Qiagen). Oligonucleotide primers (Pyeon et al., 2009) were designed using the Primer3 primer design program, (Rozen and Skaletsky, 2000) synthesized by MWG and used at 0.5 μM for PCR amplification for 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and 30 s polymerization at 72 °C. Obtained values were normalized with the levels of β-actin.

2.7. Dose-dependent luciferase assays and cell viability assays

1×10^4 HaCaT cells were pre-split in 96-well plates overnight and treated with serial dilutions of compounds (Fisher Scientific Cat. #165306) (from 0 to 10 μM) for 4 h. 1×10^5 GFP transducing units of HPV16:LucF pseudovirus (10 M.O.I.) were then added in the cells. At 48 h post-pseudovirus infection, cells were lysed, and the infectivity was measured by quantitative luciferase assays (Promega, Luciferase Assay System Cat. #1501 and #1531). Parallel cell viability assays (Promega, Luminescent Cell Viability Assay Cat. #G7571) were performed to monitor cytotoxicity of the compounds. Alternatively, experiments above were repeated by using HPV11:LucF and HPV31:LucF instead of HPV16:LucF in HaCaT cells.

2.8. Transient transfection

1×10^4 HaCaT cells were pre-split in 96-well plates overnight and transfected with 100 ng pLucf for 2 h. Cells were then treated with serial dilutions of PFL#13 or #14 (from 0 to 30 μM). At 48 h post-treatment, cells were lysed, and the luminescence was quantified.

2.9. Time of compound addition

1×10^4 HaCaT cells were pre-split in 96-well plate overnight, then treated or mock-treated with 1×10^5 HPV16:LucF (10 M.O.I.), and added 3 μM compound at different time points or a period of time in reference to the time of exposure to pseudovirus. Time of HaCaT cells receiving HPV16 pseudovirus was set as 0 h. At 48 h post-infection, the infectivity was determined by performing luciferase assays.

2.10. Confocal microscopy

HaCaT cells grown on glass coverslips were treated with 10 μM PFL #13, 10 μM PFL #14, or DMSO (vehicle) for 4 h, and infected with AlexaFluor594 capsid-labeled wild-type HPV16 (Smith et al., 2007) for 6 h in the presence of these compounds. Cells were washed, fixed with 4% paraformaldehyde and mounted on ProLong Gold antifade reagent with DAPI (Invitrogen). Imaging analysis was done using the Olympus FluoView 1000 confocal microscope at the Advanced Light Microscopy Core Facility (University of Colorado School of Medicine).

3. Results

3.1. High throughput screen for compounds inhibiting HPV infection

We developed a cell-based high throughput screen (HTS) for identifying potential HPV antivirals (Fig. S1). This assay makes use of the efficient production technique for generating virus like particles established by Dr. Chris Buck and colleagues in Dr. John Schiller's laboratory in which one encapsidates plasmids that contain reporter genes in self-assembling particles composed of the major (L1) and minor (L2) capsid proteins (Buck et al., 2004, 2005; Buck and Thompson, 2007). Upon exposure of cells to these 'pseudoviruses', their entry and delivery of encapsidated DNA to the nucleus results in reporter gene expression that can be assayed quantitatively, providing a measure of the efficiency of early steps in HPV infection. We used this technology to generate HPV16 pseudovirions containing the plasmid, pSEAP-control (Clontech), which expresses a secreted form of alkaline phosphatase (SEAP). Using this secreted reporter allowed us also to use cell lysates for cell viability assay; thus the assay design permitted us to measure virus infectivity and cell cytotoxicity in the same well. This assay was initially tested using two human cell lines, 293T, which are commonly used in HTS screens, and HaCaT, an immortalized human keratinocyte cell line that is a more relevant cell type for HPV infection. Signal to noise levels were much higher in 293T cells and therefore these cells were chosen for further use in the initial HTS, while the more relevant HaCaT cells were used in subsequent screens using alternative reporters that provided acceptable signal to noise readouts in this cell line. The fact that HPV pseudoviruses infect many different mammalian cell types (Müller et al., 1995) provided us confidence that the use of 293T cells in the HTS would not be problematic.

To determine the robustness of this HTS, we screened a subset of compounds sourced from the Prestwick Chemical Library using a 384 wells screening format. A total of 960 molecules were screened along with controls (e.g. neutralizing antibody). Acceptable Z scores (range: 0.52–0.68) were obtained for all individual plates, demonstrating the robustness of the assay. We then performed a HTS of over 40,000 small molecules available at the Keck-UWCCC Small Molecule Screening Facility in the University of Wisconsin-Madison including 16,000 compounds from Chembridge DIVERSet collection, 14,400 compounds from the Maybridge HitFinder library, 5000 known bioactive compounds from NIH and from the Prestwick Chemical Library, and ~5000 compounds from smaller libraries available at the UW/Keck small molecule screening facility. The bioactive compound library screen identified a subset of cell cycle inhibitors that inhibited HPV16 infection. Follow-up studies on these lead bioactive compounds demonstrated that cell cycle progression is essential for early steps in HPV infection (Pyeon et al., 2009). From the HTS of all other libraries, an additional 104 compounds of unknown function were identified based upon their ability to selectively inhibit HPV infection at least 1.7-fold (range 2–60% infectivity/viability) at the 3 μM

HTS standard concentration. These were the focus of the current study.

3.2. Secondary/tertiary screens of the top 104 microbicidal compounds

The top 104 compounds from the HTS were retested in the primary screen assay and in a secondary screen assay in which we monitored infectivity in 293T cells using a HPV16 pseudovirus that has a different reporter gene, Renilla luciferase driven by the HPV16 promoter (data not shown). 22 compounds (PFL #1 ∼ #22) were selected for further analyses based upon their reproducibly effectiveness at inhibiting HPV16 pseudovirus infectivity. All but one of these compounds (PFL #6 is longer commercially available) were repurchased and subjected to a tertiary screen performed in the human keratinocyte cell line, HaCaT, and using HPV16 pseudoviruses (HPV16:LucF). This reporter plasmid, pLucf, provided by Dr. Chris Buck encodes a third reporter gene, firefly luciferase, transcribed from the elongation factor 1 alpha (EF1a) promoter. In this assay, dose response curves were performed at seven concentrations of each test compound from 0.1 to 100 μM, and both the efficiency of inhibiting infectivity (luciferase assay) as well as cell viability were assayed. Each dose response assay was repeated at least twice and, within each repeat, six replicates of each assay were performed. Results for a representative experiment are shown in Table 1 (see also Fig. S2). Two lead compounds, PFL #13 and #14, stood out both in terms of displaying reproducible, submicromolar IC_{50} (for PFL #13 < 0.1 μM; for PFL #14 < 1 μM) and little to no cytotoxicity (CD_{50} for PFL #13 ∼ 30 μM, CD_{50} for PFL #14 > 100 μM). Several others, (PFL #9, #12, #20, #21 and #22) also showed IC_{50} in the low to mid μM range, with acceptable cytotoxicity (i.e. CD_{50} greater than 10× their IC_{50}).

The 21 selected, commercially available compounds were also tested for their ability to inhibit infection by bona fide HPV16 virions, containing the full HPV16 genome (Pyeon et al., 2005), by measuring early viral gene expression by real time PCR (Pyeon et al., 2009) at 48 h post-infection (Fig. 1) after treating cells with 5–10 μM of each compound. Consistent with the reporter gene assays, HPV early gene expression was inhibited by PFL #4, #13, #14, #18 and #20. Other compounds, PFL #8 and #16, actually enhanced infection by the bona fide virus (Fig. 1), demonstrating the importance of not relying solely on HPV pseudovirus-based assays in identifying lead compounds. Given the large differences in IC_{50} and CD_{50}, and the reproducible capacity to inhibit infection by HPV16 pseudovirus and bona fide HPV16 virus in different cell types, PFL #13 and #14 were selected as primary leads for further analysis.

Table 1
IC_{50} and CD_{50} for lead compounds.[a]

PFL #	Chembridge No.	Viability CD_{50} (μM)	Infectivity IC_{50} (μM)
001	6164172	∼10	<0.1
002	5826431	∼30	<0.1
003	5379458	>100	<0.1
004	SPB01290	∼30	<30
005	5345246	>100	>100
007	6073118	∼0.4	>100
008	6114935	<30	<30
009	6138893	<30	<10
010	6060474	<30	<10
011	6190827	<30	<30
012	6013991	∼30	∼4
013	6228481	∼30	<0.1
014	5367380	>100	<1
015	5650630	∼10	∼100
016	6110323	<10	<10
017	5352239	<100	>100
018	6172721	∼100	<10
019	6074705	∼1	∼1
020	SPB04406	∼10	∼0.1
021	HTS07022	>100	<10
022	60794598	∼100	<10

[a] See supplemental data Fig. S2 for dose response curves for each compound.

3.3. Monitor effectiveness of top two lead microbicidal compounds on inhibiting infection by multiple anogenital HPV genotypes

The goal of this study was to identify compounds that inhibit all sexually transmitted HPVs. For this reason, we monitored the ability of PFL #13 and #14 to inhibit infection of two additional human papillomavirus pseudoviruses that were generated using the capsid proteins of two other sexually transmitted HPVs, high-risk HPV31 and low-risk HPV11. These pseudoviruses have encapsidated in them the same reporter plasmid used in our HPV16 pseudovirus-based screen used in Table 1 and Fig. S2. HPV11 and 31 pseudovirus were both efficiently inhibited by PFL #13 and #14 at concentrations commensurate with that seen for HPV16 pseudovirus (Fig. 2). PFL #13 gave IC_{50}s of <0.1 μM and ∼0.1 μM for HPV11 and HPV31 pseudoviruses, respectively. PFL #14 gave IC_{50}s of <0.1 μM for both HPV11 and HPV31 pseudoviruses.

To address whether the compounds could inhibit reporter gene expression out of the context of an infection event, we monitored their effect on luciferase expression when the reporter gene was transfected into the same HaCaT cells (Fig. S3). PFL #14 showed no inhibition of luciferase activity in this transfection experiment. PFL #13 showed a modest inhibition of luciferase at increasing concentrations. At the highest concentration tested, 30 μM, PFL #13 inhibited luciferase expression 4-fold in the context of the transfection experiment (Fig. S3A). This correlated with the modest cytotoxicity displayed by PFL #13 at >10 μM Fig. 2). Importantly, at this same concentration of 30 μM, PFL #13 inhibited infection by all three HPV pseudoviruses by over 100-fold (Fig. 2).

3.4. Defining pharmacophores for the top two HPV antivirals

The structures of compounds 13 and 14 are shown in Fig. 3A and B, respectively. Twenty-nine PFL #13-like and 15 PFL #14-like compounds were identified *in silico* based upon a screen for commercially available small molecules with similar chemical structures and tested for their microbicidal activities compared to PFL #13 and #14 (Figs. S4 and S5). These data informed on the pharmacophore for compounds 13 and 14, displayed in Fig. 3C and D, and described in detail below.

None of the 13-like compounds had IC_{50} below 10 μM besides 13-9 and 13-18, which had IC_{50}s of ∼3 μM, still over an order of magnitude worse than the IC_{50} for the lead compound 13 (see Fig. S4). Structurally, 13-18 differs from cpd 13 only in the absence of side chains at the R1 and R2 positions (Fig. 3C). 13-9 also differs in the side chains at the R1 and R2 positions, and the Cl atom on the pyridine ring is at position 4 instead of position 5. Indeed the length of the side chain at the R2 position alone appears to be critical as cpd 13-20, which only differs in the length of this side chain, has an IC_{50} > 10 μM. Replacement of the Cl atom on the pyridine ring with a methyl group also eliminates activity (cpd 13-3). All other 13-like compounds either had IC_{50}s ⩾ 30 uMor were cytotoxic. Many of these were sufficiently different from the lead compound to make it difficult to gain additional pharmacophore information.

Of the cpd 14-like compounds, one, cpd 14-7, was as potent as the lead compound 14, with an IC_{50} below 0.1 μM (Fig. S5). Remarkably it differs structurally from cpd 14 in many regards. Firstly, the fluorophenyl group at position X is replaced with aryl ether (Fig. 3D). Secondly, the benzene rings at the R1 and R2 positions are di-methylated. Thirdly, the amide groups linking the benzene rings to the phenyl rings Ph1 and Ph2 are reversed in their

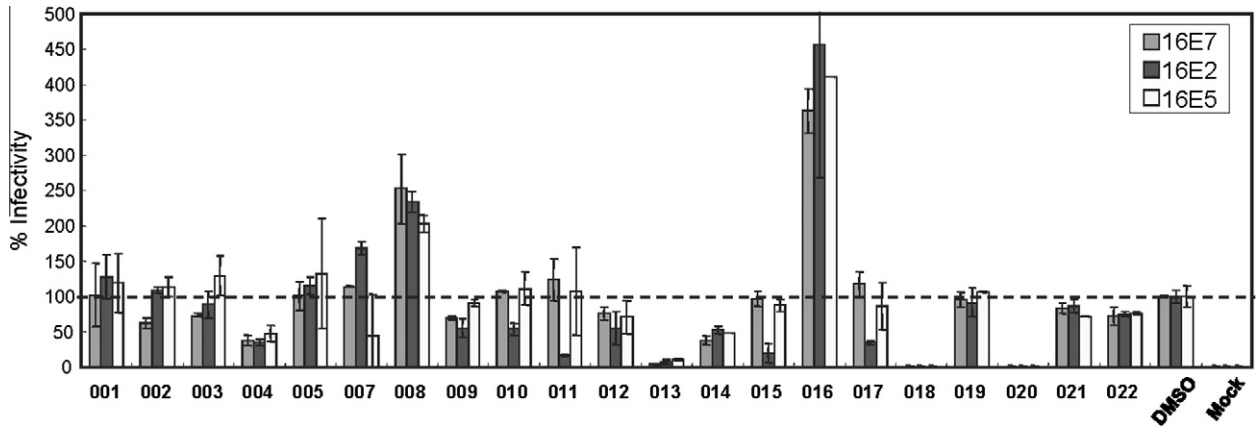

Fig. 1. Inhibition of infection by HPV16 virus. Shown is the relative efficiency of infection by HPV16 virus in 293T cells as scored by quantifying early viral transcription using real time PCR for E2 (black bars), E5 (white bars) and E7 (gray bars) containing mRNAs at 48 h post-infection in the absence (DMSO – vehicle only, values set at 100%) or the presence of indicated compound (10 μM concentration). Data points shown are the average of triplicate sample values normalized by β-actin mRNA expression. Dotted line indicates the normalized value for no compound (set as 100% infection value). Note PFL #4, #13, #14, #18 and #20 all indicated reproducible and significant inhibition of HPV16 early gene expression.

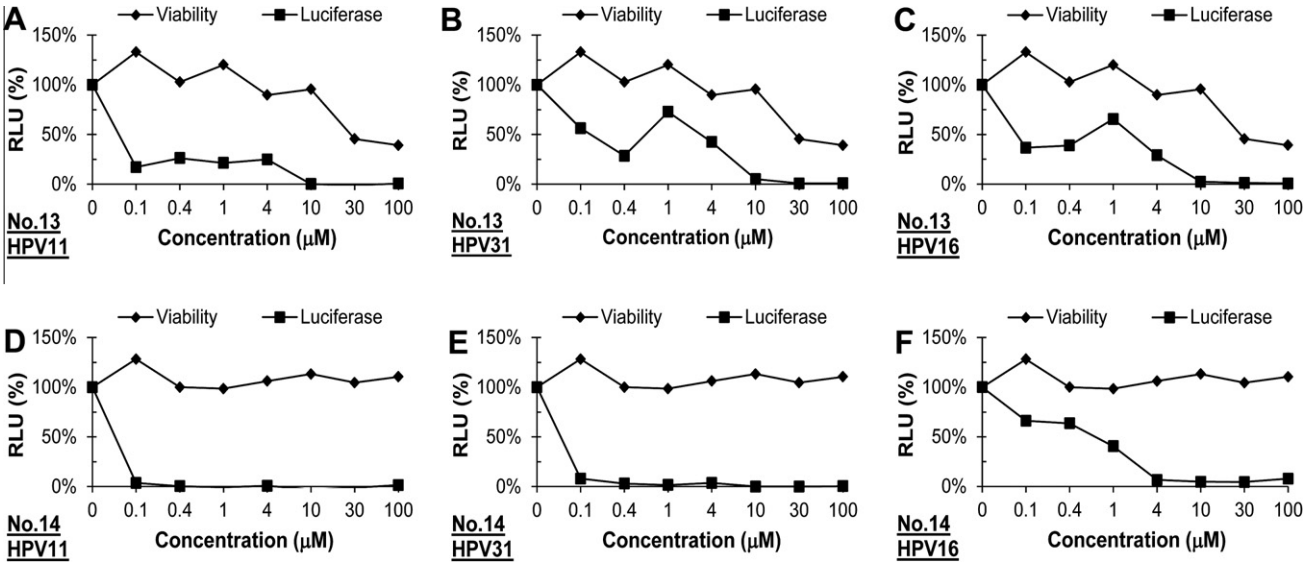

Fig. 2. Compounds PFL #13 and #14 can inhibit infection by multiple anogenital HPVs. Shown are results from infection experiments in HaCaT cells monitoring the efficiency of infection by HPV16, HPV11 and HPV31 pseudovirus carrying the firefly luciferase gene in the absence (0 μM, vehicle only) or increasing concentrations (0.1–100 μM) of PFL #13 or #14. The *Y* axis is the relative efficiency of infection (square symbols) or cell viability (diamond symbols) as a function of compound concentration (*X* axis). All data points represent the average of values from triplicate samples. Note that both PFL #13 and #14 displayed submicromolar IC_{50}s for all three HPV pseudovirus infections.

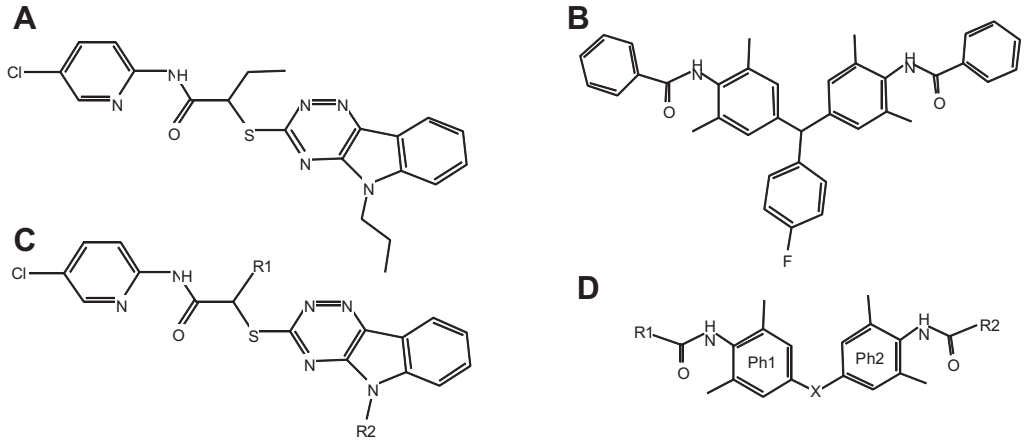

Fig. 3. Pharmacophores deduced for compounds PFL #13 and #14. Shown are the chemical structures of PFL #13 (A), #14 (B), and the pharmacophores for PFL #13 (C), and PFL #14 (D). See text for further description.

orientation. Fourthly, the methyl groups on the phenyl rings Ph1 and Ph2 are lost. What does remain constant between cpd 14-7 and the lead compound 14 is their bis structures. On the other hand, Cpd 14-5, which is nearly identical to the lead compound 14 including its bis structure but has methyl groups in place of the benzene rings at positions R1 and R2, has an IC_{50} of 30 μM, over two orders of magnitude worse than the lead compound 14. Another cpd, 14-11, that is highly similar to the lead compound 14 and retains the benzene rings at R1 and R2 but is missing the fluorine atom in the central fluorophenyl group at position X likewise has an IC_{50} of ~30 μM. All other 14-like compounds either were cytotoxic or had IC_{50}s ⩾ 30 μM. Of these most had structures sufficiently different from the lead compound to make it difficult to gain additional pharmacophore information.

3.5. Investigating mechanism of action

To begin to learn how PFL #13 and #14 act to inhibit HPV infection we performed time of addition experiments wherein compound was added to cells for different periods of time prior to, at, or after exposure of the cells to HPV16 pseudoviruses. HaCaT cells were exposed to 3 μM of each compound for the indicated period of time in reference to the time of exposure to pseudovirus, and 48 h post-infection cell lysates were harvested and luciferase activity measured to determine the infectivity (Fig. 4). Both compounds were optimal in their effectiveness when delivered at the time of, or before, cells were initially exposed to HPV pseudovirus. Initiating delivery of either compound at 8–12 h after exposure to HPV limited its effectiveness, which was completely lost if compound was delivered at 20 or later hours after exposure to HPV. Maintaining compound presence during just the first 12 h after exposure to the virus was sufficient to give protection to the majority of cells. These results suggest that the compounds both work at an early step in HPV infection, however they do not define what step is inhibited.

HPV is known to enter cells slowly as evidenced by the fact that neutralizing antibodies can inhibit infection when added up to 4 h after exposing cells to the virus (Day et al., 2003). Thus the results of the time of compound addition studies (Fig. 4) do not allow us to deduce whether the compounds work prior to entry, at the time of

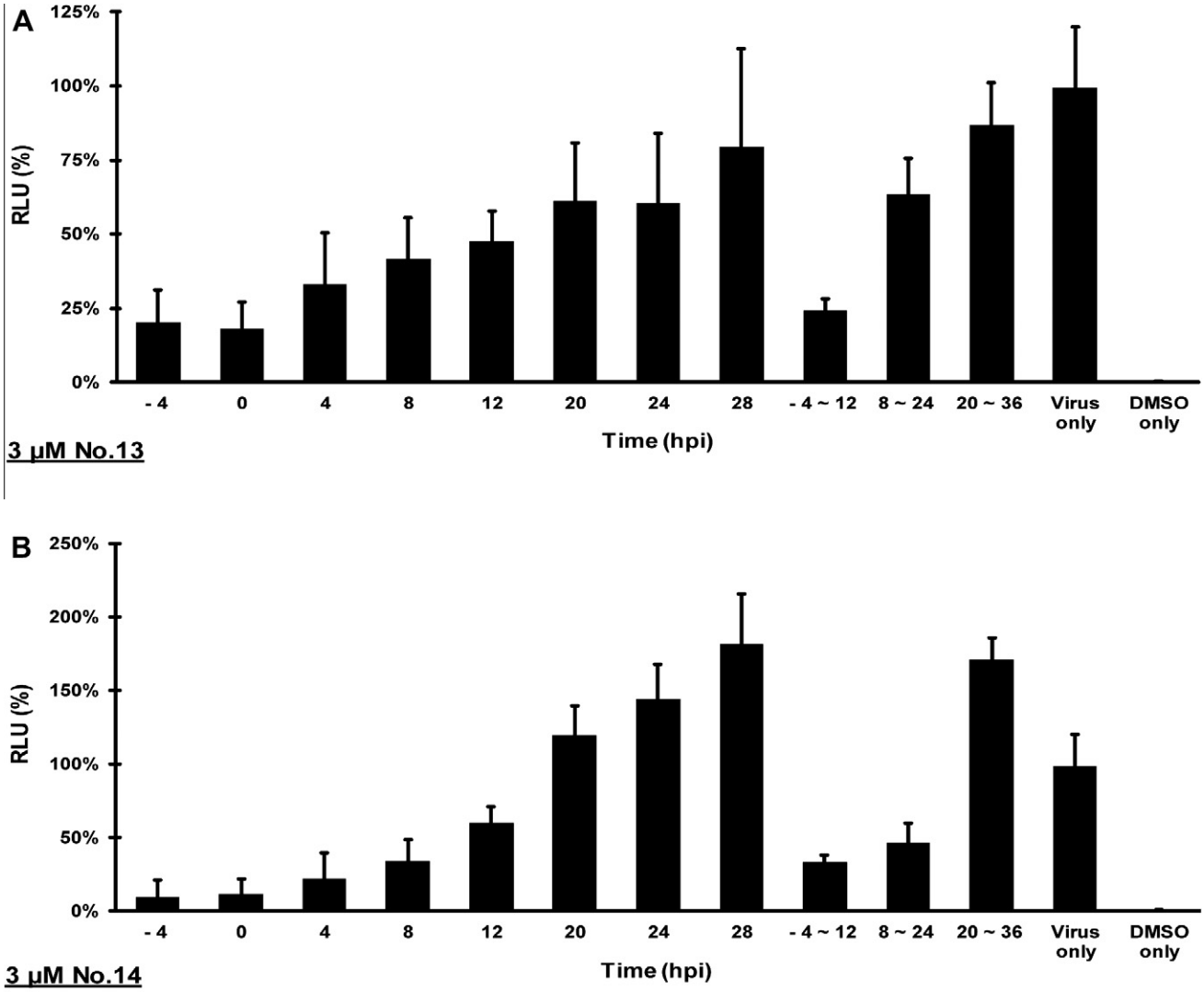

Fig. 4. Time of compounds addition. 4 h prior HPV16 pseudovirus exposure, HaCaT cells were treated with 3 μM of (A) PFL #13 or (B) PFL #14. The time point at which HaCaT cells were exposed to HPV16 pseudovirus was set as the 0 h. Labels on *X* axis indicate time in hours at which compound was added to the media pre-(negative number) or post-(positive numbers) exposure of the cells to the pseudovirus. Where two numbers are separated by "~", the cells were exposed to the compound for the indicated span of time in hours. Forty-eight hours post-infection, cells were lysed and luciferase assays were performed to measure the efficiency of infection. Relative luminescent unit (RLU) of cells with mock treatment (virus only) was set as 100%. DMSO treatment only was used as negative control. $N = 12$.

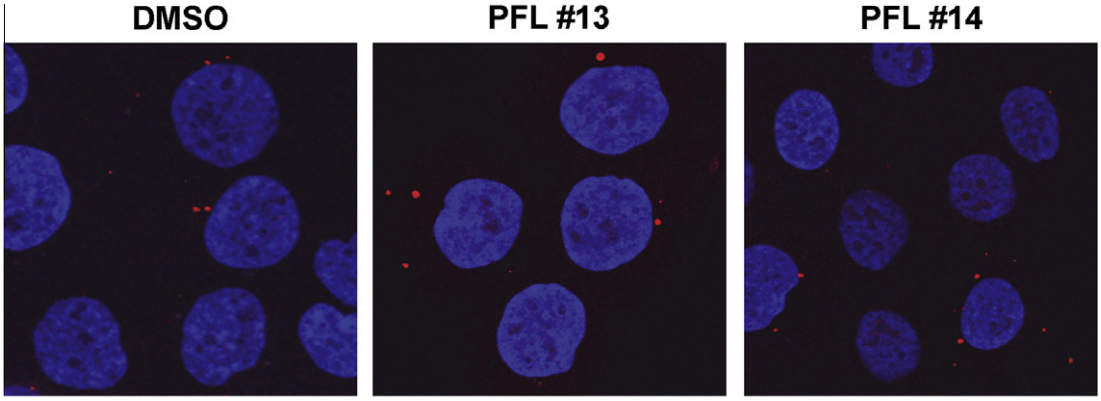

Fig. 5. Effect of PFL #13 and PFL #14 on HPV16 entry in HaCaT cells. HaCaT cells were treated with 10 μM PFL #13, 10 μM PFL #14, or DMSO (vehicle) for 4 h, and infected with AlexaFluor594 capsid-labeled wild-type HPV16 (red) for 6 h in the presence of these compounds. Cells were washed and the nucleus was stained with DAPI (blue). Confocal microscopy was performed using the Olympus FluoView 1000 microscope. Note the presence of virus particles (red signal) primarily within the cytoplasm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

entry, or post-entry. To distinguish between these three possibilities, we performed confocal microscopy analysis with cells treated or not treated with PFL #13 and #14 that were infected with wild-type HPV16 virions in which the capsid was labeled with a fluorescent dye. As shown in Fig. 5, wild-type HPV16 virions were able to enter cells treated with either compound indicating that they function post-entry.

4. Discussion

Antivirals can provide a cost effective approach to inhibit virally induced STDs. In this study we report on the use of a robust HTS to identify small molecules that can efficiently inhibit HPV infection. Two lead compounds with low to submicromolar IC_{50}'s and little to no cytotoxicity were characterized in detail, providing evidence that they can inhibit multiple genital HPV genotypes. Initial information on their pharmacophores and mechanism of action was provided. We consider these two compounds to be potentially valuable new antivirals for inhibiting HPV infection.

The value of inexpensive and highly efficient antivirals cannot be underestimated for virally induced STDs for which vaccines are not available or too costly for general use worldwide. Recent trials of tenofovir gel showed promising efficacy and safety of anti-retroviral compounds for the prevention of HIV infection in women (Abdool Karim et al., 2010). Sexually transmitted HPVs are the most common STD and the recognized cause of not only anogenital cancers but also a subset of head and neck cancers. In recent years several other HPV antivirals have been described including carrageenan (Buck et al., 2006; Roberts et al., 2007), and gamma secretase inhibitors (Huang et al., 2010; Karanam et al., 2010). These examples provide evidence that HPV inhibitors of critical steps in early HPV infection will be an effective means of preventing HPV infection that cannot be stopped by current vaccines either because of their limited access or limited breadth of HPV genotypes for which they provide protection. Thus, novel small molecule compounds could be used as more accessible, broad spectrum HPV inhibitors designed for application in the vaginal or rectal area by gel, cream, or implant, and in the oral cavity by spray or foam.

In the same HTS screens, we previously identified cell cycle inhibitors that prevent cells from progressing through mitosis and inhibit HPV infection (Pyeon et al., 2009). In the context of studying these cell cycle inhibitors, we observed that virus particles accumulated within treated cells in the cytoplasm (DP, PA and PFL: unpublished data), as seen for PFL #13 and #14 (Fig. 5). This prompted us to investigate whether PFL #13 and #14 function by inhibiting the cell cycle. They were not found to inhibit the cell cycle (data not shown). Thus, compounds PFL #13 and #14, likely act by some other mechanism(s) post-entry to inhibit HPV infection. It is also possible that PFL #13 and/or 14 alter the mechanism by which HPVs enter cells causing them to enter through a process that leads to unproductive infections. Further studies are necessary to define the critical step in infection disrupted by these HPV antivirals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.12.007.

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