Title
Novel Inhibitors of the Human Papillomavirus

Author
Walhart, Tara

Publication Date
2016

Peer reviewed|Thesis/dissertation

Novel Inhibitors of the Human Papillomavirus

by

Tara Walhart

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nursing

in the

GRADUATE DIVISON

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

I would like to thank and acknowledge all the members of the Palefsky lab and Small Molecule Discovery Center at the University of California, San Francisco with whose support and collaboration make this dissertation possible.

I would also like to thank the members of my dissertation committee for the constant support and guidance throughout this process.

My dissertation includes previously published material: Walhart, T. (2015). "Human Papillomavirus Biology, Pathogenesis, and Potential for Drug Discovery: A Literature Review for HIV Nurse Clinical Scientists." The Journal of the Association of Nurses in AIDS Care: JANAC, doi: 10.1016/j.jana.2015.07.001. ·

I could not have completed my doctoral education without extensive financial support form the following UCSF sources: Chancellor's Graduate Research Fellowship; Graduate Dean's Health Science Fellowship; and the HIV/AIDS Nursing Care and Prevention training program (T32 NR07081).

In addition, I would like to thank the many other people who supported me in various ways throughout this process: Carol Dawson-Rose, Joel Palefsky, Erin Isaacson-Wechsler, my fellow nursing students, and my friends and family.

Abstract

Infection with human papillomavirus (HPV) represents a complex presentation of symptoms, from benign warts to cancer. Prevention options for sexually active individuals outside the nonavalent HPV vaccine range are limited. Thus, the overarching aim of this dissertation work was to prevent HPV at the cellular level by investigating FDA- and internationally-approved drugs with the potential to prevent HPV infection. To this end, I conducted three research studies: a) a review of the literature focusing on human papillomavirus (HPV) biology, pathogenesis, and potential for drug discovery; b) developed a HPV-16 cell based assay for high-throughput screening (HTS) to conduct a pilot screen of 1906 compounds the Small Molecule Discovery Center at UCSF, and c) performed dose-response activity confirmation and investigated mechanisms of action for top candidate drugs. The results of these three studies demonstrate that the identification of novel anti-HPV drugs to be used for HPV prevention in the sexually active population is linked to understanding HPV biology; and using a HTS HPV-16 cell based assay is a valid and reliable tool for identifying potential anti-HPV to prevent HPV infection. Overall, my goal was to identify previously approved FDA- and internationally-approved drugs that have the potential to be repurposed for HPV prevention in sexually active individuals outside the of nonavalent vaccination range.

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I. Introduction

Statement of the Problem

Like cervical cancer, anal cancer is caused by human papillomavirus (HPV). HPV is the most common sexually transmitted agent and is found in almost all HIV-positive men who have sex with men (MSM) (Centers for Disease Control and Prevention, 2016). This combination results in HPV-related cancers disproportionately affecting MSM living with HIV. In the 1990's the widespread availability of highly active antiretroviral therapy (HAART) dramatically decreased mortality rates among MSM living with HIV (Duncan, Chan, Chiu, Montaner, Coldman, et al., 2015, Glick, Feng, Popov, Koutsky, Golden, 2014). Paradoxically, the incidence of anal cancer among MSM living with HIV has increased since the introduction of HAART, most likely due to increased survival time (Pokomandy, Rouleau, Ghattas, Trottier, Vezina, et al., 2011).

Currently, there are limited interventions to prevent HPV-related infections and cancers. A leading preventative intervention is the nonavalent HPV vaccine. The HPV vaccination is effective against nine strains of HPV, including some strains that cause cancer and other benign strains of common warts. The HPV vaccine is approved for individuals prior to sexual debut and between the ages of nine to 26 years old. In the USA, uptake has been poor (Centers for Disease Control, 2014). The suggested reasons for the poor uptake are many including, lack of knowledge about the vaccine, risk of increasing sexual promiscuity in adolescents, and the three-dose administration series (Walhart, 2012). Individuals outside the HPV vaccination guidelines have few options to protect themselves against HPV-related infections and cancers.

Other methods of preventing HPV infection are clearly needed. The identification of novel compounds from drug libraries with the potential to prevent infection is one such solution.

Drug libraries can be found in both the academic and industry setting and contain tens to hundreds of thousands of drugs including FDA- and internationally-approved drugs. Utilizing a technique called high-throughput screening (HTS); a researcher can quickly scan a library with a target specific assay and the assistance of liquid robot handling arms. Once identified, additional investigation into the mechanisms by which the drug(s) prevent infection will ensure safe administration by confirming the drugs works in the body the way it is thought to work. Taken together, the identification of novel compounds will be critical to reducing the burden of HPV-related anal (and likely cervical) pre-cancers and cancers among MSM living with HIV, as well as those in the general population.

Traditionally, the field of nursing has not been associated with conducting basic scientific research. However, clinical nurse scientists are in a unique position to design and implement scientific studies focused on improving the health outcomes for HPV-related infections based on the intimate lens of caring for patients in their beds. Clinical nurse scientists are researching ways to improve quality of life for patients suffering from chronic illnesses (i.e., HIV and cancer), encouraging patients to make healthy choices about safe sexual practices and decreasing the risk of acquiring a sexually transmitted infection (STI), such as HPV.

Clinical nurse scientists are in a position to conduct basic scientific research to aid in the development of therapeutic interventions aimed at protecting both MSM living with HIV and the general sexually active population from HPV-related infections and cancers. Although clinical nurse scientists conducting basic science are still in the early stages of integration with more established fields such as medicine and biology, the patient-focused nursing lens puts the field of nursing in a position to excel using these techniques to ask and answer questions. This lens helps

clinical nurse scientists use basic scientific techniques and biology at the cellular level to improve patient health outcomes.

Thus, the overarching goal of this dissertation is to prevent HPV at the cellular level by developing a HPV cell-based assay able to identify novel drugs that prevent HPV infection in MSM living with HIV as well in all sexually active people. In order to examine different aspects of this goal, I have conducted three research studies;

1. Human Papillomavirus Biology, Pathogenesis, and Potential for Drug Discovery: A Literature Review for HIV Nurse Clinical Scientists;
2. HPV-16 Cell Based Assay for High-throughput Screening Anti-HPV Drug Discovery
3. High-throughput Screening for Novel Anti-HPV Drug Inhibitors

Epidemiology of HPV Infection

HPV infection is stunningly common—the most prevalent sexually transmitted infection in the United States of America (USA) (Centers for Disease Control, 2014). Every year, 14 million new people get infected, with an estimated 79 million people infected in total. There are over 150 different kinds of HPV spread through skin to skin contact—about 40 of which infect the genital tract. 13 strains of HPV are classified as oncogenic and have the ability to cause infections that may lead to cancer in the anus, cervix, head and neck regions. The ubiquity of HPV and ease of transmission means that people who end up getting HPV usually do so when they are young and with their first sexual partners (Markowitz, Dunne, Saraiya, Chesson, Curtis et al., 2014).

HPV-16 and-18 are responsible for 70% of anal high grade squamous intraepithelial lesion (aHSIL) in men and women (Palefsky, Gioloano, Goldstone, Moreira, Aranda, et al, 2011). Among the MSM living with HIV, the incidence of anal cancer is more than 80-fold greater than that of the general population. The annual incidence is as high as 131/100,000 in HIV-infected MSM (National Cancer Institute at the National Institutes of Health, 2014; Shiels, Pfeiffe, Gail, Hall, Li, et al, 2011; Silverberg, Lau, Justice, Engels, Gill, 2012). Over time, persistent infection with HPV-16 and -18 can increase the probability of the progression to cancer (Palefsky et al, 2011). Despite the introduction of HAART, the incidence of anal cancer in PLWH has continued to increase (National Cancer Institute at the National Institutes of Health, 2014). A likely explanation is increased survival rates (Pokomandy et al., 2011).

HPV vaccination has emerged as an important strategy for prevention of HPV infection. Recently, the FDA approved the nonavalent HPV vaccine. While it is effective and safe (Merck and Co, 2013), it is expensive and adherence to vaccination schedule will likely be difficult (Palefsky et al., 2011). The difficulty of HPV vaccination administration is multifaceted. HPV vaccine is optimally administered prior to sexual debut or up to the age of 26 years. A limitation to this strategy is that a large percent of the current population is over the age of 26 (approximately 98% of the population) and past their sexual debut (U.S. and World Population, 2016).

The social stigma surrounding intercourse in adolescents has prevented some parents from allowing their children to be vaccinated (Walhart, 2012). Thus, the administration rate of the HPV vaccine is well under expected goal rates (Centers for Disease Control, 2014). Four out of ten adolescent girls and six out of ten adolescent boys' have not started the HPV vaccine series, and are vulnerable to cancers caused by HPV infections (CDC, 2014).

In addition, a high proportion of young men and women at risk for HPV infection live in countries where vaccination is not available for a variety of reasons, including high cost. Other preventive methods such as condoms demonstrate limited efficacy in preventing HPV transmission (Hessol, Holly, Minkoff, Schowalter, Garragh, et al., 2009). This is in part due to the likelihood that condoms do not fully cover the penis and perianal areas allowing for transmission to occur by exposure of these areas when in contact with a sexual partner (Brewer, Fazekas, 2007). The combination of these factors decreases the effectiveness of the vaccine. It is therefore critical to investigate novel compounds that can prevent HPV infections and cancer.

The benefits to identifying additional compounds are to provide a full spectrum of protective and preventive interventions to MSM living with HIV as well as the entire sexually active population. Once identified, this compound would have the potential to be combined with a lubricant that could be used before anal or vaginal intercourse. Since many if not most MSM who engage in anal intercourse use lubricant, this intervention would allow for decreased risk of acquisition of HPV infection and development of aHSIL and ultimately anal cancer in the MSM population (Carballo-Diéguez, Stein, Sáez, Dolezal, Nieves-Rosa, et al., 2000; Shiels et al., 2011, Silverberg et al, 2012)

High-throughput Screening in the Literature

High throughput screening **(**HTS) allows a researcher to quickly scan thousands of compounds in a compound library. Through this process, one can rapidly identify active

compounds that modulate a particular HPV bio-molecular pathway. Few studies have utilized HTS to identify novel compounds capable of inactivating or inhibiting HPV viral infection.

There are limited studies using HTS to identify novel inhibitors of HPV infection. One example of an HTS assay was the production of high-titer HPV pseudovirion (PsV) assay developed to perform HTS in vitro screens to identify compounds to prevent HPV infection (Buck et al., 2006; Roberts, et al., 2007). This assay used flow cytometric analysis to assess the inhibition of PsV-mediated delivery of a green fluorescent protein reporter plasmid into HeLa cells. A wide variety of compounds were screened using the inhibition assays and they identified carrageenan, a sulfated polysaccharide extracted from red algae, as a potent infection inhibitor for a broad range of sexually transmitted HPVs (Buck et al., 2006).

A recent clinical study by Marais et al., investigated carrageenan as a potential inhibitor for HIV transmission and their results suggested that carrageenan may have increased the ability of HIV to infect the host. They also performed a post-hoc secondary analysis to investigate carrageen's usefulness to prevent HPV infection. After adjusting for risk factors, their results showed that women who used carrageenan prior to vaginal intercourse were less likely to have a prevalent oncogenic HPV infection (OR 0.62, 95% CI 0.41–0.94) than compliant placebo users. However, this study has several major limitations: 1) there was no baseline or intermediate measurement of genital HPV infection and 2) prevalence of HPV was only measured at study end. Given its potential to increase HIV transmission, it remains important to identify other compounds with the potential to be potent inhibitors of HPV infections (Marais, Gawarecki, Allan, Ahmed, Altini, et al., 2011).

Two additional compounds have been identifies using HTS (Huang et al., 2012). A cell-based HTS assay was developed using HPV16 PsV expressing a reporter gene. Two lead

compounds were chosen for further analyses based upon the structure-activity relationship (SAR) (e.g., relationship between the chemical of a molecule and its biological activity), scaffold diversity, strength of the inhibitory activity and low cytotoxicity. These compounds, identified as #13 and #14 by the authors, showed low to submicromolar inhibitory concentration (IC_{50}) and little to no cytotoxicity. The IC_{50} is the half maximal inhibitory concentrations, which is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. The authors stated this provided evidence that compound #13 and #14 can inhibit multiple genital HPV types (Huang et al., 2012). In general, it is difficult to directly compare these studies since information on assay development, composition of compound libraries, and Z-factor parameters are limited in the literature or not provided.

A number of studies have used HTS assays to identify compounds able to prevent viral infections other than HPV, including filoviruses and Epstein-Barr virus (Thompson, Messick, Schultz, Reichman, and Lieberman, 2010; Basu, Li, Mills, Panchai, Cardinale, Butler, et al., 2011). Additionally, HTS have been used effectively to screen and identify potential antimetastatic and anti-inflammatory agents for cancer treatments (Reddanna, 2010).

Methodological Approaches / Three Papers Overview

In the first study, I examined the literature to determine the known factors of HPV biology, pathogenesis, and potential for drug discovery using HTS. This review was aimed at informing HIV clinical nurse scientists to increase their awareness of inter-disciplinary collaboration of molecular biology and nursing. The purpose of this review is to provide clinical nurse scientists information about HPV-related cancer and highlight the connection between: a)

HPV biology and pathogenesis and b) the development of drugs and novel therapeutic methods using high-throughput screening. PubMed and CINAHL were used to search the literature to determine HPV-related epidemiology, biology, and use of high-throughput screening for drug discovery. The results of this study suggest that several events in the HPV life cycle have the potential to be developed into biologic targets for drug discovery. The biological targets can be developed into assays for use with HTS. The HTS technique has been successfully used to identify compounds to inhibit HPV infections (Buck et al., 2006; Huang et al., 2012).

In the second study, I report the development of a HPV-16 cell-based HTS assay to identify drug inhibitors of HPV infection. In collaboration with the Palefsky lab and the Small Molecule Discovery Center (SMDC) at University of California San Francisco (UCSF), we developed and validated a cell-based HTS assay to identify compounds able to prevent HPV infections. The SMDC Screening Core performs biochemical and cell-based assays using liquid-handling robots and a screening library of >150,000 compounds. The SMDC compound collection library was purchased from various commercial vendors and represents a diverse collection of compounds including the kinase-targeted, carboxamide, and bioactives collections.

We have developed a HPV-16 cell-based assay in several steps. First, we produced PsV with the large T antigen SV40 promoter (SV40). PsV are HPV capsids expressed in vitro that include the papillomavirus capsid proteins L1 and L2 and when they are co-expressed in mammalian cells, they co-assemble and package heterologous nonviral DNA plasmids into infectious particles that resemble authentic HPV virions. We optimized HPV PsV for maximum inhibitory signal expression in relative light units (RLU) and selected the SV40 promoter to be used in the plasmid to drive renilla expression given its high strength in RLU and the availability of the 293 FT cell line. The 293 FT cell line is a fast-growing highly transfectable clonal isolate

derived from human embryonal kidney cells transformed with the SV40. Second, we determined the optimal ratio of PsV to 293 FT cells needed to produce an inhibitory signal (i.e., preventing PsV entry) by performing 3-fold serial dilutions of PsVs in 293 FT cells in 384-well plates. We determined that approximately 400 PsV/cell produced a magnitude of expression at least ten times greater than the control, a number that is considered ideal for this assay (Huang et al., 2012). We also determined that the optimal concentration of cells is 30,000 cells per well.

We determined our assay procedure using HTS in the following steps. First, we seeded six 384-well plates with 50ul of 293 FT cells (30,000 cell/ml) in G418 media per well over night incubated at 37^{O}C. Seeding the cells overnight allows the cells to 'rest' and to adhere to the surface to the 384-well plates creating an optimal environment for cell growth. The following morning, the cells are removed from 37^{O}C and allow equilibrating at room temperature. Third, we added 8.3uM of each compound and allow incubating at 37^{O}C for 2 hours. Forth, we added 10ul PsV (400 PsV/cell) to each well. Fifth, the plates were incubated incubate at 37^{O}C for 72 hours. After 72 hours the plates were read using Progemga DualGlo Reporter Assay System according to industry protocol. The results were analyzed using GraphPad Prism 6 software and IC50 calculated using 4-parameters non-linear regression analysis of the same software.

The assay was then validated using HTS parameters in conjunction with the SMDC to detect hits (Zhang, Chung, Oldenburg, 1999). The parameters include the gold standard z-prime and the secondary parameters % coefficient of variation (%CV) and signal to background (s/b). The %CV number indicates the percent of variability within the replicates. A %CV of <15% is considered statistically significant. The signal to background (S/B) is the ratio of (-) control (cells + PsV without drug) and (+) control (cells only), which indicates the assay window. S/B ratio higher than 1:1 indicates more signal than noise suggesting a high quality of the

measurement. Finally, z-prime is an indication of how well the assay will perform in an HTS. Z-prime is a dimensionless calculation used to assess the robustness of the HTS assay with a statistically significant range of 0.5 to 1 (Zhang et al., 1999). These parameters define the time point when the signal is the strongest before it plateaus, measuring RLU after they are saturated can provide misleading data on how effective different compounds are at preventing HPV entry into cells. After the optimization steps described above, we determined that the parameters of our PsV HTS assay were; z-prime 0.67, %CV 10, and S/B 424, indicating that the assay was robust enough to proceed with the pilot screen.

Finally, in the third study, we report the results of the pilot screen using the HPV-16 cell-based assay for HTS. The pilot screen was conducted using a small validation library of 1906 diverse classification of FDA- and internationally-approved pharmacologically active drugs selected by SMDC. A validation library is standard protocol for a pilot screen because it helps to control for statistical variability and identify potential sources of interference.

A "hit" was defined using the parameters as any drug that showed > 77% inhibition of Renilla expression following PsV entry (mean + 2 standard deviation cutoff). 98 hits were selected from the 1906 drugs screened for dose-response activity confirmation. Some of the hits were likely to be false-positives due to drug-induced cell toxicity. To minimize false-positives (i.e., if the PsV inhibition was the result of cell death) we investigated cell viability by determining the number of viable cells in culture based on quantification of the ATPase, an indicator of metabolically-active cells. To help distinguish false-positives from false-negatives we also required a "hit" to have a minimal decrease (> 0.7 fold change) in cell viability. We used these parameters to select the top hit and drugs with interesting pharmacological properties (i.e., plant based biologicals) from the 98 hits. We narrowed the 98 drugs to 25 drugs. The HTS

infection assay and cell-viability assay was then repeated with these 25 compounds to confirm the original findings. The 25 compounds were repurchased to control for quality. The dose-response activity, infection, cell viability were all confirmed with the repurchased drugs in the Palefsky lab. The results of the pilot screen suggest that the HTS assay we developed was successful at identifying hits when used with HTS automation in the compound library at SMDC.

An example of one of the hit drugs that inhibits PsV infection and has no cell toxicity at the concentration used is doxifluridine. Doxifluridine is a fluoropyrimidine derivative and oral product of the antineoplastic agent 5-fluorouracil (5-FU). 5-FU is currently one of the drugs used to treat aHSIL. Doxifluridine showed 79% inhibition at 10 uM assay concentration during the primary screen. In the follow up cell-viability dose response study, doxifluridine exhibited more than 125-fold selective inhibition of PsV infection (IC50 = 0.4 uM) compared with cell viability (IC50 > 50 uM).

Human Papillomavirus Biology, Pathogenesis, and Potential for Drug Discovery: A Literature Review for HIV Nurse Clinical Scientists

Tara Walhart, RN, MSN, MPH, NP-C, PhD(c)*

Persistent oncogenic human papillomavirus (HPV) infection increases the probability that precancerous anal high-grade squamous intraepithelial lesions will progress to invasive anal cancer. Anal neoplasia associated with HPV disproportionately affects HIV-infected individuals, especially men who have sex with men. Prevention is limited to HPV vaccine recommendations, highlighting the need for new treatments. The purpose of this review is to provide HIV information to nurse clinical scientists about HPV-related cancer to highlight the connection between: (a) HPV biology and pathogenesis and (b) the development of drugs and novel therapeutic methods using high-throughput screening. PubMed and CINAHL were used to search the literature to determine HPV-related epidemiology, biology, and use of high-throughput screening for drug discovery. Several events in the HPV life cycle have the potential to be developed into biologic targets for drug discovery using the high-throughput screening technique, which has been successfully used to identify compounds to inhibit HPV infections.

(Journal of the Association of Nurses in AIDS Care, 26, *693-702*) *Copyright © 2015 Association of Nurses in AIDS Care*

Key words: *anal cancer, high-throughput screening, HIV, human papillomavirus*

Human papillomaviruses (HPV) represent a diverse anthology of medical diseases. The spectrum ranges from benign plantar warts to invasive cancers. The emerging burden of cancer in people living with HIV (PLWH) draws attention to HPV as a growing public health concern. Several factors contribute to the burden, including the lack of screening guidelines, absence of knowledge regarding transmission, minimal prevention methods, and limited clinical signs and symptoms of infection (Brabin, Roberts, Farzaneh, & Kitchener, 2006; Brewer & Fazekas, 2007; Walhart, 2012), which highlights the need to understand the biology and pathogenesis of HPV in order to identify novel interventions to prevent high-risk groups such as PLWH and men who have sex with men (MSM) from developing anal cancer and HPV-related infections.

The purpose of this literature review is to provide HIV nurse clinical scientists with an epidemiology background of HPV-related cancer to highlight the connection between: (a) understanding HPV biology and pathogenesis and (b) its role in drug discovery and the development of novel therapeutic methods. The information provided will help HIV nurse clinical scientists contribute to the development of novel treatment interventions. HIV nurse clinical scientists are in a unique position to translate basic science into therapeutic interventions because they address the individual from multidimensional perspectives, and the

*Tara Walhart, RN, MSN, MPH, NP-C, PhD(c), is a doctoral candidate, School of Nursing, Department of Community Health Systems, University of California, San Francisco, California, USA. (*Correspondence to: tara.walhart@ucsf.edu).*

JOURNAL OF THE ASSOCIATION OF NURSES IN AIDS CARE, Vol. 26, No. 6, November/December 2015, 693-702
http://dx.doi.org/10.1016/j.jana.2015.07.001

questions that they generate from clinical settings are often grounded in basic biology. This enables HIV nurse clinical scientists to combine clinical and translational research to directly contribute to the patient's continuum of care.

Background

HIV nurse clinical scientists need to recognize the important role HPV plays in the development of cancer, specifically anal cancer, in PLWH. There are more than 100 types of HPV; subsets of high-risk HPV, specifically HPV-16 and -18, have the ability to induce several types of cancer, including anal cancer (Chen et al., 2014; Da Costa, Hogeboom, Holly, & Palefsky, 2002; Morshed, Polz-Gruszka, Szymanski, & Polz-Dacewicz, 2014). For the purpose of this article, HPV-16 and -18 will be synonymous with HPV.

HPV Epidemiology

HPV is the most common sexually transmitted infection (Hessol et al., 2009). At least 75% of individuals will acquire a genital HPV infection in their sexual lifetimes (Buck et al., 2006; Hessol et al., 2009). In a recent meta-analysis of 157,879 women, the prevalence of oncogenic HPV was reported to be as high as 10% in women with normal cytology, giving an estimate of 600 million infected people (Chen et al., 2014). HPV has the ability to cause precancerous changes in the anal canal, or anal high-grade squamous intraepithelial lesions (aHSIL; Hessol et al., 2009; Palefsky et al., 2011). aHSIL is believed to be the precursor to anal cancer and it has been suggested that persistent infection with HPV can, over time, increase the probability of aHSIL progressing to invasive anal cancer (Hessol et al., 2009; Palefsky et al., 2011). In 2014 it was estimated that, in the United States, the incidence rate of anal cancer was 7,270 (4,630 in women and 2,640 in men) and there were 1,010 deaths (American Cancer Society, 2014; National Cancer Institute, 2014, n.d.).

The presence of HPV is nearly universal within the MSM population and, as a result, aHSIL disproportionately affects MSM who have HIV (Glick, Feng, Popov, Koutsky, & Golden, 2014; Hessol, et al., 2009; Koutsky, 1997). In a meta-analysis by Machalek and colleagues (2012), the prevalence of aHSIL in HIV-infected MSM proved by biopsy was 29%, compared with 22% in HIV-uninfected MSM. In the same meta-analysis, anal cancer incidence was 46/100,000 in HIV-infected MSM per year, versus 5.1/100,000 in HIV-uninfected MSM (Machalek et al., 2012). Additional incidence surveillance data have suggested that HIV infection rates of people with aHSIL are 10-50 times greater in MSM when compared with HIV-uninfected MSM (Hessol et al., 2009). There are several reasons for these data, including sexual behavior, immunosuppression, HIV viral load, length of time on antiretroviral therapy (ART), and prolonged duration of HIV infection (Duncan et al., 2015; Guiguet et al., 2009; Silverberg et al., 2012; Simard, Pfeiffer, & Engels, 2011).

HPV infection is associated with higher incidence rates of anal cancer in PLWH and MSM, but the direct relationship between HIV and anal cancer has been difficult to separate from the prevalence of HPV in this population (Stanley, Winder, Sterling, & Goon, 2012; Uronis & Bendell, 2007). One reason for this may be that HIV-infected individuals have higher HPV viral loads than HIV-uninfected individuals, with more frequent infections with concomitant multiple HPV types. With multiple HPV types, there is less HPV clearance and thus, more ubiquitous HPV persistent infection (Strickler et al., 2005). As a consequence, HPV incidence rates and prevalence are higher in HIV-infected individuals. This is evidenced by increasing incidences of anal cancer in HIV-infected MSM over the last 2 decades (Da Costa et al., 2002; Hessol et al., 2009; Schwartz et al., 2013; Shiels et al., 2011).

The widespread availability of effective ART beginning in 1996 dramatically decreased the mortality rate of HIV-infected individuals (Simard et al., 2011). Despite overall improved survival, PLWH remain at an increased risk for anal cancer because of prolonged HIV-induced immune suppression and longer life expectancies (Pokomandy et al., 2011). Longer life expectancies suggest PLWH are more likely to be affected by diseases that manifest more slowly, such as anal cancer, while maintaining a regimen of ART (Duncan et al., 2015; Pokomandy et al., 2011).

HPV Prevention

Current HPV prevention methods for sexually active individuals are limited. This is due, in part, to the lack of signs and symptoms of HPV infection and a general absence of understanding and knowledge about the transmission of HPV infection in the general public (Morshed et al., 2014; Palefsky et al., 2011; Palefsky, Berry, & Jay, 2012). What we do know is that HPV is transmitted through microtraumas or tears in the epithelium during intercourse. A practical HPV prevention intervention would be the use of a lubricant to help reduce the risk of microtraumas associated with intercourse. However, if one does not know s/he is infected with HPV, because of the lack of presenting signs and symptoms, or understand how easy it is to transmit HPV from one sexual partner to the next, there is simply no consideration to protect him-/herself and others.

The recent availability of several HPV vaccines (i.e., quadrivalent and 9-valent) have emerged as potentially important strategies for HPV prevention (Merck and Co., Inc., 2014; Palefsky et al., 2011). Commercially available HPV vaccines can provide coverage against HPV types 16, 18, 31, 33, 45, 52, and 58 for prevention of cancer, and HPV types 6 and 11 for prevention of genital warts (Merck and Co., Inc., 2014). They are considered to be safe, easy to produce, and stable (Palefsky et al., 2011). However, there are several limitations to the vaccine. It is expensive, and it is recommended that it be administered between the ages of 9 and 26 years, and prior to sexual debut (Huang et al, 2012; Palefsky et al., 2011). The age and sexual debut restrictions on the administration of HPV vaccines create a gap in the continuum of care for anyone ages 26 years and older and anyone who has achieved sexual debut (Palefsky et al., 2011). Additionally, condoms demonstrate limited efficacy in preventing HPV transmission because condoms do not fully cover the penis and perianal areas, allowing for contact transmission on exposed areas with a sexual partner (Brewer & Fazekas, 2007). It remains worthwhile to investigate other interventions to provide individuals with protection against HPV, particularly therapeutic compounds that target HPV types not currently covered by vaccine.

High-Throughput Screening Techniques

One way to identify new prevention strategies is to build on advances in the field of HPV biology at the cellular and molecular levels. HPV biologists now have models to describe the complex life cycle and pathogenesis of HPV. HIV nurse clinical scientists can use knowledge of the complex HPV life cycle to aid in the discovery of novel therapeutic compounds by high-throughput screening (HTS) techniques. HTS is a proven pharmacology technique to identify small molecules and compounds that have the potential to be developed into new therapeutic interventions. The process involves developing a measurement tool (referred to as an assay) designed for a specific biologic target (i.e., kinase-inhibitor, binding sites, or enzyme) in a 96-well plate. Once the assay has been optimized in a 96-well plate, it is scaled up or miniaturized to a 384-well plate format. The miniaturization of the assay allows automated liquid-handling robots to rapidly prepare the plates and scan a library of thousands and thousands of small molecules and compounds.

HTS is becoming increasingly more common among clinicians and scientists interested in identifying small molecules or bioactive compounds appropriate for drug development and clinical application. HTS has the potential to be used to identify compounds able to modulate HPV infection (Buck et. al., 2006; Huang et al., 2012). Identification of these compounds is the first step in the development of new drugs for treatment and prevention. An example of a prevention intervention in sexually active individuals would be a personal lubricant or mouthwash combined with a compound able to block HPV from infecting cells. Novel interventions such as a personal lubricant or mouthwash would expand clinical abilities to provide prevention and patient care for both PLWH and MSM.

Summary

HPV is the most common sexually transmitted infection. The HPV co-infection in PLWH, especially in MSM, increases the risk of acquiring aHSIL and its development into anal cancer. The epidemiology of HPV is critical to framing the answer to "why we care" and promoting the discovery of new therapeutic

Table 1. Review of the Literature

Type	Database	# Articles Retained
Literature search	PubMed	23
	CINAHL	5
References		
HPV biology and pathogenesis	Burk et al., 2009; Da Costa et al., 2002; Day & Schelhaas, 2014; Doorbar, 2006; Duncan et al., 2015; Guiguet et al., 2009; Hessol et al., 2009; Joyce et al., 1999; Kajitani et al., 2012; Machalek et al., 2012; Matthews et al., 2003; Middleton et al., 2003; Morshed et al., 2014; Munger et al., 2004; Pokomandy et al., 2011; Pyeon et al., 2009; Roden et al., 2001; Schiller et al., 2010; Stanley et al., 2012; Strickler et al., 2005; Uronis & Bendell, 2007	
Use of HTS for drug discovery	Basu et al., 2011; Buck et al., 2006; Huang et al., 2012; Kumar et al., 2011; Mahida et al., 2013; Marin et al., 2015; Thompson et al., 2010	

Note. HPV = human papilloma virus; HTS = high-throughput screening techniques.

methods. The key to discovering new therapies is to understand the intricate and complex biology and pathogenesis of HPV. The knowledge of HPV biology and pathogenesis will help identify potential biologic targets (i.e., phases in the cell cycle, gene expression, or capsid formation). Biologic targets can be developed into assays that can be used with HTS to assist in drug discovery.

Methods

The field of HPV research is large and multifaceted, represented by HPV biologists, clinicians, and researchers. This review of the literature focused on the specific niche between the relationship of HPV biology/pathogenesis and the use of HTS for drug discovery. The literature will be grouped according to: (a) biology/pathogenesis of HPV, and (b) use of HTS screening for HPV drug discovery. The databases PubMed and CINAHL were selected because of PubMed's broad scope of basic science research and CINAHL's focus on nursing research. PubMed and CINAHL were searched using the following inclusion criteria search and MESH terms: HPV, aHSIL, anal cancer, HIV, HPV biology, HPV pathogenesis, high-throughput screening, and HPV drug discovery. Articles focusing on the following were excluded: condylomata acuminata, abnormal cytology, vaccine titers, genotyping, HPV biology only in females, HPV head and neck cancers, basic viral infection, proctology, and herpes simplex virus. Table 1 summarizes the relevant literature applicable to the purpose of this article.

Results

HPV Biology and Pathogenesis

A brief primer of HPV biology will be presented to lay the foundation to discuss the relationship between HPV biology/pathogenesis and drug discovery. HPV is a small double-stranded DNA nonenveloped virus and has a unique and complex viral life cycle among viruses. This is because HPV viral replication is tightly linked to the differentiation of epithelial cells in the host (Day & Schelhaas, 2014; Pyeon, Pearce, Lank, Ahlquist, & Lambert, 2009). In order for progeny virus to form, HPV must: (a) establish viral DNA in mitotically active basal cells in the stratified epithelium, (b) facilitate maintenance of viral DNA at low copy numbers in dividing epithelial cells, and (c) promote high copy number DNA amplification and encapsidation as the cells migrate up the epithelium where terminally differentiated (nondividing) epithelial cells are located to produce viral progeny (Doorbar, 2006; Middleton et al., 2003; Pyeon et al., 2009).

Several naturally occurring events in the HPV life cycle have the potential to be developed as biologic targets for HTS drug discovery. The HPV viral gene, E2, is one such example. E2 plays a critical role in viral transcription, replication, and maintenance of the viral genome. E2 is a DNA-binding site that recognizes E2 binding during transcription. Additionally, E2 has transcriptional trans-activator activity as well as the capacity to bind viral DNA replication factor E1 (Kajitani, Satsuka, Kawate, & Sakai, 2012). The E2 gene has four binding sites on

the HPV-16 long control region (Kajitani et al., 2012). A binding site represents a potential attachment point that could be blocked by a drug, thereby preventing the completion of the HPV replication life cycle. Because E2 plays an intricate and important role in the replication life cycle of HPV, it is a potential target to be used with HTS for drug discovery.

In the normal cell cycle, uninfected basal cells in the epithelium exit the cell cycle soon after migrating to the suprabasal cell layers and undergo terminal differentiation (Doorbar, 2006). Cells in a terminally differentiated state are not mitotically active. Accordingly, the cell's DNA replication activity is suppressed in differentiated cells that exit from the cell division cycle (Kajitani et al., 2012). In order to sustain replication, HPV must reactivate the cell division process in differentiated cells. It does this with the expression of E6 and E7. E6 and E7 are the primary oncogenes in HPV and together they are responsible for dysplastic changes at the cellular level and the progression to invasive cancer. E6 and E7 function by inactivating p53 and retinoblastoma protein (pRb; Matthews et al., 2003). The normal functions of p53 and pRb are to enable cells to maintain cell cycle DNA potential (Munger et al., 2004). Through the transcription process, E6 and E7 prevent the normal function of p53 and pRb, thus allowing the cell to support HPV DNA replication (Doorbar, 2006). This suggests that the expression of E6 and E7 are predisposing factors in the development of HPV-associated cancer (Doorbar, 2006). The ability of E6 and E7 to commandeer the cell's natural cell life cycle makes these oncogenes ideal targets for drug discovery. A drug that could interrupt E6 and E7 inactivation of p53 or pRb would have great therapeutic value to prevent HPV-related infections.

In addition to gene expression, proteins have the potential to make ideal biologic targets because they tend to be associated with antibody expression. HPV encodes two structural proteins, L2 and L1, which are expressed in the upper layers of infected tissue (Doorbar, 2006; Joyce et al., 1999, Middleton et al., 2003). L1 and L2 have evolved to fulfill multiple roles that are critical to establishing HPV infection. L2 is often characterized as a good candidate for prophylactic vaccine development because L2-specific antibodies have cross-neutralizing activity against diverse HPV types (Schiller, Day, & Kines, 2010). For nonenveloped viruses, such as HPV, the protein coat encases and protects viral nucleic acid as well as provides the initial interaction site of the viral particle with the host cell (Horvath, Boulet, Renoux, Delvenne, & Bogers, 2010). This allows assembly of infectious HPV particles in the upper layers of the epithelium (Doorbar, 2006; Roden et al., 2001), which is why L2 was chosen as the biologic target for the development of the HPV vaccine (Merck and Co., Inc., 2014).

HPV Pathogenesis and Progression

This section ties together the pathogenesis of HPV and its progression to cancer. HPV forms high-grade lesions at sites where the productive life cycle cannot be completed, and this process is referred to as an abortive infection. High-grade lesions have a more extensive proliferative phase than normal cells and the productive stages of the viral life cycle are either not supported or supported only poorly (Burk, Chen, & Van Doorslaerb, 2009; Doorbar, 2006). The key event in the progression of productive lesions to aHSIL may result from a deregulation of the expression of the E6 and E7 proteins (Middleton et al., 2003). Decreased expression of these two viral transforming proteins leads to increased cell proliferation in the lower epithelial layers and an inability to repair mutations in the host cell's DNA (Doorbar, 2006).

The transformation zone or squamous-columnar junction is a particularly susceptible site for anal cancer to develop. A predominate theory is that high-risk HPV types such as HPV-16 cannot reliably complete the life cycle at this site, which occasionally leads to abortive infections (Doorbar, 2006). Progression from high-grade aHSIL to cancer usually occurs in lesions that contain integrated copies of viral genome in which E7 expression is elevated (Doorbar, 2006). This again highlights the need for HIV nurse clinical scientists to recognize HPV-related disease burden in PLWH and to identify novel interventions to prevent high-risk groups and the general population from developing anal cancer and HPV-related infections. HTS plays a critical role in the discovery of compounds able to prevent HPV infection.

HTS is an important step in the discovery of new therapeutic modalities. The primary advantage to HTS is that it allows a researcher to quickly scan thousands of compounds in a compound library.

The automated process uses liquid handling and robotic arms to inject small volumes of drugs or molecules into 384-well plates in several hours. Through this process, one can rapidly identify active compounds that modulate a particular HPV biomolecular pathway (Huang et al., 2012). The alternative method involves manual pipetting of each compound into an individual well on a 384-well plate. This labor-intensive method would render drug discovery nearly impossible. In addition to the physical labor required, a high degree of error occurs when a researcher pipettes less than 1 μL. However, HTS does have several drawbacks: it is expensive, results are generalizable only to the library screened, and few studies have used HTS to identify novel compounds capable of inactivating or inhibiting HPV viral entry (Buck et al., 2006; Huang et al., 2012).

High-Throughput Screening for HPV Drug Discovery

The use of HTS in the field of virology is a relatively new idea. One reason for this may be that HTS is dependent on a biologic target to identify drugs. Viruses are complex living organisms that interact with host cells to establish infection. The biology underlying the infectious process must be defined and quantified first, which may take years to fully comprehend. The contributions of HPV biologists to the field of virology have made it possible to identify biologic targets within HPV (i.e., L2 and E2) to be used with HTS for drug discovery. Currently, only two articles have reported on the use of HTS for HPV drug discovery (Buck et al., 2006; Huang et al., 2012).

Buck and colleagues (2006) developed an inhibition assay by optimizing a pseudovirions (PsV) plasmid expressing the biologic targets of L1 and L2. PsV are noninfectious HPV plasmids that can be created to express L1 and L2. The PsV acts as a segregate maker for HPV infection. The inhibition assay was developed to perform HTS in a library of several thousand compounds to identify hits or compounds able to prevent HPV infection. The assay used flow cytometric analysis to assess the inhibition of PsV-mediated delivery of a green fluorescent protein reporter plasmid into HeLa cells. Any compound that prevented the green fluorescent protein expression was identified as a hit (i.e., compounds able to prevent PsV from entering the cell and preventing infections). Buck and colleagues (2006) identified carrageenan, a type of sulfated polysaccharide extracted from red algae, as an extremely potent infection inhibitor for a broad range of sexually transmitted HPVs.

Recently, HTS assay was used to identify two additional compounds able to inhibit HPV infection (Huang et al., 2012). A cell-based HTS assay was developed using HPV16 PsV plasmid expressing L2. Huang and colleagues (2012) screened a compound library of 40,000 elements consisting of commercially available bioactives, U.S. Food and Drug Administration-approved drugs, natural organics, and predesigned drug-like small molecules. Two lead compounds were chosen for further analyses based on the structure-activity relationship (e.g., relationship between the chemical of a molecule and its biologic activity), scaffold diversity, strength of the inhibitory activity, and low cytotoxicity. The compounds, identified as #13 and #14 by the authors, showed low to submicromolar inhibitory concentration (IC_{50}) and little to no cytotoxicity. IC_{50} is the half maximal inhibitory concentration that is a measure of the effectiveness of a compound in inhibiting biologic or biochemical function. This can be interpreted as compounds #13 and #14 at their optimal micromolar (μM) concentration prevented 50% of PsV inhibition while exhibiting little to no cell toxicity. Preliminary results suggested that compounds #13 and #14 could inhibit multiple genital HPV types (Huang et al., 2012). The road to bringing a novel drug or compound to the clinical setting is long and arduous; the initial discovery is only the first step. Although the results from Huang and colleagues (2012) seem promising, they will need to be biologically reproduced; additional experiments to identify mechanism of action for #13 and #14 need to be performed. This process can take years.

Other High-Throughput Screening Drug Discovery

A number of studies have used HTS assays to identify compounds able to prevent viral infections other than HPV, including filoviruses (Basu et al.,

2011), HIV (Marin et al., 2015), and Epstein-Barr virus (Thompson, Messick, Schultz, Reichman, & Leiberman, 2010). Additionally, HTS has been used effectively to screen and identify potential anti-metastatic drugs (Mahida et al., 2013) for cancer, and for anti-inflammatory disorders (Kumar et al., 2011).

Summary

The Results section emphasized the complex and intricate events in the life cycle of HPV, pathogenesis to aHSIL, and use of HTS for drug discovery. Several naturally occurring events in the HPV life cycle have the potential to be developed into biologic targets for HPV HTS including: (a) E2, (b) E6 and E7, (c) L1, and (d) L2. The HPV life cycle helps to keep developing infections under the radar of the human body's robust immune system. Upon infection, HPV does not present with typical signs and symptoms (i.e., pain, fever, or cough). This adds to the barriers of introducing population-level interventions to reduce the risk of HPV infections. This highlights the need for HIV nurse clinical scientists to investigate and understand HPV biology and pathogenesis at the cellular and molecular levels in order to develop potential biologic targets into HTS assays to discover new compounds that may be developed into treatment interventions. Novel treatment interventions help high-risk individuals such as PLWH and HIV-infected MSM reduce exposure to HPV-related infections and cancer.

Discussion

HIV nurse clinical scientists are on the forefront of molecular health research focused on evaluating the relationships between the HIV-infected patients' continua of health care and prevention of opportunistic oncogenic HPV infections (Palefsky et al., 2012; Rudy & Grady, 2004). HPV-related infections have the potential to cause precancerous lesions in the anal canal that may progress to anal cancer. Although most people will be exposed to HPV in their sexual lifetimes, certain high-risk groups, including PLWH and MSM, are at increased risk for developing HPV-related anal cancer (Glick et al., 2014). The current prevention options for sexually active people are limited. However, through the use of HTS for drug discovery, several compounds with the potential to inhibit HPV infection have been identified. It remains worthwhile to mine additional compound libraries to identify novel compounds able to prevent HPV infections. The identification of these compounds creates an opportunity to develop prevention interventions such as a personal lubricant or mouthwash that can be administered before intercourse as an intervention to help protect all people from HPV-related infections and the development of precancerous lesions.

HIV nurse clinical scientists are in a unique position to translate basic science into viable clinical applications. Nursing science addresses the individual from a multidimensional perspective, and the questions they generate from clinical settings are often grounded in basic biology. The ability to combine clinical and translational research enables nurse clinical scientists to directly contribute to the patient's continuum of care. Additionally, nurse clinical scientists have the potential to contribute to a diverse body of basic science knowledge from a perspective driven from the nursing scope of practice.

There are several limitations to this review of the literature. HTS is a well-established technique used in pharmaceutical industries for small molecule and drug discovery. HTS is a relatively new method being used in the field of virology for drug discovery, and at this time there are few published articles to validate the process (Basu et al., 2011; Buck et al., 2006; Huang et al., 2012). Additionally, few published articles using HTS for HPV drug discovery limit biologic reproducibility, making it hard to directly compare results (Buck et al., 2006; Huang et al., 2012). In general, it is difficult to directly compare studies because information on assay development, composition of compound libraries, and z-prime parameters are limited in the literature or not provided. There is also a lack of contributions from the clinical nursing perspective by nurse clinical scientists. The patient care component of a nurse's body of knowledge is incredibly powerful, and nurse-scientists have the potential to develop future research questions guided by clinical insight. HIV

nurse clinical scientists have the capability to integrate basic science with patient-centered care, and intimate knowledge of HPV biology and patient care is a major asset. Its contribution may help to inform future development of new therapeutic methods.

Key Considerations

- Collective efforts will allow HIV nurse clinical scientists to provide care and advise patients on treatment options.
- Knowledge gained from research will fuel additional efforts in the development of HPV prophylactic interventions for all sexually active individuals.
- Translating research into clinical practice will provide more robust prevention methods for sexually active individuals, specifically, people living with HIV and HIV-infected men who have sex with men, to reduce the risk of exposure to HPV-related infections, anal high-grade squamous intraepithelial lesions, and anal cancer.

Disclosures

The author reports no real or perceived vested interests that relate to this article that could be construed as a conflict of interest.

Acknowledgments

Support for this manuscript was provided by: University of California, San Francisco School of Nursing Training Program in HIV/AIDS Nursing Care and Prevention (T32 NR007081), the American Cancer Society Doctoral Degree Scholarship in Cancer Nursing (DSCN-13-271-01 SCN), the Ruth L. Kirschstein National Research Service Award/National Institute of Nursing Research (5 F31NR01 3863-02), the Palefsky Laboratory, and Dr. Carol Dawson Rose.

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A High-throughput Screening Cell Based Assay to Identify Inhibitors of HPV-16 Infection

Tara Walhart, Erin Isaacson-Wechsler, Sharof Tugizov, Kean-Hooi Ang, Carol Dawson-Rose, Michelle Arkin, Joel Palefsky

University of California, San Francisco

Abstract

Like cervical cancer, anal cancer is caused by human papillomavirus (HPV). HPV is the most common sexually transmitted agent. HPV infections are found in almost all men who have sex with men living with HIV. Rates of HPV-related cancers are disproportionately higher in this population when compared with the general public. The nonavalent HPV vaccine is one preventative option. However, limitations on administration guidelines including up to the age of 26 years and prior sexual debut, leave a large portion of the population at risk for HPV exposure and the development of cancer. This continues to make HPV-related infections a public health concern worldwide. Thus, it is important to identify new drugs able to prevent HPV infection. Currently, the identification of drugs to prevent HPV has been hindered by the lack of robust assays for high-throughput screening. We report on the development of a high-throughput cell-based assay using HPV-16 pseudovirions with a luminescence-reporter. We conducted a novel screen of 1906 FDA- and internationally-approved drugs. The assay performance was consistent and reliable at scale. Assay control statistics show an average Z' of 0.67± 0.04, coefficient of variation of 10%, and signal-to-background ratio of 424 ± 8, indicative of a robust assay in this cell-based format. Using a threshold of ≥ 77 % HPV inhibition with < 20% cellular cytotoxicity, 152 compounds were initally classified as active compounds. A follow-up screen confirmed 98 compounds with > 90% HPV inhibition and little to no cellular cytotoxicityat a concentration of 30 μM. Taken together, this assay offers the opportunity to screen large libraries of drugs to identity and develop novel drug candidates to prevent HPV infection.

A High-throughput Cell Based Assay to Identify Inhibitors of HPV-16 Infection

Introduction

Human papillomavirus (HPV) is the causative agent of several types of cancer including anal cancer. About 95 percent of anal cancers are caused by HPV. Most of these are caused by HPV type 16 (National Cancer Institute, 2016). HPV is the most commonly transmitted sexual infection worldwide and disproportionally affects men who have sex (MSM) living with HIV. Among the MSM living with HIV the risk of developing anal cancer is more than 80-fold greater when compared with the general public (Palefsky et al., 2008, Shiels, Pfeiffer, Gail, Hall, Li, Chaturvedi, 2011). The annual incidence is as high as 131/100,000 in this population (American Cancer Society, 2016; National Cancer Institute, 2016). Overall treatment options for anal cancer are limited and associated with substantial morbidity. Thus, to decrease rates of morbidity and mortality due to HPV infections and related cancers the focus needs to shift to prevention. Fortunately, most cancers related to HPV infection are preventable with the administration of the nonavalent HPV vaccine. However, the HPV vaccine has several limitations including administration prior to sexual debut up to the age of 26 years. The HPV vaccination guidelines create a gap leaving approximately 50% of the adult population between the ages of 26 and 65 years old vulnerable to HPV related infections and development of cancer (United States Census Bureau, 2016). Clearly, alternatives are needed, particularly for high-risk individuals including MSM living with HIV and outside the vaccination range.

Anal high-grade squamous intraepithelial lesion (aHSIL) is the clinical term to describe changes at the cellular level that may progress into anal cancer. Therapeutic options for aHSIL are limited. Those available are caustic, often causing skin ulceration, or involve burning the affected area. Individuals who experience adverse medication affects are less likely to continue with aHSIL treatment regimens. Untreated or under treated aHSIL increases the risk of aHSIL progressing to cancer. There is a critical need to identify new preventative treatment options.

To facilitate drug discovery for prevention of HPV infection, we developed and validated an automated, high-throughput screen (HTS) for HPV-16. The ultimate goal is to identify a novel drug able to prevent HPV infection that can be combined with a lubricant. The lubricant would be used before anal or vaginal intercourse, thus empowering individuals to reduce their own risk of repeated HPV infections and development of cancer.

HTS is one of the most powerful tools available for drug discovery. HTS use of liquid handling automation allows researchers to quickly screen thousands of compounds. The compounds, collectively called a drug library, can be evaluated for biological activity (i.e. antiviral activity) and cytotoxicity in living cells. Therapeutic agents, discovered with HTS have found their way into the clinic for treatment of many different diseases including transplantation, inflammatory and infectious diseases as well as cancer (Macarron, Banks, Bojanic, Burns, Cirovic, et al., 2011, Haung, Holmgren, Reik, Lucas-McGady, Roberts, et al., 2004, Haung, Holmgren, Reik, Lucas-McGady, Roberts, et al., 2004).

We developed and validated a cell-based HPV-16 assay suitable for HTS. The assay was validated using HTS parameters in conjunction with the Small Molecule Discovery Center (SMDC) at the University of California, San Francisco (UCSF) to detect active compounds.

Active compounds are defined as drugs preventing HPV-16 infection. The assay validation parameters include; the gold standard Z' and the secondary parameters % coefficient of variation (%CV) and signal to background (s/b) (Zhang, Chung, Oldenburg, 1999). The %CV number indicates the percent of variability within the replicates. A %CV of <15% is considered statistically significant. The signal to background (s/b) is the ratio of (-) controls and (+) controls, which indicates the assay window. S/B ratio higher than 1:1 indicates more signal than noise suggesting a high quality of the measurement. Finally, Z' is an indication of how well the assay will perform in a HTS. Z' is a dimensionless calculation used to assess the robustness of the HTS assay with a statistically significant range of 0.5 to 1.0 (Zhang et al., 1999).

Material and Methods

Cells

Human embryonal kidney cells (293FT) transformed with the SV40 large T antigen are a line of fast-growing highly transfectable clonal isolate cells. 293 FT were routinely cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin 100x, 1% G418 50mg/ml (Life Technologies). The cells were cultured at 37^0C in a humidified atmosphere of 5% CO_2.

Production of Pseudovirions

Pseudovirions (PsV) are plasmid shells, that encapsidate a reporter gene in self-assembling particles containing the two HPV-16 capsid proteins, the major L1 and minor L2 proteins (Buck, Pastrana, Lowy, Schiller, 2004, Buck, Thompson, 2007). When they are co-

expressed in mammalian cells they co-assemble and package heterologous nonviral DNA into infectious particles that resemble authentic HPV virions. Given that HPV-16 is the most common cause of cervical and anal cancer, we chose to synthesize HPV-16 PsV. HPV-16 PsV were used in all various model systems.

PsV were produced in our lab as previously described by Buck et al., (2004). Briefly, PsV were produced in 293 FT cells. An expression plasmid encoding the renilla reporter gene *pRL-S*V40 (Promega) was co-transfected into 293 FT cells, along with a second plasmid, p16L1L2 (Addgene), which drives expression of the HPV-16 L1 and L2 capsid proteins leading to capsid formation. The resultant crude PsV stock was then purified by Opti-prep as previously described by Buck et al., (2007). Briefly, the crude PsVs stock was layered onto an Opti-prep gradient and the purified PsV fractions were collected following ultracentrifugation. Opti-prep fractions were quantified in relative light units (RLU) by measuring renilla expression. Fractions with the highest magnitude of renilla expression were pooled together. The viral copy number was quantified from the pooled PsV stock.

Cell and Viral Titer

We developed a HPV-16 cell-based assay in several steps. First, SV40 promoter was used in the plasmid to drive renilla expression in 293 FT cells. We chose SV40 given its high promoter strength of renilla expression in 293 FT cells. Second, an optimal ratio of 400 PsV/cell produced a magnitude of expression at least ten times greater than the control, a number that is considered ideal for a luminescent assay (Buck et al., 2006, Huang, Pyeon, Pearce, Simon,

Griffin, et al., 2012). We also determined that the optimal concentration of cells to be 30,000 cells per well in the 384-well format.

Compound Library

The SMDC has a library of over 150,000 compounds. The screen was conducted in a smaller subset of this library, containing 1906 clinical drugs. The 1906 drugs were compiled by the SMDC and contained primarily FDA- and internationally-approved drugs. The formation of a small subset of drugs for initial screening is a common practice in the drug discovery field. FDA- and internationally-approved drugs help to control for systematic error in the initial assay validation process (Engle, Ang, Chen, Arkin, McKerrow, et al., 2010). The SMDC's library was assembled from commercially available Pharmakon 1600 collection (Micro Source Discovery Systems, Gaylordsville, CT) and supplemented with the addition of approved small molecule drugs sourced directly from vendors (Enzo Life Sciences, Farmingdale, NY; Tocris Bioscience, UK) and smaller historical collections (Iconix FDA drug set).

HPV entry assay

We developed a cell-based assay for identifying drugs to prevent HPV-16 infection. The assay makes use of the efficient production technique for generating PsV established by Buck et al. (2004). Upon exposure to 293 FT cells PsV enter and deliver encapsidated DNA to the nucleus resulting in renilla luminescent reporter gene expression. The renilla luminescence can be assayed quantitatively, providing a measure of the efficiency of the early steps in HPV infection. We used renilla expression to measure PsV infectivity in RLU.

384-well assay plates (Greiner BioOne CellStar cat. no. 781080) were filled with 50 µL of 293 FT cells (30,000 cell/ml) in G418 media (DMEM + 10% FBS + 1% P/S + 1% 50 mg/ml of G418) using WellMate microplate dispenser (Thermo Scientific) and left incubated overnight at 37^{o}C in 5% CO_2 (culture condition). An aliquot of 50nl (10mM stock in DMSO) of each compound was then transferred to 384-well plate with fixed volume pin tool (V&P Scientific) loaded to the BioMek-FXP liquid handling automation workstation (Beckman Coulter). Assay plates were pre-incubated for two additional hours before 10 µl PsV (400 PsV/cell) were added. The positive control (no infection) wells received only 10 µL of growth medium. The final assay volume was 60 µL, with an assay concentration of test compounds of 8.3 µM. After an additional 72 hours of incubation in culture condition, reagents from the DualGlo Reporter Assay System (Promega) were added to the assay plates in accordance with the manufacture's protocol. Renilla luminescence was measured with an Envision multimode microplate reader. A parallel counter assay of cell viability, as determined by the ATP content of living cells, was also carried out using the Cell Titer Glo Assay Kit (Promega). The luminescent signals of test compounds were normalized as percent inhibition relative to both positive (no infection) and negative (infection) controls. Dose-response of active compounds was analyzed using GraphPad Prism 6 software. The IC_{50} was calculated using 4-parameters non-linear regression analysis of the same software.

A 4-parameter nonlinear regression analysis provided a mathematical model to describe the assay's biological process. The 4-parameters include hill slope, log $IC_{50,}$ bottom plateau, and top plateau. This model provided a framework to fit the data, allowing for best-fit values that were used to interpolate the data in context of the model. A non-linear regression analysis was

chosen over a linear-regression analysis because it was more general than linear-regression. Non-linear regression found the best-fit values for the 4-parameters that generated a curve that came closest to describing to the data (GraphPad Software, 2016).

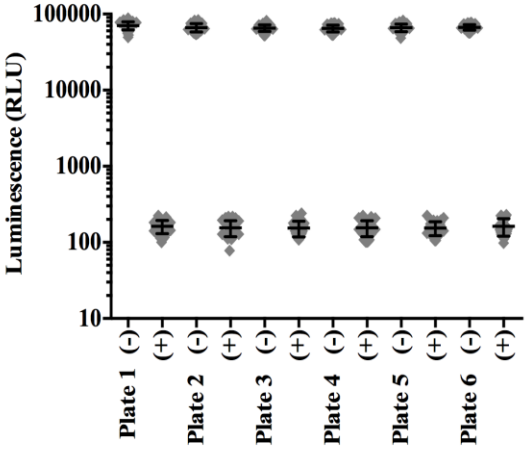

Plate	1	2	3	4	5	6
S/B	435	426	424	416	428	413
Z'	0.63	0.62	0.70	0.68	0.65	0.74

Figure 1: Screen performance of the HPV infection assay. Luminescence signals (gray diamonds) of positive and negative controls in six plates assayed during the screen (n = 32 for each control per plate; with mean and standard deviation bars). Table shows the signal-to-background window (S/B) and assay performance parameter Z' of controls in each assay plate.

This assay was initially optimized using two human cell lines, 293 FT, which are commonly used in HTS, 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 293 FT cells when compared with HaCat cells and therefore these cells were chosen for further use in the validation screening process. Additionally, it was beyond the scope of this screen to validate HTS active compounds in HaCat cells.

Results

HPV-16 cell-based HTS assay

To determine the robustness of this screen, we optimized a 384-well plate format using HPV-16 PsV and 293 FT cells. A diverse collection of 1906 clinical drugs at 10 μM and controls were screened. The assay showed excellent discrimination between active and inactive

compounds with a Z′ (a dimensionless calculation used to assess the quality of a high-throughput assay) of = 0.67 ± 0.05 and a large assay window (signal-to-background = 424 ± 8) (Figure 1).

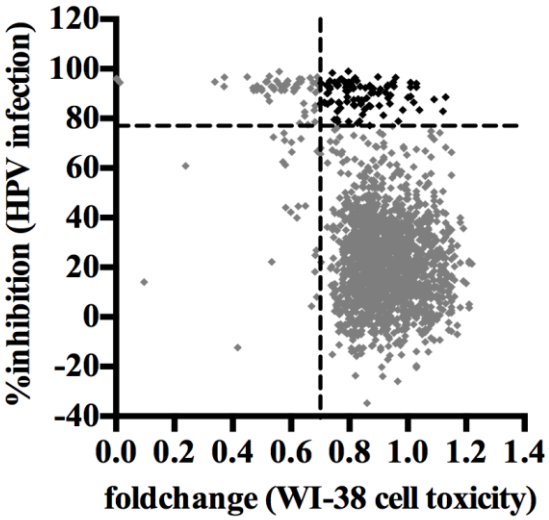

Figure 2: Hit selection for inhibitors of HPV infection. Screening of 1906 clinical drugs and bioactive molecules (gray diamonds). A total of 98 hits (black diamond) were selected based on mean + 2 standard deviation cutoff (≥ 77 % inhibition) in HPV infection assay and viability in 293 FT cells (≥ 0.7 fold-change relative to negative controls).

We identified 152 compounds as 'active', causing statistically significant inhibition, ≥ 77% inhibition (mean + 2 sd of total compounds screened) with 98 exhibiting low cytotoxicity against human WI-38 normal lung cells (Figure 2). After further prioritization on chemical properties of interest (i.e., GABA antagonist, purine antimetabolite, topoisomerase inhibitor), a smaller set of 80 active compounds was selected for reproducibility in dose-response tests. 25 active compounds showed dose-dependent 90%-100% inhibition of HPV infection (Supplemental Data Table 1).

Applying the thresholds of ≥ 77 % HPV inhibition with < 20% cellular cytotoxicity we narrowed the 98 drugs to 25 drugs. The 25 compounds were repurchased to control for quality. The infection assay and cell-viability assay was then repeated with the 25 repurchased compounds to confirm the original findings.

Control Validation

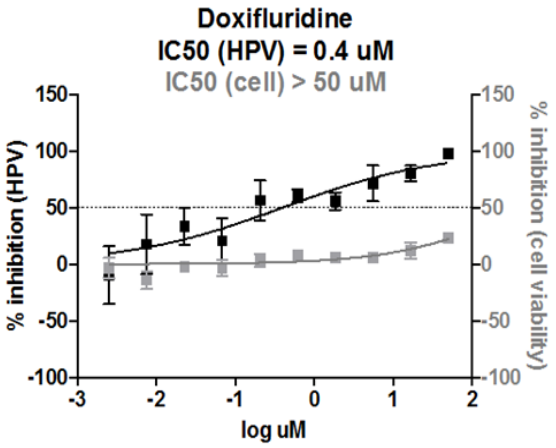

Figure 3: Doxifluridine is one example of several hit drugs from the pilot screen that prevents HPV infection and has negative cell toxicity.

Several compounds are known to prevent HPV infection including carrageenan (Buck, Thompson, Roberts, Muller, Lowry, et al., 2006) and heparin (Giroglou, Florin, Schafer, Streeck, Sapp, 2001). Carrageenan and heparin were added as positive controls, to determine if our HPV-16 cell-based assay was able to detect known drugs able to prevent HPV infection.

Additionally, in the Palefsky lab, we work with sera from donors vaccinated with the quadrivalent HPV vaccine and known to contain high titers of neutralizing antibodies against the HPV 16 L1 protein (Merck and co., 2009). Sera were provided by the National Institute of Health (NIH).

The results of carrageenan, heparin, and sera were consistent in our assay with previous studies demonstrating HPV inhibition (Buck et al, 2006, Huang et al., 2012, Pastrana, Gambhira, Buck, Pang, Thompson, 2005) and consistent with the literature (Buck et al., 2006, Pastrana et al., 2005, Huang et al., 2012). Carrageenan showed 95% inhibition at 10 µM concentration, heparin showed 80% inhibition at 10 µM concentration, and the sera showed 98% inhibition at 1:2560 µl dilution. The compounds also exhibited an excellent cell toxicity profile with little to no cell death. These results suggest the active compounds identified in our screen are inhibiting HPV-16 infection at a concentration that induces little or no cell toxicity.

Currently there are several drugs in the clinical setting used to treat aHSIL, a precursor to anal cancer, including 5-fluorouracil (5-FU). Our assay identified doxifluridine as an active compound that inhibits PsV infection and has negative cell toxicity (Figure 3). Doxifluridine is a fluoropyrimidine derivative and oral prodrug of the antineoplastic agent 5-fluorouracil (5-FU). Doxifluridine showed 79% inhibition at 10 uM assay concentration during the pilot screen. In the follow up dose response study, doxifluridine exhibited more than 125-fold selective inhibition of PsV infection (IC_{50} = 0.4 uM) compared with cell viability (IC_{50} > 50 uM). The identification of doxifluridine represents proof of concept.

Discussion

The HPV-16 cell-based assay described here constitutes a rapid and reliable screening method for inhibitors of HPV-16 infection in mammalian cells. The assay evaluates two of the major criteria for drug candidate identification, namely high efficacy with minimal cell toxicity, and is amenable to evaluating a large number of compounds in a mechanized and consistent manner. The screen was performed in human embryonal kidney cells transformed with the SV40 large T antigen with HPV-16 PsV. In our screening system, compounds of interest be could rapidly identified by their ability to inhibit PsV infection without producing cyctotoxicity. 293 FT cells were selected because they are of human origin, are highly infectable by PsV, grow rapidly, and can endure the mechanical challenges of HTS format.

PsV infection and cell viability were monitored using renilla and ATP, respectively. We screened 1906 drugs and 98 active compounds were found to inhibit HPV infection with low cell

toxicity. The 98 active compounds were retested for dose-response confirmation and 25 drugs met the threshold of ≥ 77 % HPV inhibition with < 20% cellular cytotoxicity. The 25 active compounds displayed abilities to inhibit HPV infection and little to no cyctotoxicity.

Recently, a small number of reports have appeared documenting the use of HTS formats to identify inhibitors of HPV. One study, used HTS to explore different types of biological compounds able to prevent HPV infection and identified carrageenan, a sulfated polysaccharide extracted from red algae (Buck et al. 2006). A second study by Huang et al., (2012), used a cell-based HTS assay to identify two lead compounds. The compounds, identified as #13 and #14 by the authors, showed low to submicromolar inhibitory concentration (IC_{50}) and little to no cytotoxicity. Currently, carrageenan and the other compounds are still in early stages of development and there have not yet been any reports of prospective randomized clinical trials testing its efficacy to prevent anal or cervical HPV infections (Roberts, Buck, Thompson, Rhonda, Bernardo, et al., 2007).

MSM living with HIV remain at an increased risk of developing HPV related aHSIL and anal cancer. The nonavalent HPV vaccination has restrictions as to who can receive the vaccine. This highlights a continued need to identify additional compounds able to prevent HPV infection to provide a full spectrum of preventive interventions to the sexually active population. Ideally, once identified, this compound would have the ability to be combined with a lubricant for anal and vaginal prior to intercourse. A drug that can be applied before intercourse may help prevent HPV infection (Buck et al., 2006; Marais, Gawarecki, Allan, Ahmed, Altin, et al., 2011). The development of an anti-HPV lubricant has particular relevance to the MSM living with HIV population. Since many if not most MSM who engage in anal intercourse use lubricant, this

intervention would allow for decreased risk of HPV exposure, development of aHSIL, and ultimately anal cancer (Pokomandy et al., 2011).

Our next step is to screen a larger sample of the compound library at the SMDC to identify compounds able to prevent HPV infection. Additionally, we plan to investigate the mechanism of actions based on what is known about HPV biology (i.e., cell surface receptors and pathways of entry). The HPV cell-based assay developed in the study provides a valid and reliable platform to use with HTS in drug libraries to potentially help accelerate discovery of inhibitors of HPV infection, ultimately preventing the development of cancer.

Supplemental Table 1: List of 25 active compounds with IC50 showing selective inhibition for HPV infection in comparison to viability in 293FT human embryonal kidney cells)

No.	SMDC ID	Name	HPV infection: IC50 (μM)	293FT cell viability: IC50 (μM)
1	131248	Floxuridine	< 0.007	> 15
2	735569	Ancitabine	< 0.007	5
3	131375	Bleomycin	< 0.007	2
4	255720	Etoposide	0.01	1
5	735623	Amsacrine	0.01	1
6	915004	Clofarabine	0.02	2
7	285708	Cladribine	0.05	4
8	285803	Irinotecan	0.06	3
9	735553	Thiostrepton	0.1	3
10	131546	Trifluridine	0.1	> 15
11	131324	Hycanthone	0.1	6
12	756738	Clofarabine	0.4	2
13	285957	Rubitecan	0.8	> 15
14	285779	Oxaliplatin	1	8
15	130676	Fluorouracil	1	13
16	131779	Trichlormethine	1	9
17	757032	Tolonium Chloride	2	4
18	130032	Ciclopirox	2	8
19	130690	Mechlorethamine	2	> 15
20	130651	Pentamidine	2	9
21	757019	Bronopol	3	7
22	285650	Fluorodeoxyuridine	4	13
23	131711	Doxifluridine	5	16
24	41379	Ondansetron	5	> 15
25	130726	Nortriptyline	6	> 15

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A High-throughput Drug Screen for HPV-16 Identifies Two New Leads

Tara Walhart, Erin Isaacson-Wechsler, Kean-Hooi Ang, Carol Dawson-Rose,

Michelle Arkin, Sharof Tugizov, Joel Palefsky

University of California, San Francisco

Abstract

Anal cancer, caused by human papillomavirus (HPV), is a major cause of cancer in HIV-positive men who have sex with men (MSM) population. HIV-positive MSM are 80-times more likely to develop anal cancer when compared to MSM who are HIV negative. HPV-related cancer can be prevented with the administration of nonavalent vaccine. However, vaccine administration is limited to individuals under age 26 and ideally before their sexual debut. Few preventive options are available to individuals outside the vaccination range. Alternatives to vaccination are needed to prevent HPV infection. Ready access to effective HPV antivirals represents one potentially valuable approach to the prevention of HPV infections. Identification of drugs that efficiently prevent HPV infection has been hindered by lack of robust and appropriate assays for high-throughput screening (HTS). To facilitate discovery of drugs that prevent HPV infection, we developed and validated a high-throughput screening assay. We screened 1906 FDA- and internationally-approved drugs. The top 25 compounds were chosen for further analyses based upon pharmacological properties, strength of inhibitory activity, novel association with HPV antiviral prevention, and lack of cytotoxicity. Of these compounds, the antifungal pentamidine and GABA receptor agonist securinine stood out as drugs best able to prevent HPV infection. Pentamidine and securinine both displayed superior biological reproducibility, >90% inhibition of HPV-16 pseudovirion infection, and little to no cytotoxicity. The use of pentamidine and securinine represents promising therapy for reducing HPV-related infections and cancers for individuals outside the vaccination range or for whom vaccination is not available.

A High-throughput Drug Screen for HPV-16 Identifies Two New Lead Compounds

Introduction

HPV is a small double-stranded DNA non-enveloped virus. At present, well over 100 different HPV types have been described, but only a small proportion of these types have been demonstrated to contribute to the development of human malignancies (Culp and Christensen, 2004). Of these, a subset of high-risk HPV types -16 and -18, cause virtually all cases of cervical cancer and about 95 percent of anal cancers (National Cancer Institute, 2016). Most anal cancers are caused by HPV type 16 (NCI, 2016).

Anal high-grade squamous intraepithelial lesion (aHSIL) can be a precursor to anal cancer. It is estimated that 1 in 10 HIV-positive MSM diagnosed with aHSIL will get anal cancer over their lifetime. Recent surveillance data suggest that HIV-positive incidences of anal cancer are 80 times greater in MSM when compared with the HIV-negative population. The incidence rate of anal cancer in the MSM HIV-positive population is 131/100,000. This number is higher in comparison with the incidence of cervical cancer before widespread pap screening guidelines were in place. Therefore, anal cancer incidence rates among HIV-positive MSM are even higher than the rates of much more common cancers that are routinely screened for such as cervical, colon, and breast cancers (Silverberg, Lau, Achenbach, Althoff, et al., 2015). Additionally, the widespread availability of antiretroviral therapy (ART) beginning in 1996 dramatically decreased the mortality rate of HIV-positive MSM. Although the exact reason for the increase is unclear, it this thought that the prolonged HIV-induced immune suppression of ART therapies improves life expectancies and

therefore increasing the risk of developing anal cancer (Pokomandy, Rouleau, Ghattas, Trottier, Vezina, Cote, et al., 2011). The Centers for Disease Control again ranked HIV as one of the biggest public health issues (Centers for Disease Control and Prevention, 2016). This re-enforces the needs to address the gaps in preventative care for HIV-positive individuals.

The recent availability of nonavalent HPV vaccine has emerged as an important strategy for HPV prevention (Merck 2013; Palefsky et al., 2011). However, there are several limitations to the vaccine including administration up to age 26 years, the need to initiate vaccine administration as early as possible around or before the time of sexual debut, and stigma around increasing adolescent promiscuity (Walhart, 2012). The restriction on HPV vaccine administration creates a gap in preventative care that may include as much as 50% of the adult population between the ages of 26 and 65 years old (United States Census Bureau, 2016). There are limited HPV prevention options for this population. Therefore, it remains worthwhile to investigate other interventions to provide individuals with protection against HPV.

A practical alternative intervention would be to add a drug with HPV inhibitory properties into a personal lubricant (Carballo-Diéguez, Stein, Sáez, Dolezal, Nieves-Rosa et al., 2000). Drug delivery would focus on specific tissue types HPV infects within the body including anal, cervical, and mouth, or throat (NCI, 2016).To accomplish this, researchers require ready access to effective antivirals to facilitate drug discovery. One of the most powerful tools available for drug discovery is high-throughput screening (HTS). HTS allows for rapid identification of novel drugs by using liquid handling automation to evaluate large libraries of compounds for both inhibitory efficacy and cytotoxicity.

The overarching purpose of this article is to present novel drugs able to prevent HPV infection. In the article we describe the validation, screening of a cell-based assay against a library of FDA- and internationally approved drug at the University of California, San Francisco (UCSF) Small Molecule Discovery Center (SMDC), and novel findings. We developed a HPV-16 pseudovirion cell based assay. Our assay utilizes renilla readout to directly monitor pseudovirion (PsV) inhibition and ATP to measure cell viability to assess cytotoxicity (Walhart, Isaacson-Wechsler, Ang, Dawson-Rose, Arkin, et al., 2016). We identified drugs as 'active' if they were able to prevent at least 70% PsV infection and exhibited little to no cytotoxicity. Several prominent classes of drugs were identified including antibiotic, antihypertensive, antidepressant, antiprotozoal drugs, and a γ-amino butyric acid (GABA) antagonist. Active drugs were repurchased for activity dose-response confirmation. Two lead compounds, pentamidine and securinine, were subsequently selected to investigate the mechanisms of action.

Materials and Methods

Cells

293 FT cells are a line of fast-growing highly transfectable clonal isolate derived from human embryonal kidney cells transformed with the SV40 large T antigen (Invitrogen). 293 FT cells were maintained in DMEM containing 10% FBS supplemented with 0.1mM MEM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 2mM l-glutamine (Invitrogen) and 500 ug/ml Geneticin (Invitrogen).

Reagents

Production of Pseudovirions

Pseudovirions (PsV) are HPV capsids expressed in vitro that include the papillomavirus capsid proteins L1 and L2 and when they are co-expressed in mammalian cells, they co-assemble and package heterologous nonviral DNA plasmids into infectious particles that resemble authentic HPV virions. PsV can be engineered to encapsidate a reporter plasmid that expresses the reporter gene upon cell entry (Buck, Pastrana, Lowy, Schiller, 2004, Buck, Thompson, 2007). PsVs have been produced in our lab as previously described by Buck et al. (Buck et al., 2004). Given that HPV-16 is the most common cause of cervical and anal cancer, we chose to synthesize HPV-16 PsVs and use these for our various model systems. Briefly, HPV-16 PsVs were produced in human embryonal kidney 293 FT cells. An expression plasmid encoding the renilla reporter gene under the control of the *pRL-SV40* (Promega) was co-transfected into 293 FT cells, along with a second plasmid p16L1L2 (Addgene), which drives expression of the HPV-16 L1 and L2 capsid proteins, leading to capsid formation. The resultant crude PsV stock was then purified

by Opti-prep as previously described by Buck et al. (Buck et al., 2007). Briefly, the crude PsV stock was layered onto an Opti-prep gradient and the purified PsV

Statistical parameter	**Statistical significance**
Z'	0.67 +- 0.04
%CV	10
s/b	424 +- 8

Table 1: Assay validation parameter. A) Z' is a dimensionless calculation used to assess the robustness of the HPV HTS assay with a statistically significant range of 0.5 to 1, B) % coefficient of variance. The number indicates the percent of variability within the replicates. A %CV of <15% is considered statistically significant, C) The signal to background (S/B) is the ratio of (-) control (cells + PsV without drug) and (+) control (cells only), which indicates the assay window. S/B ratio higher than 1:1 indicates more signal than noise suggesting a high quality of the measurement.

fractions were collected following ultracentrifugation. Opti-prep fractions were quantified in relative light units (RLU) by measuring renilla expression. Fractions with the highest magnitude of renilla expression were pooled together. The PsV copy number was quantified from the pooled PsV stock.

Statistical Significance

Several parameters are used in the HTS to determine statistical significance. The gold standard parameter to assess assay performance is Z'. The Z' constitutes a dimensionless parameter that ranges from 1.0 (infinite separation) <0.0. It is defined as follows: $Z' = 1 - (3\sigma_{c+} + 3\sigma_{c-})/[\mu_{c+} - \mu_{c-}]$, where σ_{c+} , σ_{c-}, μ_{c+}, and μ_{c-} are the standard deviations (σ) and the averages (μ) of the high (c+) and low (c-) control (Zhang, Chung, Oldenburg, 1999). Z' between 0.5 and 1.0 indicates an excellent assay with good separation between the controls. Z' between 0.0 and 0.5 indicated a marginal assay and <0.0 signifies a poor assay with overlap between controls. Our assay has a Z' of 0.67 +- 0.04. IC_{50} curve fitting was carried out using GraphPad Prism 6 software (GraphPad Software, Inc). The overall statistical significance of our HPV HTS cell-based assay is summarized in Table 1.

Viral Assay Titer

We determined the PsV viral titer by performing 2-fold serial dilutions in 293 FT cells. 293 FT cells were seeded onto 384-well plates in at 30,000 cells/well. Each 2-fold serial dilution of PsV was applied in triplicate and allowed to incubate for 72 hours. We selected 420 viral particles per cell (1:1600 dilution) as the viral titer

because it produced a magnitude of expression ten times that of the control (Huang, Pyeon, Pearce, Simon, Griffin, et al., 2012)

High Throughput Screening: Infection and Cytotoxicity

The development of the HPV cell-based assay was previously described by Walhart et al. (Walhart et al., 2016). Briefly, 384-well assay plates (Greiner BioOne CellStar cat. no. 781080) were filled with 50 µL of 293 FT cells (30,000 cell/ml) in G418 media (DMEM + 10% FBS + 1% P/S + 1% 50 mg/ml of G418) using WellMate microplate dispenser (Thermo Scientific) and left incubated overnight at 37oC in 5% CO2 (culture condition). An aliquot of 50µl (10mM stock in DMSO) of each compound was then transferred to 384-well plate with fixed volume pin tool (V&P Scientific) loaded to the BioMek-FXP liquid handling automation workstation (Beckman Coulter). Assay plates were pre-incubated at culture condition for two additional hours before 10 µl PsV (400 PsV/cell) were added. The positive control (no infection) wells received only 10 µL of growth medium. The final assay volume was 60 µL, with an assay concentration of test compound of 8.3 µM. After a further 72 hours of incubation in culture condition, reagents from the DualGlo Reporter Assay System (Promega) were added to the assay plates in accordance with the manufacture's protocol. Renilla luminescence was measured with Envision multimode microplate reader. A parallel counter assay of cell viability, as determined by the ATP content of living cells, was also carried out using the Cell Titer Glo Assay kit (Promega). Luminescent signals from wells of test compounds were normalized as percent inhibition relative to both positive (no infection) and negative (infection) controls. Dose response of active compounds were first tested in singlicate, eight

concentrations in 3-fold serial dilution from 0.007 – 15 µM. Select active compounds were subsequently repurchased from chemical vendors and re-tested in triplicate dose responses, from 0.00152 – 30 µM. Supplemental data Table 1 provides a list of 25 active compounds with IC_{50} showing selective inhibition for HPV infection in comparison with viability in 293FT human embryonal kidney cells.

Naked Transfection

293 FT cells were seeded overnight at 30,000 cells/well in 96-well plates. Cells were incubated at $37^{O}C$. The next day, cells were co-transfected with SV40 plasmid and a carrier plasmid, Lipofectamine2000 was used in accordance with manufacturer's specifications. Each plate contained positive (SV40 + drug) and negative (CP only and cells only) controls. Naked transfection was allowed to incubate at $37^{O}C$ for 6 hours. After 6 hours, 30 uM of drug were applied to each well. Plates were than incubated at $37^{O}C$ for 72 hours. After 72 hours, plates were read using Dual-Glo assay (Promega) and promoter activity was measured in renilla expression.

Compound Library

The SMDC has a library of over 150,000 compounds. The screen was conducted in a smaller subset of this library, containing 1906 clinical drugs. The 1906 drugs were compiled by the SMDC and contained primarily FDA- and internationally-approved drugs. The formation of a small subset of drugs for initial screening is a common practice in the drug discovery field. This is because the mechanism of action, structural scaffolding, as well as, pharmacodynamics and

pharmacokinetic is known in most FDA- and internationally-approved drugs.

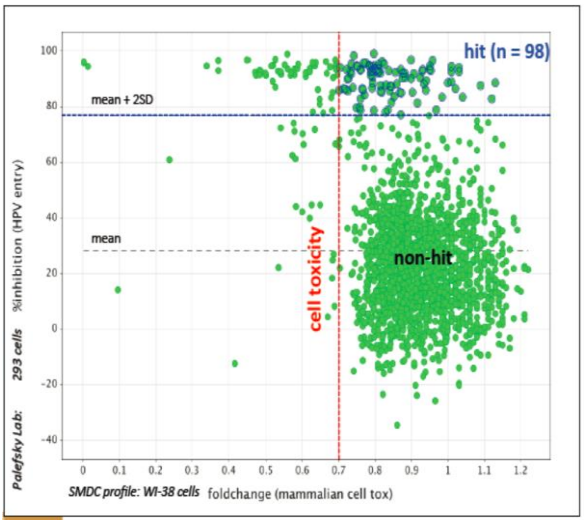


Figure 1: A hit was defined by as any drug that showed > 77% inhibition of renilla expression following PsV entry (mean + 2 standard deviation cutoff) and minimal decrease (> 0.7 fold change) in cell viability in the mammalian primary fibroblast cell line WI-38 toxicity assay provided by the SMDC. The right upper quadrant of the graph represents the hits.

Subsequently, this helps to control for systematic error in the initial validation of the assay (Engle, Ang, Chen, Arkin, McKerrow, et al., 2010). The SMDC's library was assembled from the commercially available Pharmakon 1600 collection (Micro Source Discovery Systems, Gaylordsville, CT) and supplemented with the addition of approved small molecule drugs sourced directly from vendors (Enzo Life Sciences, Farmingdale, NY; Tocris Bioscience, UK) and smaller historical collections (Iconix FDA drug set). The library was dissolved as 10 mM stock in DMSO.

Criteria for Active Compound Selection

In collaboration with the SMDC we identified criteria to determine 'active' compounds in several steps. In the primary screen of 1906 drugs, the initial criterion was based on a dichotomous prevention outcome of at least 70% of infection or not. 152 compounds were selected for a secondary screen to confirm dose-activity response and investigate cytotoxicity. Drugs that demonstrated at least 70% reduction in infection and at least 90% cell viability were selected. 98 drugs met these conditions. For feasibility we then examined the remaining 98 drugs and selected 25 drugs to characterize further.

The selection of these 25 drugs was based on overall performance (inhibit infection by at least 70% and little to no cell toxicity) as well as representing interesting drug classes (antihypertensive, antidepressant, antiemetic). We de-selected known anti-HPV and anti-neoplastic drugs. The drugs were repurchased to control for quality and potency. The drugs were retested in the Palefsky laboratory in which we monitored infectivity in 293 FT cells using HPV-16 PsVs. The criteria for compound selection are summarized in Supplemental Data Table 2.

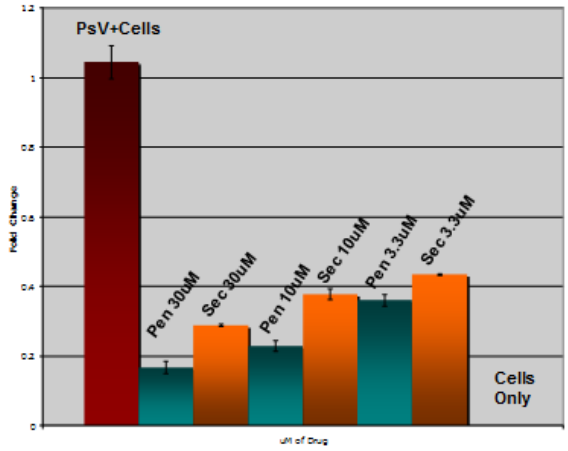


Figure 2: Infection Assay for Pentamidine and Securinine. Pentamidine and Securinine were tested for their ability to prevent HVP infection with 3-fold dose-response activity. The positive control is cells+PsV.

Results

We performed a high-throughput screen of FDA- and internationally-approved drugs to identify novel inhibitors of HPV-16 infection. We screened 1906 drugs in a 384-well plate format. 152 compounds showed ≥ 77% inhibition (mean + 2 sd of total compounds screened) with 98 exhibiting low or no cytotoxicity against human WI-38 normal lung cells (Figure 1). After further prioritization on selection criteria of interest, a smaller set of 98 active compounds were selected for reproducibility test in 10-fold dose-response concentrations. As many as 25 hits showed dose-dependent

inhibition of HPV infection with $IC_{50} \leq 15$ uM with comparatively lower cytotoxicity (see Supplemental Data Table1 and Supplemental Data Figure 6a and 6b).

Dose-response activity confirmation with repurchased drugs in the Palefsky laboratory

To first identify the best candidate drugs, we increased the PsV infection inhibition parameter from 70% to 85% with little to no cell toxicity and we performed both dose response experiments for PsV infection and cell toxicity. Dose-response curves were performed at 3-fold concentrations of each compound from 0.00152 uM to 30 uM in triplicate. Both the efficiency of infection inhibition and cell viability was assayed. Each dose response assay was repeated at least three times and, within each repeat, six replicates of each assay were performed. All 25 repurchased active compound inhibited infection. However, not all of the 25 drugs were able to prevent at least 85% of PsV infection at a concentration associated with an acceptable level of cell viability (see Supplemental Data Table 1 and Supplemental Figure 6a and 6b). We chose to proceed with two lead compounds with the highest level of inhibition and cell viability securinine and pentamidine (Figure 2).

Securinine and Pentamidine

Two lead compounds, securinine and pentamidine stood in terms of displaying biological reproducibility, >90% inhibition of HPV-16 PsV infection, and little to no cytotoxicity (see Supplemental Data Figures 4 and 5). In addition to exceeding selection criteria parameters, securinine and pentamidine met our criteria for novelty

because neither drug is classified as an antiviral or known to have HPV inhibitory properties.

Securinine is the major alkaloid natural product from the root of the plant *securinega suffruticosa*. It was originally discovered over 50 years ago and reported to have potent biological activity (Iaa, Turova, 1956). Though this compound has not been utilized in the United States, it has been used clinically in several other countries particularly China and Russia. In China it is considered one of the 50 fundamental Chinese herbs and is used in Chinese herbal medicine (Duke, 1985). Securinine has been found to be active as a GABA receptor antagonist. Its activity as a GABA antagonist likely explains its reported clinical success in limited studies for the treatment of neurological conditions such as amyotrophic lateral sclerosis (ALS), poliomyelitis and multiple sclerosis (Copperman, Copperman, Marderosian, 1973).

Though securinine has never been previously tested clinically as an anti-cancer agent, there is a single report that demonstrates that it can induce apoptosis at high doses in a leukemic cell line (Dong, Gu, Chou, Kwok, 1999). Securinine has also been reported to induce macrophage activation and was therefore proposed as a potential therapeutic for infectious processes (Lubick, Radke, Jutila, 2007). However its exact mechanism of action is unknown. To date, securinine has not been evaluated as a therapeutic agent for HPV prevention.

Pentamidine

Pentamidine is an antiprotozoal agent. It is an aromatic diamidine, and is known to have activity against the fungus *Pneumocystis carinii*. The mode of action of pentamidine is not fully understood. *In vitro* studies with mammalian tissues and

the protozoan *Crithidia oncopelti* indicate that the drug interferes with nuclear metabolism producing inhibition of the synthesis of DNA, RNA, phospholipids and proteins. The effect of pentamidine on DNA, RNA, phospholipid, and protein synthesis was evaluated in 6C3HED ascites tumor of C3H mice by incorporation *in vitro* of 3H-thymidine, 3H-uridine, 3H-algal protein hydrolysate, and 32P-orthophosphate. Inhibition of all parameters measured was observed, suggesting that pentamidine does not, as has been suggested, selectively inhibit DNA synthesis or act as an antifungal. A reduced incorporation of 32P into charcoal adsorbable acid soluble phosphate was observed, suggesting that pentamidine may impair nucleotide formation (Bornstein, Yarbro, 1970). Little is known about the drug's pharmacokinetics. The medication is also useful in leishmaniasis and in prophylaxis against sleeping sickness caused by Trypanosoma brucei gambiense.

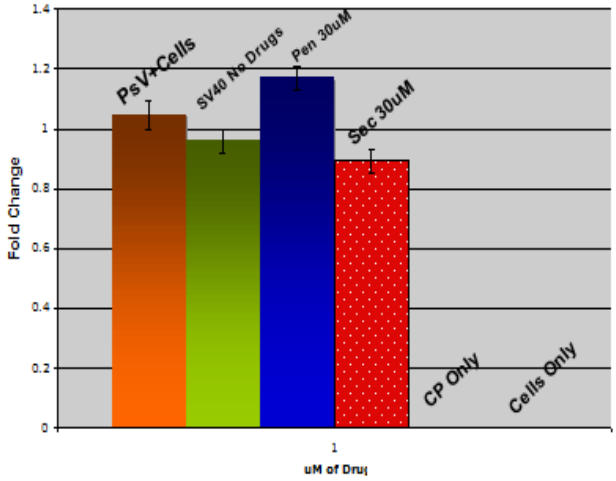


Figure 3: Naked Transfection of Pentamidine and Securinine. When compared to SV40 promoter activity with no drug (positive control) there not a significant decrease in pentamidine and securinine expression. CP = carrier plasmid.

Investigating Mechanism of Action

To begin to learn how pentamidine and securinine act to inhibit HPV infection we performed an experiment to investigate the effects of the drugs on the SV40 promoter. The SV40 promoter enhances and drives expression of the luciferase gene renilla. If the drug(s) works by inhibiting promoter activity this would suggest that the

effect on HPV inhibition is not specific. Therefore, drug(s) that affect promoter activity may have limited therapeutic value.

We performed a transfection of the SV40 promoter in 293 FT cells. Instead of inserting genetic material, such as p16L1L2 (Addgene), into mammalian cells, we inserted the SV40 promoter plus a carrier plasmid to perform a "naked" transfection.

We selected 30uM concentration for each drug. This concentration prevented 90% of HPV infection and showed little to no cell toxicity (see Supplemental Data Figure 4 and 5 for Cell Viability).

We compared SV40+ pentamidine and SV40+ 30uM securinine to the positive control of SV40+no drug (PsV+cells was included as a second positive control). Our results suggest 30uM of Pentamidine and Securinine do not significantly affect SV40 promoter activity (Figure 3). This may suggest Pentamidine and Securinine inhibit HPV infection but another mechanism; however they do not define which mechanism is operative. The drug(s) may act by directly disrupting capsid formation or preventing the binding of PsV to cell surface receptors, therefore disrupting viral entry. Further studies are necessary to define the critical step in infection disrupted by these potential HPV antivirals.

Discussion

Pentamidine and securinine are interesting drug candidates to prevent HPV-16 PsV infection. Pentamindine is classified as antiprotozoal drug. In the clinical setting, it is used to treat *Pneumocystis carinii pneumonia* and other infections. Securinine is a plant-derived alkaloid popular in folk medicine that has been used clinically as a therapeutic for primarily neurological-related diseases. Neither drug has previously

been associated with HPV prevention or treatment regimens. In order to understand how these two drugs might prevent HPV infection, we proceeded to investigate potential mechanisms of action. Our results suggest that both pentamidine and securinine are able to prevent at least 90% of HPV-16 PsV infection at 30uM and show little to no cell toxicity at this concentration. It is possible concentrations of 30uM may cause cell death *in vivo*. However, given that the mechanism of action in preventing pseudovirion infection is not yet known and requires additional investigations.

There are several ways that a drug could potentially prevent HPV infection. It could kill the host cells, disrupt HPV capsid protein coat formation, interfere with cell surface attachment sites, affect gene expression, and interfere with viral entry pathways. Ideally, the best drug(s) candidates will work up-stream to cellular entry by either degrading the virus or destroying the viral capsid. We first chose to investigate if the drug(s) affect promoter activity since promoter inhibition occurs after the PsV enters the cell. For non-enveloped viruses, such as HPV, the protein coat encases and protects viral nucleic acid as well as provides the initial interaction site of the viral particle with the host cell allowing for the assembly of infectious HPV particles in the upper layers of the epithelium. Our results suggest neither 30uM of pentamidine or securinine, in our current *in vitro* model system affect SV40 promoter activity (Figure 3). This would suggest the drugs act on another part of the HVP life cycle or viral entry. Likewise, additional studies are needed to confirm cell toxicity within *in vivo* models.

Further studies are necessary to define the critical step(s) in infection disruption by pentamidine and securinine. Additional experiments to investigate

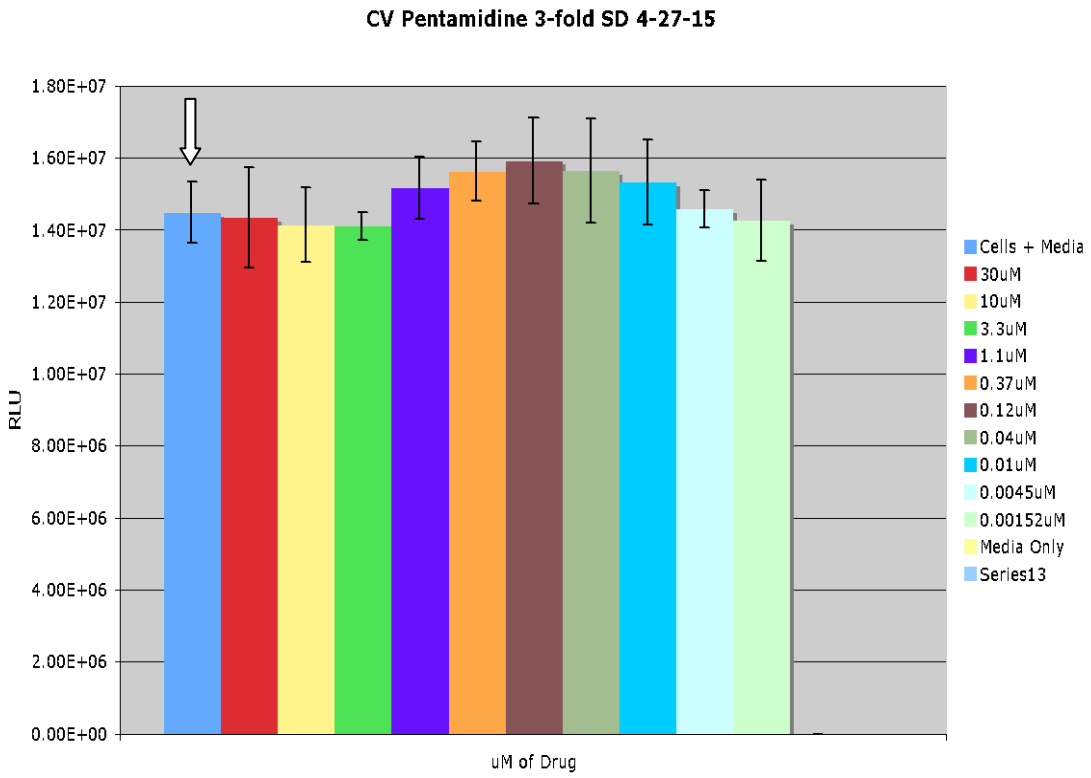
mechanisms of action would include a) degradation of the L1 and L2 proteins leading to disruption of PsV capsid formation, b) the alteration of HPV-16 viral epitopes, c) drug binding to the cells surface and altering the epithelial cell surface receptors, d) alteration to the cellular receptor(s) for HPV-16 therefore preventing attachment of PsVs to the cell surface, and finally, e) the drug(s) may reduce or inhibit HPV entry by modifying endocytosis and macropinocytosis pathways and/or cell recycling mechanisms.

The value of inexpensive and highly efficient drugs to prevent infection cannot be underestimated for virally-induced sexually transmitted diseases (STD) and for which vaccines are not available, too costly, or contraindicated for certain populations. HPV is the most common sexually transmitted infection and recognized as the leading cause for anal and cervical cancer. 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). At this time using HTS for HPV drug discovery is still limited (Buck et al., 2006; Huang et al., 2012). Currently, carrageenan and the other compounds are still in early stages of development and there have not yet been any prospective randomized clinical trials testing their efficacy to prevent anal or cervical HPV infections (Roberts, Buck, Thompson, Rhonda, Bernardo, et al., 2007). However, overall these studies provided evidence that HPV inhibitors of critical steps in the HPV infection process will be an effective means of preventing HPV infection. They also add valuable information for the use of HTS from HPV drug discovery by validating assays and demonstrating proof of concept.

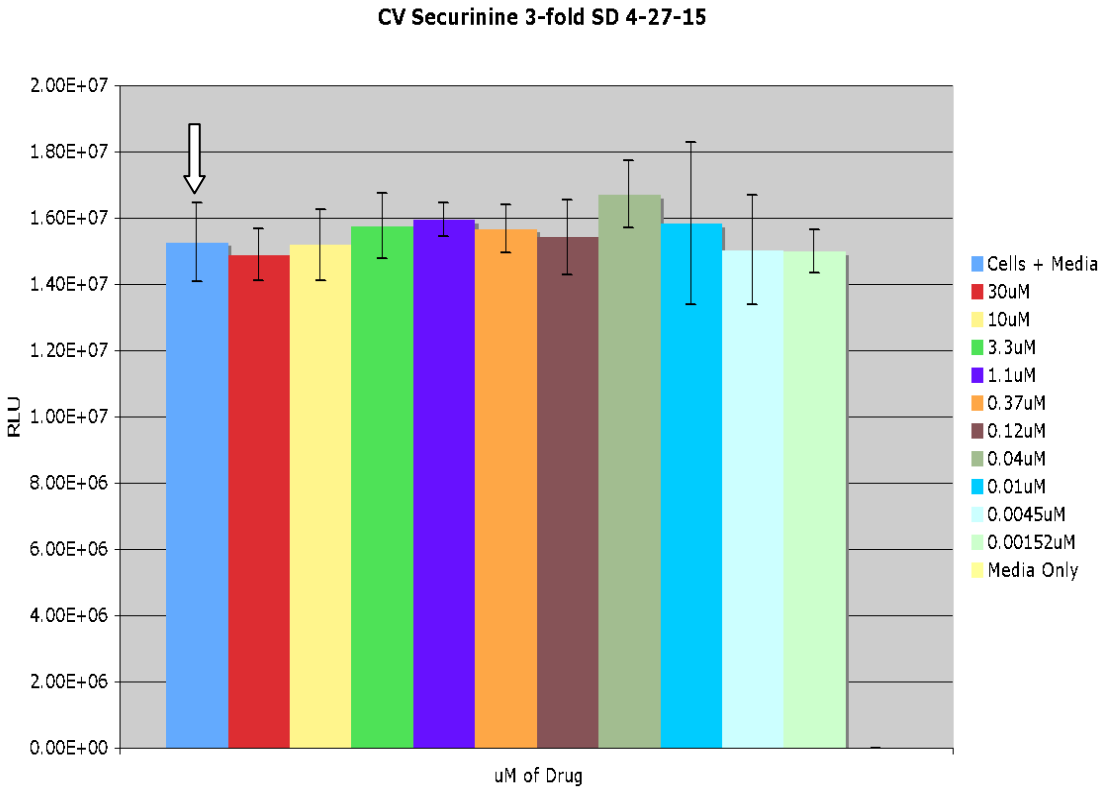
Taken together, our results indicate that HPV cell-based assay for HTS can be deployed as a primary method in the drug discovery pipeline for novel drugs to prevent HPV infection. We have shown that it is feasible to screen a large number of drugs in the HTS format for effectiveness against HPV-16. The repurposing of FDA- and internationally-approved drugs for HPV prevention has the potential to significantly decrease the costs and delivery time associated with bringing a new drug to market. This offers a promising drug-repurposing opportunity for the prevention of HPV-related infections and cancer in sexually active individuals.

Supplemental Data

Supplemental Data Figure 4: Cell Viability for Pentamidine. Cell viability was measure by ATPase. Even at highest concentration of 30uM Pentamidine so little to low cell death. The positive control is cells+media (white arrow).



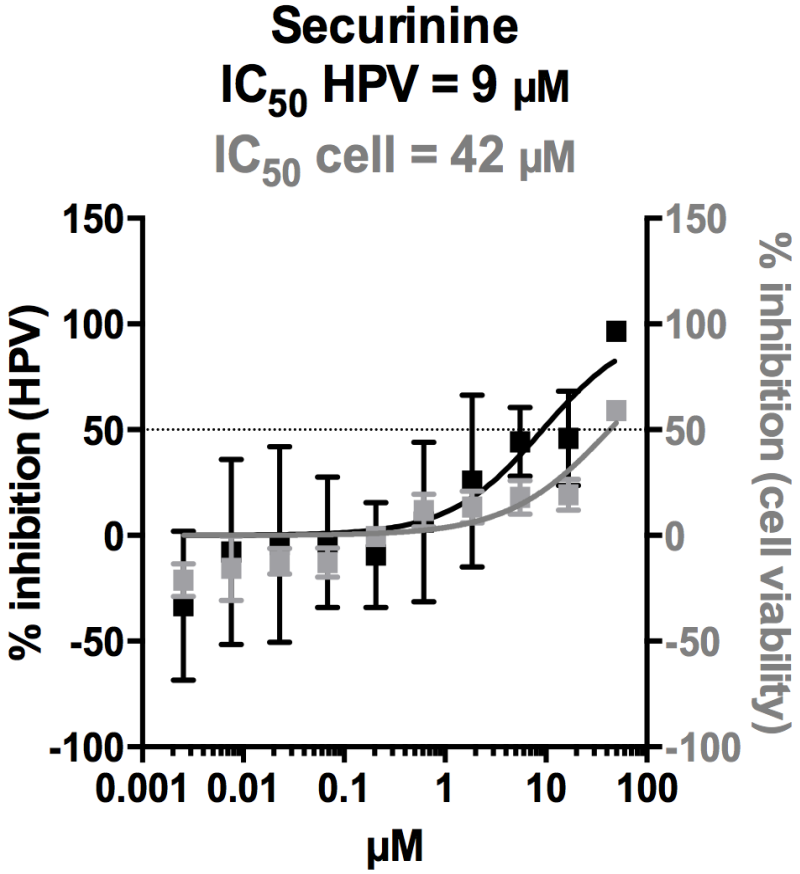
Supplemental Data Figure 5: Cell Viability for Securinine. Cell viability was measure by ATPase. Even at highest concentration of 30uM Pentamidine so little to low cell death. The positive control is cells+media (white arrow).



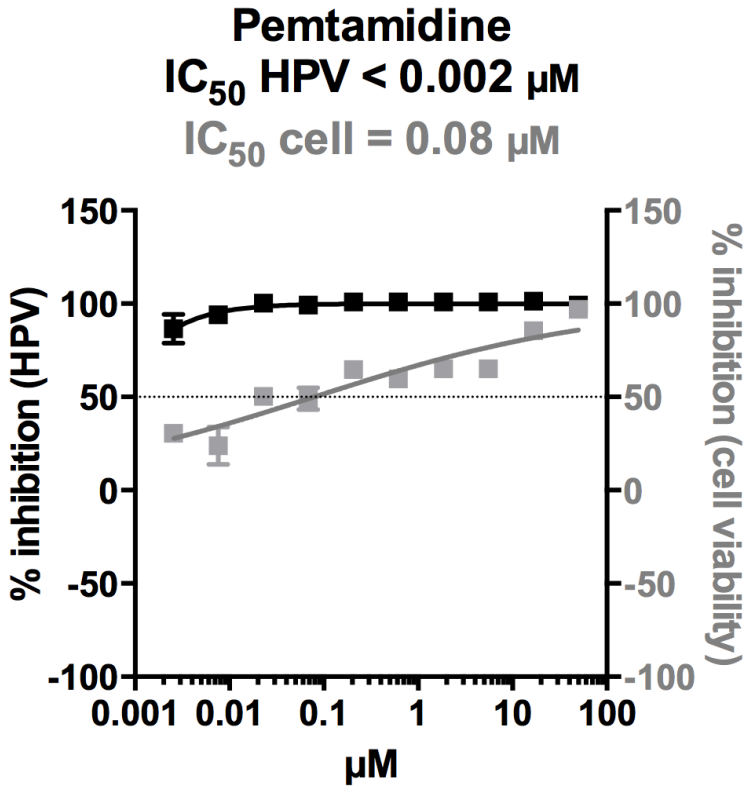
No.	SMDC ID	Name	HPV infection: IC50 (μM)	293FT cell viability: IC50 (μM)
1	131248	Floxuridine	< 0.007	> 15
2	735569	Ancitabine	< 0.007	5
3	131375	Bleomycin	< 0.007	2
4	255720	Etoposide	0.01	1
5	735623	Amsacrine	0.01	1
6	915004	Clofarabine	0.02	2
7	285708	Cladribine	0.05	4
8	285803	Irinotecan	0.06	3
9	735553	Thiostrepton	0.1	3
10	131546	Trifluridine	0.1	> 15
11	131324	Hycanthone	0.1	6
12	756738	Clofarabine	0.4	2
13	285957	Rubitecan	0.8	> 15
14	285779	Oxaliplatin	1	8
15	130676	Fluorouracil	1	13
16	131779	Trichlormethine	1	9
17	757032	Tolonium Chloride	2	4
18	130032	Ciclopirox	2	8
19	130690	Mechlorethamine	2	> 15
20	130651	Pentamidine	2	9
21	757019	Bronopol	3	7
22	285650	Fluorodeoxyuridine	4	13
23	131711	Doxifluridine	5	16
24	41379	Ondansetron	5	> 15
25	130726	Nortriptyline	6	> 15

Supplemental Data Table 1: List of 25 hits with IC_{50} showing selective inhibition for HPV infection in comparison to viability in 293FT human embryonal kidney cells

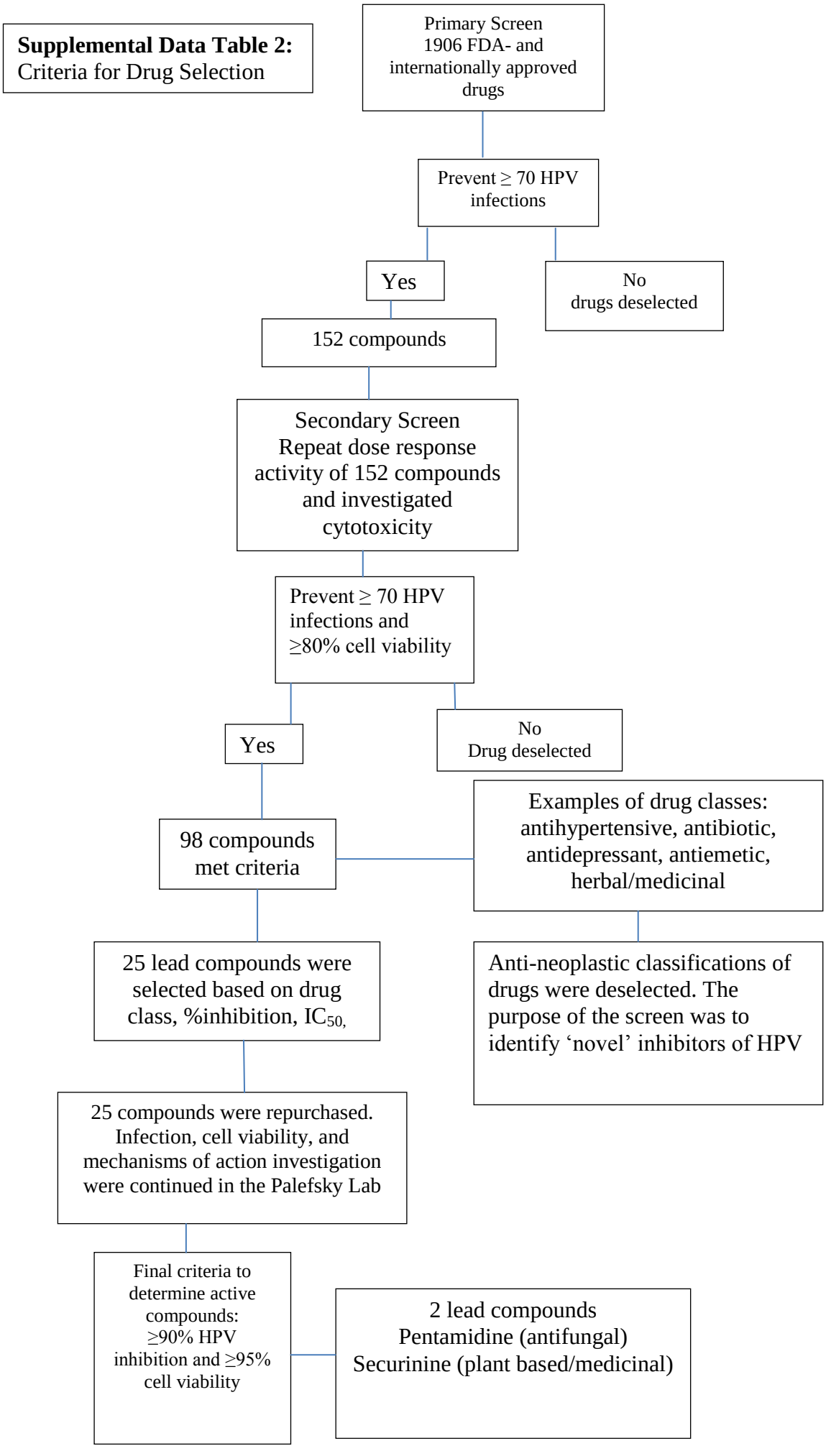
Supplemental Data Figure 6a: Dose-Response Plots for Securinine: Securinine showed 70% inhibition at 10 uM assay concentration during the pilot screen. In the follow up dose response study, securinine exhibited more than 125-fold selective inhibition of PsV infection (IC50 = 0.9uM) compared with cell viability (IC50 > 42 uM).



Supplemental Data Figure 6b: Dose-Response Plots for Pentamidine: Securinine showed >90% inhibition at 10 uM assay concentration during the pilot screen. In the follow up dose response study, securinine exhibited more than 150-fold selective inhibition of PsV infection (IC50 = <0.002uM) compared with cell viability (IC50 =0.08 uM).



Supplemental Data Table 2: Criteria for Drug Selection



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IV. Conclusion / Implications

The purpose of these three papers was: a) to develop a basic science resource for clinical nurse scientists caring for patients with HPV infections and cancer, b) describe the development and validation of a HPV-16 cell based assay to be used with HTS in drug libraries to identify drugs with anti-HPV properties, and c) to present the results from the HTS and begin to identify the mechanism of action of the drugs.

The shared purpose of the three papers that constitute this dissertation is focused on the prevention of HPV at the cellular level. Preventing HPV infections will greatly reduce the risk of developing cancer, especially in the high risk groups such as HIV-positive MSM and those individuals outside the vaccination range. The goal of this work is to identify drugs with the potential to prevent HPV infections. The end goal is to develop the drug into a lubrication to be used before intercourse. The research presented in this dissertation provide proof of concept, feasibility, validation that an HPV-16 cell-based assay is an appropriate screening tool to use with HTS for drug discovery. Although the three papers vary in terms of both context and methodology, they bring together overarching analytics for understanding how this information is important to clinical nurse scientists, the development of a HPV-16 cells based assay suitable for HTS, and understanding the mechanism as to how drug(s) may prevent HPV infection.

Summary of the Research

The goal of this dissertation was to prevent HPV infections and cancers. This research will expand the current body of nursing and basic science knowledge by contributing three manuscripts. The research addressed this goal by conducting a review of the literature,

describing the methods for the development and validation HPV-16 cell based assay, and presenting the results of the HTS and being to investigate the drugs mechanism of action. It is important to point out the prevention of HPV infections and cancers rely on a collaborative approach between clinicians and scientists. This research contributes to this collaboration by being generalizable to those that investigate HPV prevention at the cellular level and those that help to directly prevent infection in patients.

Human Papillomavirus Biology, Pathogenesis, and Potential for Drug Discovery: A Literature Review for HIV Nurse Clinical Scientists

In the first paper, I conducted an extensive review of the literature in the PubMed and CINAHL databases. This study focused on the biology, pathogenesis, and drug discovery for HPV aimed at informing HIV Nurse Clinical Scientists (Walhart, 2015). The goal of this manuscript was to increase the knowledge of clinical nurse scientists to better understand the development of new treatment modalities for HPV in people living with HIV. The results suggest HPV has a complicated life cycle that is dependent on differentiated cells, making it unique among viruses. There are several naturally occurring events in the HPV lifecycle that have the potential to be developed into biological drug targets, including E2, E6/7, and L2/L1 (Buck et al., 2006). The research also presents HTS as relatively new technique being applied to the field of virology (Macarron et al., 2011). A potential reason for this is HTS is dependent on a biological target to identify drugs. The biology underlying of the infectious process must be defined and quantified first. Viruses are complex living organisms that interact with the host cells to establish infection. The process of understanding viral infection is slow and complicated.

HPV is an excellent of example of this point. It has only be recently that studies by Doorbar, investigating the molecular biology of human papillomavirus infection and cervical cancer (2006), Horvath et al., investigating mechanisms of cell entry by human papillomavirus (2010), and Middleton et al., investigating the organization of the human papillomavirus productive cycle during neoplastic progression (2003), and have greatly contributed to our understanding of the HPV lifecycle.

Although this study provided important insight to clinical nurse-scientists, the study has several limitations. HTS is a well-established technique used in pharmaceutical industries for small molecule and drug discovery. HTS is a relatively new method being used in the field of virology for drug discovery, and at this time there are few published articles to validate the process for HPV (Buck et al., 2006 and Huang et al., 2012). The Buck et al., and Huang et al., articles used developed vastly different HPV HTS assay. Although both assays were validated, it makes it hard to directly compare results because different cell types, promoters, HPV types, and compound libraries were screened (Buck et al., 2006 and Huang et al., 2012).

The final limitation on this study is a lack of contributions from the nursing profession (Dole, 2001). The patient care component of a nurse's body of knowledge is incredibly powerful, and nurse-scientists have the potential to develop future research questions guided by clinical insight. Nurse clinical scientists have the capability to integrate basic science with patient-centered care. They should be encouraged to use this unique lens to drive questions based in basic science to formulate questions which can inform future development of new therapeutic methods.

Methods Paper: HPV-16 Cell Based Assay for High-throughput Anti-HPV Drug Discovery

In the second paper, I present the methods used to develop and validate the HPV-16 cell-based assay for the HTS. We collaborated with the SMDC to develop the assay. We conducted a pilot screen of 1906 FDA- and internationally approved drugs. 98 active compounds were selected based on 70 % inhibition HPV infection and little to no cytotoxicity. The assay was validated using the gold standard statistic parameter Z', and secondary parameters %CV and s/b ratio. The results were found to be statistically significant (Z' factor = 0.67 ± 0.05, %CV 10, s/b = 424 ± 8). We developed 'selection criteria' to narrow down the 98 active compounds to a more reasonable number to work with in the Palefsky lab. The selection criteria included % inhibition, cytoxicity, and non- anti-neoplastic drugs. Twenty-five compounds were selected and repurchases for dose-response confirmation studies. The data presented in the Methods paper suggests the HPV-16 cell-based assay we developed was validate and robust to meet the parameters required to run a high-throughput screening.

HTS is an efficient technique to help identify potential therapeutic drugs to prevent HPV infection and cancer in HIV-positive MSM and sexually active people. Our study built on the previous knowledge gained from using HTS from drug discoveries (Buck et al., 2006; Huang et al., 2012). Likewise, HTS screening for drug discovery is becoming an increasing popular method to identify drugs and repurpose them as treatment options (Buck et al., 2006; Huang et al., 2012; Macarron et al., 2011; Plant, Stacey, tiong-Yip, Walsh, Yu, et al.; 2015; Zhang, et al., 2014; Marin, Du, Girou, Kim, Qui, 2015). A number of studies have used HTS assays to identify compounds able to prevent viral infections other than HPV, including filoviruses (Basu,

Li, Mills, Panchai, Cardinale, Butler, et al., 2011) and epstein-barr virus (Thompson, Messick, Schultz, Reichman, and Lieberman, 2010). Additionally, HTS have been used effectively to screen and identify potential antimetastatic and anti-inflammatory agents for cancer treatments (Macarron et al., 2011).

This study too had limitations. This study included only one type of HPV. There are over 200 types of HPV and 13 strains have the ability to cause cancer. We chose to focus on HPV-16 because it is responsible for approximately 90% of all HPV cancers. For example, about 5,010 people are diagnosed with anal cancer each year, and about 91% of anal cancers are thought to be caused by HPV (CDC, 2016). This means about 4,600 diagnoses of anal cancer are caused by HPV-16. The other 8.0% is thought to be caused by HPV-types 31, 33, 45, 52, and 58 (CDC, 2016). This makes a strong case to include other types of HPV in future HTS assay development.

HTS is a key part of the drug discovery process during which thousands of chemical compounds are screened and their activity levels measured in order to identify potential drug candidates. Many technical, procedural or environmental factors can cause systematic measurement error or inequalities in the conditions in which the measurements are taken. Such systematic error has the potential to critically affect the hit selection process (Dragiev, Nadon, Makarenkov, 2011). Systematic error may be caused by various factors, including robotic failures and reader effects, pipette malfunction or other liquid handling anomalies, unintended differences in compound concentrations due to agent evaporation or variation in the incubation time and temperature differences, and lighting or air flow present over the course of the entire screen. Unlike random error that produces measurement noise and usually has minimal impact

on the whole process, systematic error produces measurements that are systematically over- or underestimated, leading to false-positive and false-negative (Dragiev et al., 2011). False-positives and false-negatives are a limitation in all high throughput screening research (Buck et al., 2006; Huang et al., 2012; Macarron et al., 2011; Plant, et al., 2015; 2014; Marin, Du, Girou, Kim, Qui, 2015, Basu, et al., 2011; Thompson, et al., 2010). Although our assay was developed with several systematic controls (i.e., negative control = cells + PsV without drugs, and positive control = cells only) on each plate erroneous hits could not be fully avoided. We addressed this error by repeating dose-response activity and lowering the submicromolar inhibitory concentration and increasing cell viability curves.

Several models in the HTS literature have been presented to control for systematic error including the t-test, Kolmogorov-Smirnov (K-S) and χ 2 goodness-of-fit tests in most of the practical situations (Dragiev et al., 2011, Kevorkov, Makarenkov, 2005). However, special attention should be paid to control the results of aggressive data normalization procedures that could easily do more damage by introducing biases in raw HTS data and, therefore, lead to the selection of many false positive hits even in the situations when the data don't contain any kind of systematic error (Kevorkov et al., 2005).

Results Paper: A High-throughput Drug Screen for HPV-16 Identifies Two New Leads

In the last paper, I reported on the results of the pilot screen and begin investigation into the drugs mechanism of actions. The pilot screen used the HPV-16 cell-based HTS assay described in the methods paper. We screened 1906 FDA- and internationally- approved drugs. A total of 152 compounds showed $\geq$ 77% inhibition with 98 among them exhibited low

cytotoxicity. The 98 hits were retested for reproducibility of dose-response activity. As many as 25 hits showed dose-dependent inhibition of HPV cytotoxicity ranging 90-100% (Supplemental Data Table 1). Additionally, we reviewed the selection window of the remaining 73 drugs. The selection window, or activity window, is an indication of how a drug performs at a specific uM concentration. Although securinine had a narrow selection window, it showed 79.0% inhibition at 2.2 uM assay concentration during the pilot screen. In the follow up dose response study, securinine exhibited more than 125-fold selective inhibition of PsV infection (IC50 = 0.4 uM) compared with cell viability (IC50 > 50 uM). Securinine also peaked out intellectual curiosity because of its use in traditional Chinese and Mexican folk-medicine.

We narrowed the 98 drugs to 25 drugs. The HTS infection assay and cell-viability assay was then repeated with these 25 compounds to confirm the original findings from the pilot screen. Next the 25 compounds were repurchased to control for quality. Dose-response activity, infection, cell viability were all confirmed with the repurchased drugs in the Palefsky lab. Although several of the 25 repurchased compounds looked promising in their ability to prevent HPV-16 infection and not cause cell death, two drugs stood out pentamidine and securinine. Both drugs were able to prevent 90.0% of HPV-16 infection and cause little to no cytotoxicity. Currently, neither drug is associated with anti-viral properties. In fact, pentamindine is classified as an anti-protozoal and anti-fungal and used in the treatment of *Pnuemocystis carinii pneumonia*. Securinine is classified as a GABA agonist and derived from the *Securinega suffruticosa* plant. It has a long history of being used in folk medicine to treat neurological disorders.

We next began our investigation into the drugs mechanism of action. We developed an *in vitro* model system using 293 FT to investigate the effects of the drugs on SV40 promoter. The SV40 promoter enhances and drives expression of the luciferase gene renilla. If the drug(s) affects promoter activity this would suggest the PsV may not be intact and unable initiate the infectious process. Therefore, drug(s) that affect promoter activity may have limited therapeutic value. We performed a 3-fold serial dilution of the two drugs in a range of 30 uM to 0.00152 uM. The range of effective uM concentration vs inhibition and cytotoxicity was similar for the both drugs. For this reason, we selected 30uM concentration for each drug as our normalized dose in order to directly compare the results (see Supplemental Data Figure 4 and 5 for Cell Viability).

We compared SV40 + 30 uM pentamidine and SV40 + 30 uM secuinine to the positive control of SV40+ no drug (PsV + cells was included as a second positive control). Our results suggest 30 uM of pentamidine and securinine do not significantly affect SV40 promoter activity (Figure 3). This may suggest Pentamidine and Securinine inhibit HPV infection but another mechanism; however they do not define which mechanism is inhibited. The drug(s) may act by directly disrupting capsid formation or preventing the binding of PsV to cell surface receptors, therefore disrupting viral entry. Further studies are necessary to define the critical step in infection disrupted by these potential HPV antivirals.

This study has several limitations. SV40 is not the native HPV promoter. We optimized both the SV40 and p97 (native HPV promoter). SV40 was a more robust promoter compatible with HTS. The SV40 promoter was chosen because of its ability to produce a magnitude of expression at least ten times greater than the control (Huang et al., 2012). Confirmatory results

will be tested with p97, HPV's native promoter. A second limitation was screening a known library of FDA- and internationally approved drug libraries. Screening a known library of results might introduce selection "hit" bias, potentially increasing the assays' ability to detect hits from already FDA- and internationally approved drugs versus screening a library of compounds and small molecules.

Several studies have used HTS to identify novel anti-HPV drugs. Buck et al. (2006) identified carrageenan, a type of sulfated polysaccharide extracted from red algae, as an extremely potent infection inhibitor for a broad range of sexually transmitted HPVs. Additionally, Huang et al., (2012), used a cell-based HTS assay to identify two lead compounds. The compounds, identified as #13 and #14 by the authors, showed low to submicromolar inhibitory concentration (IC50) and little to no cytotoxicity. The benefits to identifying additional compounds are to provide a full spectrum of protective and preventive interventions to the sexually active population. Once identified, this compound would have the potential to be combined with a lubricant that could be used before anal or vaginal intercourse (Carballo-Diéguez et al., 2000). The progression of a drug identified by HTS with potential anti-HPV properties to a preventative lubricant requires understanding how the drug(s) work at the cellular level to prevent infection.

Understanding the mechanism of action or how the drugs work is essential to providing safe and accurate care. Both clinicians and basic scientist benefit from understanding how a drug works in vivo to combat disease. These results present proof of concept. Understanding the mechanism of action will require a more elaborate characterization of cell surface attachment and viral entry investigation.

Implication for Nursing Practice

The three studies presented in this dissertation have potential to influence nursing practice in numerous ways. Nurses are the front line in providing patient care. The diverse clinical presentation of HPV infections and cancer require a polygonal approach to patient care. Nursing scholarship is strongly influenced and modified by the ever-increasing body of knowledge drawn from basic science research in the fields of molecular biology and infectious diseases. The knowledge contributed from the review of the literature will inform clinical nurse-scientist about the biology, pathogenesis, and drug discovery potential in the treatment of HPV.

Biologically-based research approaches contribute to this scholarship by expanding the scope for present and future research, education, and practice in nursing. The methods paper addresses these concepts by translating a gap in patient care in the clinical setting (i.e., limited HPV preventative treatments for sexually active people) into a tool, referred to as an assay, that can be used in conjunction with HTS to identify new anti-HPV drugs.

Nursing science addresses the individual from a multidimensional perspective, and the questions they generate from clinical settings are often grounded in basic biology. The results paper identifies two new anti-HPV drug candidates. The investigation into to mechanism of action, that is, ensuring the drugs work how we think they work, is a quintessential core of the nursing practice, patient safety. The ability to combine clinical and translational research enables nurse clinical scientists to directly contribute to the patient's continuum of care.

Discussion

The information learned from this research should be applicable to most if not all HPV types and potentially to prevent HPV infection at other epithelial sites such as the cervix, in both people living with HIV and HIV-negative individuals. This research has the potential to impact both clinical and translational science. Clinically, the therapies available to prevent HPV infection are limited to a vaccine that is contraindicated to most of the population since they are over the age of 26 and past their sexual debut. The development of a personal lubricant would empower sexually active individuals to protect themselves from exposure to cancer causing strains of HPV. The novel cell-base assay will contribute to translation science by providing a validated tool to screen additional compound libraries to identify compounds able to prevent HPV infection. Ideally, the compound(s) identified will use different mechanisms to inhibit HPV infection. This would potentially provide protection from multiple strains of HPV in a single lubricant application to increase their efficacy.

The body of research presented here has the potential to contribute to the existing body of knowledge on drug discovery for HPV in several ways. First by adding to the limited pool of anti-HPV drugs discovered using HTS. Carrageenan has previously been identified as once such compound having anti-HPV properties and was identified by HTS (Buck et al., 2006). It has widespread commercial use as a thickening agent in both the food and cosmetic industries. Carrageenan is thought to be capable of acting as a broad-spectrum topical microbicide (Buck et al., 2006; Marais, 2011). The results from this research support the previous anti-HPV findings. We used carrageenan as a positive control on our HPV-16 cell based assay and our results suggest Carrageenan showed 95% inhibition of HPV infection. However, carrageenan is still in

very early stages of development and there have not yet been any prospective randomized clinical trials to test its efficacy to prevent anal or cervical HPV infection (Roberts, Buck, Thompson, Rhona, Bernardo, Choyke, et al., 2007). Therefore it remains important to identify other compounds with the potential to be developed into HPV preventative interventions.

This research will also contribute to the body of knowledge focused on the molecular techniques to developed HTS assays. Currently, there are limited studies using HTS to identify novel inhibits of HPV infection. One example of a HTS assay was the production of high-titer HPV PsV assay developed to perform HTS in vitro screens to identify compounds to prevent HPV infection (Buck et al., 2006; Roberts, Buck, Thompson, Rhona, Bernardo, Choyke, et al., 2007). This assay used flow cytometric analysis to assess the inhibition of PsV-mediated delivery of a green fluorescent protein reporter plasmid into HeLa cells. A wide variety of compounds were screened using the inhibition assays and they identified carrageenan, a type of sulfated polysaccharide extracted from red algae, as an extremely potent infection inhibitor for a broad range of sexually transmitted HPVs (Buck et al., 2006).

Recently, a HTS assay was used to identify two additional compounds able to inhibit HPV infection (Huang et al., 2012). A cell-based HTS assay was developed using HPV16 PsV expressing a reporter gene. Two lead compounds were chosen for further analyses based upon the structure-activity relationship (SAR) (e.g., relationship between the chemical of a molecule and its biological activity), scaffold diversity, strength of the inhibitory activity and low cytotoxicity. These compounds, identified as #13 and #14 by the authors, showed low to submicromolar inhibitory concentration (IC50) and little to no cytotoxicity. The IC50 is the half maximal inhibitory concentration which is a measure of the effectiveness of a compound in

inhibiting biological or biochemical function. The authors stated this provided evidence that compound #13 and #14 can inhibit multiple genital HPV types (Huang et al., 2012). In general, it is difficult to directly compare these studies since information on assay development, composition of compound libraries, and Z-factor parameters are limited in the literature or not provided.

This body of research will build on these efforts by helping to, 1) inform the development of a cell-based assay appropriate for HTS to identify novel compounds able to prevent HPV infections, 2) increase the knowledge of therapeutic discovery for clinical nurse-scientist caring for HIV- and HPV- positive patients and, 3) inform the investigation into determining the HPV mechanisms of viral entry. The knowledge gained from the research will fuel additional efforts into the development of HPV prophylactic interventions for all sexually active individuals. Finally, translating the research into clinical practice will provide more robust preventative modalities for sexually active individuals to reduce the risk of exposure to HPV-related infections, aHSIL, and anal cancer.

Remaining Questions / Further Research

While these three studies contribute to our understanding of HPV cell based assay development and drug discovery, a number of questions remain.

High-risk HPV-related infections have the potential to cause pre-cancerous lesions in the anal canal that may progress to anal cancer. Although most people will be exposed to HPV in their sexual lifetime, certain high-risk groups including HIV-positive individuals and MSM are at an increased risk for developing HPV-related anal cancer. The current preventative options for

sexually active people are limited. However, through the use of HTS for drug discovery, several compounds have been identified with the potential to inhibit HPV infection. There are remaining questions around the potential for additional drugs discovery in other compound libraries. Therefore, it remains worthwhile to mine additional compound libraries to identify novel compounds able to prevent HPV infections. Once identified the efficacy of the compound has the potential to be combined with a personal lubricant that can be applied before intercourse as an intervention to help protect all people from HPV-related infections and the development of pre-cancerous lesions.

Another important question that remains is how the drugs prevent infection and what their mechanisms of action are. The drugs were not found to inhibit the cell cycle (based on negative cell toxicity results). Thus the drugs likely act by some other mechanism(s) that potentially is post-entry to inhibit HPV infection. It is possible that pentamidine and securinine alter the mechanism by which HPV enters cells causing them to enter through a process that leads to unproductive infections. Further studies are necessary to define the critical steps in the infection disruption by these repurposed drugs.

Although many other research questions remain, one that is particularly related to the research presented here deals with screening additional compound libraries. The field of drug discovery using HTS is rapidly developing into a viable option to identifying drugs that have potential to be repurposed for therapeutic administration. How many other drugs might be identified for the treatment of infections? Drug discovery for HPV and potentially other viruses using HTS is primed and ready for future research. The screening capacity of HTS helps researchers to quickly narrow down 100,000's of drugs into a select few. This allows the

researcher to focus on ascertaining how the drugs prevent infection (or other biological action) by identifying the mechanism of action (i.e., viral entry into the cells or viral attachment onto a cell surface receptor). Taken together the outstanding questions and future research represent a power driving force to seek answers that is enabled by technology of HTS.

The nature of this research easily falls under the scope of drug discovery. However, I would like to expand the scope of this research. My dissertation research has a strong relationship to HPV prevention. My goal is to use a nursing lens to expand the scope of my current work by investigating the symptom of anxiety as it related to HPV infection. I would like to examine a) how the symptom of anxiety relates to HPV diagnostic testing in men and women of diverse populations across the illness trajectory in a variety of health care settings, b) how sexually active men and women outside of the HPV vaccination range feel about therapeutic gels and foams to be inserted into the vagina or anus prior to intercourse to prevent HPV infection and c) would an intervention like this reduce the symptom of anxiety of becoming infected with cancerous strains of HPV.

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