Induction of HPV-16 Late Gene Expression Through
Use of Small Molecule Drugs

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**ABSTRACT**

Cervical cancer is the second most common cancer in women worldwide. The principal cause of cervical cancer is infection with human papillomavirus (HPV). HPV-16 is a high-risk virus and it is responsible for a high portion of all HPV-caused cancers. The HPV-16 genome consists of early and late genes. The virus initially infects basal cells of the cervix epithelium and in these cells early genes are expressed, whilst late genes, L1 and L2, are only expressed in the upper cell layers of the epithelium. Proteins encoded by the late genes are highly immunogenic, thus it is speculated that expression of the late genes earlier in the virus life cycle could lead to clearance of the virus due to interference of the immune system.

The aim of this study was to treat reporter cell lines with three different small molecule drugs to see if they had the ability to induce HPV-16 late gene expression. The reporter cell lines used in this study had been previously created by transfecting HeLa-cells with plasmids representing the HPV-16 genome. In these plasmids, L1 is replaced with a CAT reporter gene that encodes the CAT protein, which can be easily quantified using a sandwich ELISA.

Upon treating the reporter cell lines with TPA, a significant induction of late gene expression was detected. Furthermore, treatment with valproic acid showed some induction of late gene expression. In conclusion, TPA and valproic acid was deemed to have potential to act as a candidate drugs for treatment of HPV infections.

**KEYWORDS**

Human papillomavirus type 16; Cervical cancer; Cell culture; TPA; CAT ELISA
ABBREVIATIONS

BSA  bovine serum albumin
CAT  chloramphenicol acetyltransferase
CIN  cervical intraepithelial neoplasia
DIG  digoxigenin
DMSO dimethyl sulfoxide
DNA  deoxyribonucleic acid
EDTA  Ethylenediamine tetraacetic acid
ELISA enzyme-linked immunosorbent assay
HISL high-grade squamous intraepithelial lesion
hnRNP heterogeneous nuclear ribonucleoprotein
HPV  human papillomavirus
kb  kilobase pairs
LISL low-grade squamous intraepithelial lesion
PBS  Phosphate buffered saline
POD  peroxidase
PTB  polypyrimidine tract binding protein
RA  retinoic acid
RPMI Roswell Park Memorial Institute
SA  splice acceptor
SD  splice donator
SF2/ASF splicing factor 2/alternative splicing factor
TPA  12-O-tetradecanoylphorbol-13-acetate
URR upstream regulatory region
VPA  valproic acid
INTRODUCTION

Next to breast cancer, cervical cancer is the second most common cancer in women worldwide, with 80% of the cases occurring in developing countries [1]. During their lifetime most sexually active women will be infected with HPV, however only a few of these infections will lead to invasive cervical cancer. In fact, most of the infections are cleared within 2 years, which is believed to be a result of intervention of the immune system [2]. On the other hand, if the infection is not cleared it may lead to dysplasia and eventually invasive cancer. Cervical dysplasia, also known as cervical intraepithelial neoplasia (CIN), is classified into 3 grades: CIN1 (mild dysplasia), CIN2 (moderate dysplasia) and CIN3 (severe dysplasia/carcinoma in situ). To detect a pre-cancerous or cancerous process in the cervix, Papanicolaou test, also known as Pap smear, are usually carried out. Cells are collected from the cervix, then stained and examined in microscope. The result of a Pap smear is reported using the Bethesda system where CIN1 is reported as low-grade squamous intraepithelial lesion (LSIL) whilst CIN2 and CIN3 are reported as high-grade squamous intraepithelial lesion (HSIL) [3].

The process of an HPV infection starts with the virus infecting basal cells of the cervical epithelium in the transformation zone. The transformation zone is the site where squamous epithelium and columnar epithelium meet. The virus is believed to reach the basal cells through small wounds or abrasions in the epithelium. In the basal cells, viral gene expression is activated which leads to production of viral proteins. In the initial stages of the viral life cycle only early genes are expressed whilst late gene expression is being suppressed. Normally, uninfected basal cells migrate upwards into the suprabasal cell layers of the epithelium and exit the cell cycle, leading to terminal differentiation. In contrast, when an infected cell replicates, the viral DNA is divided between the two daughter cells. One of the daughter cells leaves the basal cell layer, starts differentiating and migrates in the epithelium.
Instead of exiting the cell cycle an infected cell stays active and the normal terminal differentiation does not occur. These active cells continue to replicate which can lead onto cancer development [4].

Human papillomavirus (HPV) is a DNA virus that belongs to the Papillomaviridae family. HPVs are divided into five genera: α-, β-, γ-, mu- and nu-papillomavirus. The α-papillomaviruses and β-papillomaviruses are the two main genera, with about 90% of the currently characterized HPVs belonging to one of these two groups. The γ-, mu- and nu-papillomaviruses usually cause cutaneous papillomas and warts that do not lead to cancer. The group of α-papillomaviruses contains HPV types that infect genital and mucosal sites. Furthermore, the α-papillomaviruses are divided into low-risk, intermediate and high-risk viruses, depending on the frequency with which they are found in cancers [5]. Infection with a high-risk HPV is the principal cause of cervical cancer, in fact, HPV virus is found in 99.7% of all cervical cancers [6]. HPV type 16 is one of the most common high-risk subtypes known to cause cervical cancer [7].

The HPV genome is approximately 7.9 kilobase pairs (kb) and encodes 8 genes; the early genes E1-E7 and the late genes L1 and L2. There is also a long control region in the genome, referred to as the upstream regulatory region (URR). The early genes are expressed in the early stages of the viral life cycle after the promotor p97 has been activated, immediately after the initial infection. The early genes encode proteins that have regulatory functions, for example proteins that are involved in the replication of the HPV genome and apoptosis control. When an infected cell differentiates and migrates to upper layers of the cervical epithelium the late promoter p670 is activated which leads to induction of the late genes L1 and L2 [8]. L1 encodes for the major viral capsid protein and L2 encodes for the minor viral capsid protein. Capsid proteins form the protein shell of a virus and encapsulate the genetic material, thus creating virions that can shed from the host cell [9]. The L1 and L2 proteins are
very immunogenic, meaning that if they were expressed in the lower part of the epithelium, where the immune system have close access to the virus, the presence of the virus would probably lead to an immune reaction. Thus, it is suggested that the suppression of the L1 and L2 gene expression in the early stages of the virus’s life cycle is a way for the virus to hide from the immune system.

The regulation of HPV gene expression is possible because of different mechanisms that take part in the transcription of DNA; one of the mechanisms is called alternative splicing. First of all, transcription is the process where DNA is copied into mRNA. The mRNA then goes through translation where it gets decoded by a ribosome to create a protein. When the mRNA is first transcribed from the DNA it contains both noncoding regions, introns, and coding regions, exons. This kind of mRNA is also known as pre-mRNA. Splicing is one of the processing events that pre-mRNA undergoes to become stable mRNA. Splicing means that a spliceosome interacts with splice sites on either end of an intron to cleave it off. Splice sites are specific sequences of an intron; there is one splice acceptor (SA) in the beginning of an intron and one splice donator (SD) in the end. Furthermore, alternative splicing means that particular exons can be excluded or included when the splicing occurs, resulting in mRNA with different looking sequences, thus encoding different proteins. In short, the process of alternative splicing means that a single gene can encode for multiple proteins. Through alternative splicing HPV can regulate which genes that should be expressed, meaning that it is possible for the virus to not express the late genes in the basal cells of the cervical epithelium, but later on, start expressing them in differentiated cells in the upper layers of the epithelium [10].

It is speculated that an earlier induction of L1 and L2 late gene expression means that the immune system could detect the virus and potentially clear the body of the infection. To induce late gene expression small molecule drugs could potentially be used. Small molecule
drugs are defined as chemical entities of low molecular weight, usually under 1000 Da\(^1\). The small molecule drugs used in this study were 12-O-tetradecanoylphorbol-13-acetate (TPA), valproic acid (VPA) and retinoic acid (RA).

TPA is a small molecule drug and a diester of phorbol that is often used in biomedical studies because of its ability to act as a tumor promotor. Furthermore, it has been demonstrated that TPA can induce HPV-31 late gene expression [11].

VPA is another small molecule drug, which is often used for treatment of epilepsy. VPA has been proven to increase the splicing factor SF2/ASF [12]. SF2/ASF is involved in pre-mRNA splicing and has been proven necessary for the function of SA3358, one of the most commonly used splice acceptor sites on the HPV-16 genome. The SA3358 splice site is also utilised to produce the mRNAs for the late genes [13].

RA is a vitamin A derivate which has the ability to down regulate hnRNP A2/B1, a protein that is involved in the RNA synthesis and is often up-regulated in many tumour cell lines [14]. Additionally, hnRNP A2/B1 normally have inhibitory interactions with DNA sequences adjacent to important splice sites and it has been shown that knock down of hnRNP A2/B1 can lead to induced expression of HPV-16 L1 mRNA [15]. To sum up, these three small molecule drugs affect different proteins that are involved in the splicing necessary to create the different proteins encoded by the HPV genome.

To be able to study late gene expression of HPV-16, and how addition of small molecule drugs affect the expression, reporter cell lines can be used. Beatrice Orrù at Dublin Institute of Technology established the reporter cell lines used in this study [16]. The cell lines are HeLa-cells, originally derived from cervical cancer, that have been transfected with reporter plasmids. These reporter plasmids are called pBELCAT and pBELMCAT and they both carry the viral early and late genes of HPV-16, except the E6 and E7 genes. The early and late

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\(^1\) Schwab M. Encyclopedia of Cancer. 3\(^{rd}\) ed. Berlin; Springer; 2012.
promoters are replaced with a strong promoter from the human cytomegalovirus. When pBELCAT is transfected into cells it mimics the expression of the HPV-16 genome during infection, meaning that high levels of early genes are expressed whilst expression of late genes is undetectable. pBELMCAT is the mutant version of pBELCAT and the difference between the two is that a splicing silencer in the L1 region have been inactivated, resulting in natural expression of the late genes. Moreover, the CAT gene is inserted in these reporter plasmids to serve as a marker for late gene expression, meaning that when the L1 and L2 genes are expressed the CAT gene will also be expressed. The CAT gene encodes for chloramphenicol acetyltransferase (CAT), a protein that can be easily detected using a CAT ELISA assay.

The CAT ELISA is based on the sandwich ELISA principle. On the surface of the microplates of the ELISA kit there are pre-bound antibodies to CAT (anti-CAT). When a sample that contains the CAT enzyme is added, the enzyme will bind to the anti-CAT. Thereafter, a digoxigenin-labeled antibody to CAT (anti-CAT-DIG) is added which binds to the CAT enzyme. An antibody to digoxigenin conjugated with peroxidase (anti-DIG-POD) is then added which binds to digoxigenin. Finally, a peroxidase substrate is added. The peroxidase enzyme catalyses the cleavage of the substrate yielding a coloured product and the absorbance can be measured using an ELISA plate reader\(^2\).

The aim of this study was to treat the stable cell lines pBELCAT and pBELMCAT with small molecule drugs to determine whether these drugs can induce HPV-16 late gene expression. These drugs could then potentially serve as candidate drugs for treatment of HPV infections.

\(^2\) Instructions for use CAT ELISA, Roche (https://www.lifescience.roche.com/shop/products/cat-elisa)
MATERIALS AND METHODS

Study Material

The cell lines used in this study were established by Beatrice Orrù at Dublin Institute of Technology. The cell lines were created by transfecting HeLa cells with pBELCAT and pBELMCAT reporter plasmids. The constructed plasmids (figure 1) were transfected using GeneJuice® Transfection reagent according to the protocol of the manufacturer3. The cell lines used in this study were pBELMCAT31, pBELCAT47, pBELCAT 51 and pBELCAT67.

No ethical approval was needed since the study was performed on commercially available HeLa cells, which then have been modified by the lab.

Figure 1. Structure of pBELCAT and pBELMCAT reporter plasmids used to transfect HeLa cells and create the stable cell lines pBELCAT and pBELMCAT. The CAT gene to the far right serves as a marker for L1 and L2 gene expression and it encodes for the CAT enzyme that can be easily detected with the CAT ELISA.

Cell Culture of Stable Cell Lines

The cells used in this study were stored in liquid nitrogen tanks and had to be thawed and cultured in flasks before further use. Cryovials containing the cells were taken out of the liquid nitrogen storage and holding a vial in the hand quickly thawed the cells. The liquid from a cryovial was then transferred into a 75 cm² cell culture flask with 10 mL RPMI-1640 complete cell medium (Sigma) containing 10 % foetal bovine calf serum (Sigma), 2 mmol/L L-glutamine (Lonza) and 40 U penicillin/streptomycin (Sigma). The flask was left to incubate at 37°C in 5 % CO₂. The next day, the medium was removed and 10 mL of fresh RPMI medium was added to remove any of the cell toxic dimethyl sulfoxide (DMSO) used in the freezing medium.

Plating Cells in 6-well plates

The cells were cultured until 80-90 % confluent before being trypsinized and plated into 6-well plates. Firstly, the medium was removed from the flask and the cells were washed with 10 mL phosphate buffered saline (PBS). Afterwards, approximately 2-3 mL of 2 % trypsin diluted in 0.02 % EDTA (Sigma) was added and left 5-10 minutes at 37°C. The flask was gently tapped to detach the cells from the surface before adding 10 mL of RPMI complete medium to wash down the cells and transfer them into a universal tube. The tube was centrifuged at 1500 rpm for 5 min to pellet the cells. The supernatant was removed and the pellet was resuspended in 2-5 mL PBS. The cells were counted using a hemocytometer and were then plated at a density of 2 x 10⁵ cells per well in 6-well plates containing 2 mL of RPMI-1640 complete cell culture medium. The cells were left to incubate at 37°C in 5 % CO₂ for 24 hours.
Treatment with Small Molecule Drugs

If cells were to be treated with small molecule drugs, the drugs were added after 24 hours at selected concentrations (table 1). The small molecule drugs used were: TPA (phorbol 12-myristate 13-acetate, Sigma), valproic acid (Sigma) and retinoic acid (Sigma). The selected concentrations were chosen based on previous work done by Orrù\textsuperscript{4} and Nolan\textsuperscript{5}. After adding the drugs the cells were incubated again for 24 hours at 37°C in 5 % CO\textsubscript{2}.

Table 1. Concentration of small molecule drugs used in this study.

<table>
<thead>
<tr>
<th>Small molecule drug</th>
<th>Concentrations</th>
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<tbody>
<tr>
<td>TPA</td>
<td>0 ng/ml, 400 ng/ml, 800 ng/ml</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>0 mM, 0.5 mM, 100 mM</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>0 nM, 1 nM, 100 nM</td>
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CAT ELISA Assay

A CAT ELISA assay kit (Roche) was used to measure CAT levels. The kit contained all reagents needed to perform the assay. The CAT ELISA is a sandwich ELISA using antibodies that binds to the CAT enzyme produced by the cells. The cultured cells were first lysed using 1 mL of lysis buffer and then the cell extract, containing the CAT enzyme, was centrifuged at maximum speed for 10 min at 4°C. Thereafter, 200 µL of cell extract was added to the wells of the microplate coated with anti-CAT. After incubation for 1 hour at 37°C the wells were washed 5 times with washing buffer. Then 200 µL anti-CAT-DIG was added to the wells and again left for incubation 1 hour at 37°C. After washing 5 times with washing buffer, 200 µL anti-DIG-POD was added to each well and left for incubation 1 hour at 37°C. After washing 5

\textsuperscript{4} Orrù, B. Analysis of HPV-16 late gene expression. Thesis [PhD] Dublin, Ireland: Dublin Institute of Technology; 2012
\textsuperscript{5} Nolan, P. The Regulation of HPV Late Gene Expression and the Potential Role of iNKT Cells in Cervical Cancer. Thesis [Master]. Dublin, Ireland: Dublin Institute of Technology; 2015
times with washing buffer, 200 µL peroxidase ABTS substrate was added to each well resulting in a coloured reaction product if CAT enzyme was present. The substrate was left to incubate at room temperature until colour development was sufficient for photometric detection. Finally, the absorbance was measured at 405 nm using a 96-well microplate ELISA reader (Labsystems Multiskan Plus). POD-substrate from the CAT ELISA kit was used as blank.

Data Analysis and Statistics
Microsoft Excel 2011 was used to create line graphs and to perform statistical analysis on the CAT ELISA data. The CAT ELISA results are presented as fold change in line graphs, meaning that the absorbance value for untreated cells were plotted as 1 and the absorbance for treated cells were compared to the untreated cells and plotted thereafter. An unpaired, two-tailed t-test was used to determine significant change in CAT expression between untreated cells and cells that had been treated with small molecule drugs. P-value < 0.05 was deemed significant.

RESULTS
In this study, reporter cell lines pBELCAT and pBELMCAT were treated with an array of small molecule drugs to determine whether or not these drugs could act as potential inducers of HPV-16 late gene expression. To measure the difference in CAT expression, and therefore late gene expression, a CAT ELISA was performed. Tests were performed on a 1:100 dilution of pBELMCAT 31 because of its natural high levels of CAT.
Determination of CAT Expression in Stable Cell Lines

To decide which cell lines to use for drug treatment, an initial test was performed to determine the natural CAT expression in the cell lines. When cell lines are transfected with reporter plasmids different clones of the cell lines may end up with variable ability of expression, thus it was of interest to examine which of the clones that seemed to behave as expected. The cell line clones tested were: pBELMCAT 31, pBELCAT 47, pBELCAT 51 and pBELCAT67. The results showed that all cell lines expressed CAT as expected. The cell line pBELMCAT 31 showed a natural high expression of CAT, whilst all the other cell lines showed a natural low expression of CAT (figure 2).

![Figure 2](image)

**Figure 2.** Natural CAT expression was measured in four stable cell lines. Quantification of the CAT enzyme was performed using a CAT ELISA. CAT unit was defined as absorbance from the CAT ELISA multiplied by the dilution factor of the cell extracts. Logarithmic scale.

Furthermore, to determine which cell line to use for further testing, initial tests also included treating pBELMCAT 31, pBELCAT 47, pBELCAT 51 and pBELCAT67 with 800 ng/mL of TPA. It was detected that pBELMCAT 31 and pBELCAT 67 were the only cell lines to show an increase in CAT expression when treated with TPA (figure 3). Due to these results, the cell
lines pBELMCAT 31 and pBELCAT 67 were chosen for further treatment with TPA, valproic acid and retinoic acid.

**Figure 3.** Four stable cell lines (pBELMCAT 31, pBELCAT 47, pBELCAT 52 and pBELCAT 67) were each treated with 0 ng/mL and 800 ng/mL of TPA. Quantification of the CAT enzyme was performed using a CAT ELISA. Induction of CAT expression is observed in cell lines pBELMCAT 31 and pBELCAT 67.

**TPA**

Treatment of the stable cell lines with TPA gave an induction of CAT expression both in pBELMCAT 31 and pBELCAT 67 (figure 4). Treatment with 400 ng/mL of TPA gave a significant increase of CAT in pBELMCAT 31 (p=0.021) and in pBELCAT 67 (p=0.031). Moreover, treatment with 800 ng/mL of TPA also gave a significant increase of CAT in pBELMCAT 31 (p=0.0034) and in pBELCAT 67 (p=0.010).
Figure 4. Treatment of stable cell lines pBELMCAT 31 (a) and pBELCAT 67 (b) with TPA at 0 ng/mL, 400 ng/mL and 800 ng/mL. Quantification of the CAT enzyme was performed using a CAT ELISA. Data is represented as fold change calculated from the average of the absorbance readings. Bars indicate mean value of the number of individual tests performed (n=3). Error bars indicate standard deviation. P-value ≤ 0.05 was deemed significant.

Valproic Acid

Treatment of the cell line pBELMCAT 31 with valproic acid showed a significant increase of CAT expression when treated with a concentration of 0.5 mM (p=0.0039). A significant decrease of CAT expression was detected when pBELMCAT 31 was treated with valproic acid with a concentration of 100 mM (p<0.001). No significant increase was detected when pBELCAT 67 was treated with 0.5 mM or 100 mM of valproic acid (figure 5).
**Figure 5.** Treatment of stable cell lines pBELMCAT 31 (a) and pBELCAT 67 (b) with valproic acid at 0 mM, 0.5 mM and 100 mM. Quantification of the CAT enzyme was performed using a CAT ELISA. Data is represented as fold change calculated from the average of the absorbance readings. Bars indicate mean value of the number of individual tests performed (n=3). Error bars indicate standard deviation. P-value ≤ 0.05 was deemed significant.

**Retinoic Acid**

Treatment of the stable cell lines pBELCAT 31 and pBELMCAT 67 with retinoic acid at a concentration of 1 nM showed no significant increase of CAT expression, neither did treatment with retinoic acid at a concentration of 100 nM (figure 6).
Figure 6. Treatment of stable cell lines pBELMCAT 31 (a) and pBELCAT 67 (b) with retinoic acid at 0 nM, 1 nM and 100 nM. Quantification of the CAT enzyme was performed using a CAT ELISA. Data is represented as fold change calculated from the average of the absorbance readings. Bars indicate mean value of the number of individual tests performed (n=3). Error bars indicate standard deviation.

DISCUSSION

In the present study, regulation of HPV-16 late gene expression was investigated by treating reporter cell lines, pBELMCAT and pBELCAT, with small molecule drugs. Small molecule drugs that induce late gene expression could have potential to act as candidate drugs for treatment of HPV infection: the principal cause of cervical cancer. The drugs used in this study were selected because of their alleged ability to interfere with splicing factors that are involved in the regulation of HPV-16 late gene expression.

First of all, initial tests were performed to determine which cell line clones to use for further examination. Natural CAT expression was examined in four cell line clones that had previously been created (pBELMCAT 31, pBELCAT 47, pBELCAT 52 and pBELCAT 67) and it was detected that all four cell lines behaved as expected. Cell lines with a transfected pBELCAT plasmid showed a natural low expression of CAT, whilst the cell line with a
transfected pBELMCAT plasmid showed a natural high expression of CAT. Furthermore, these four cell lines were treated with 800 ng/mL of TPA to examine their potential to induce CAT expression upon treatment with a small molecule drug. Cell lines pBELMCAT 31 and pBELCAT 67 were the only two cell lines to show an increase in CAT expression. Thus, pBELMCAT 31 and pBELCAT 67 were chosen for further testing.

Next on, tests were performed where pBELMCAT 31 and pBELCAT 67 were treated with TPA, valproic acid and retinoic to examine whether any of these drugs could cause a significant induction of late gene expression. The first drug examined was TPA. Treatment with TPA gave a significant induction of CAT expression in both the pBELMCAT 31 cell line and the pBELCAT 67 cell line, indicating that TPA could potentially induce HPV-16 late gene expression. A significant induction was seen when 400 ng/mL and 800 ng/mL of TPA was used. These results are in agreement with previous research performed by Orrú et al [16]. Therefore, it can be said that TPA has potential to act as a candidate drug for treatment of HPV infection.

The second drug tested, valproic acid, showed a significant increase of CAT expression only when pBELMCAT 31 was treated with the drug at a concentration of 0.5 mM. When pBELMCAT 31 was treated with valproic acid at a concentration of 100 mM a significant decrease was detected. The cause of this is not fully understood, however, it could be due to toxicity, meaning that high concentrations of the drug kills the cells before it has the ability to produce the CAT enzyme. When pBELCAT 67 was treated with 0.5 mM and 100 mM of valproic acid no significant induction was detected. In fact, the results between the individual experiments varied a lot with both decrease and increase being detected upon treating cells with the drug. It is unclear why these varying results occurred. It might be because of problems with the ELISA assay, for instance, a false increase of induction could be due to insufficient washing between the different antibody applications. Nonetheless, based on the
significant increase of induction detected when pBELMCAT 31 was treated with valproic acid, it could be said that valproic acid has potential to induce HPV-16 late gene expression, thus making it a possible candidate drug for treatment of HPV infection.

Lastly, the affect of retinoic acid was examined. Retinoic acid was tested on cell lines pBELMCAT 31 and pBELCAT 67 at two different concentrations, 1 nM and 100 nM. However, no significant change in CAT expression was detected in either pBELMCAT 31 or pBELCAT 67. Previous research performed by Nolan\textsuperscript{6} showed a significant induction of CAT expression in pBELMCAT 31 when it was treated with retinoic acid at the concentrations used in this study. It is unclear why no induction could be detected in the present work. One explanation could be that the retinoic acid solution used in this study had lost its stability. It has been reported that retinoic acid solutions can be sensitive to light, air and high temperature exposure\textsuperscript{7}. To perhaps receive better results when treating cells with retinoic acid, it might be a good idea to always prepare a fresh batch of solution before any experiment.

Moreover, in Nolan’s study no induction could be seen in the pBELCAT 67 cell line when it was treated with any of the three drugs. Whereas, in this study induction of CAT expression could be seen upon treating pBELCAT 67 with TPA and, even though there is no significant increase or decrease, a variation in expression could still be detected upon treatment with valproic acid and retinoic acid, which indicates that the pBELCAT 67 cell line can still be used for screening of small molecule drugs. In addition, when culturing cells for this study, an aliquot from the original creation of the cell lines was used, therefore indicating that earlier difficulties with tests performed on pBELCAT 67 may have been caused by a problem with that particular aliquot of cells.

\textsuperscript{6} See footnote 5
\textsuperscript{7} all trans-RETINOIC ACID Product Information, Sigma (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/1/r2625pis.pdf)
In the described work, stable cell lines with the integrated reporter plasmids pBELMCAT and pBELCAT were utilized to study HPV-16 late gene expression. Using reporter plasmids is an easy way to study the expression of particular genes of interest for which assays are not available [17]. Often a constitutive promoter is used in reporter plasmids to ensure high level of expression; in the pBELCAT and pBELMCAT plasmids a strong human cytomegalovirus (CMV) promoter was utilized [16]. When working with CMV promoters it can be useful to know that promoters can down-regulate over time [18, 19]. Down-regulation of the promoter would mean that the protein of interest cannot be produced and no detection of expression could be made. When analysing the results in this study, down-regulation of the CMV promoter could be suggested as an explanation for the insignificant change in CAT expression upon treating pBELCAT 67 with valproic acid and retinoic acid. However, it is in this case very unlikely since, just a few weeks earlier, a significant induction could be detected when treating the pBELCAT 67 cell line with TPA. Nevertheless, in the future it could be useful to perform a RT-PCR to see if the mRNAs of the early and late genes are in fact being produced and that there is no problem with the actual cell line.

Furthermore, in the reporter plasmids pBELMCAT and pBELCAT the CAT gene was used as the reporter, meaning that it is the product of the CAT gene that is measured instead of the product of the genes of interest. When creating a reporter cell line, different types of reporter genes can be used and an alternative to CAT is the gene that encodes for green fluorescent protein (GFP). GFP is a protein that was first isolated from the jellyfish Aequorea victoria and exhibits green fluorescent when exposed to UV or blue light. GFP has been proven to be useful as a quantitative reporter protein [20]. Furthermore, secreted placental alkaline phosphatase (SEAP) can also be used as a reporter. The SEAP assay is easy to perform and an advantage with this method is that the SEAP protein can be easily detected in a sample without destroying the cells [21]. Selection of a reporter depends on the type of experiment
that will be performed and what sensitivity of detection that is needed. When creating the stable cell lines used in this study several reporters were tested, however, CAT was chosen for further study because the CAT ELISA assay was believed to be sensitive, reliable and suitable for large scale screening [16].

Based on the results from this study, as well as from previous work, TPA seems to have promising chances to act as a candidate drug for treatment of HPV infection, thus it would be interesting to further investigate the workings of TPA and how it affects HPV-16 late gene expression. For instance, it can be speculated that TPA affects HPV-16 late gene expression by increasing levels of hnRNP A2/B1, a protein that is important for alternative splicing, since it has been shown that TPA can increase levels of hnRNP A2/B1 in leukemia cell lines [22] However the mechanism of action that hnRNP A2/B1 might have on late gene expression is unclear. Furthermore, it is also not clearly defined how TPA affects hnRNP A2/B1. The natural suppression and upregulation of gene expression in the cervix epithelium is controlled by cellular factors, thus it would be interesting to further investigate and define which these normal cellular factors are. This could provide a greater understanding about the regulation of HPV-16 gene expression and perhaps give information that could be used to find other small molecule drugs that could act as potential inducers of late gene expression.

Nowadays, vaccines are used for prevention HPV infections and for the last few years Gardasil and Cervarix are the two vaccines that have been available. Since 2015 there is a third vaccine on the market, Gardasil 9. This vaccine prevents against nine different HPV types (6, 11, 16, 18, 31, 33, 45, 52 and 58), compared to Gardasil that only protects against four HPV types [23]. Furthermore, surgery, radiation therapy and chemotherapy are examples of treatments available for cervical cancer. However, even though there are vaccines against HPV infection and treatment options for cervical cancer, there is no treatment against the
actual infection of the HPV virus. Thus, finding an effective antiviral agent that could be used to treat HPV infections would be a great discovery.

In conclusion, three small molecule drugs were used in this study to evaluate their effect on HPV-16 late gene expression. The results indicate that valproic acid and, in particular, TPA have the ability to induce late gene expression. Therefore, it could be stated that valproic acid and TPA has potential to act as candidate drugs for treatment of HPV infections.

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REFERENCES


