TPA and other small molecules can regulate the late gene expression in Human Papillomavirus (HPV-16)

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ABSTRACT

Cervical cancer is almost exclusively caused by the HPV virus, with HPV 16 and 18 involved in the majority of cases. The HPV virus can be divided into high risk and low risk types, where the high risk types are most associated with cancer. HPV is spread by sexual skin to skin contact, many people get infected without getting cervical cancer. HPV is also involved in the development of several other types of cancers such as oral and other genital cancers. The HPV virus infects epithelium stem cells and disrupts basic functions of the cells. A high expression of the late genes early in an infection may result in that an HPV 16 infection dies out.

The late gene expression was analysed by using a CAT ELISA method, in the cell lines used one of the late genes had been replaced by a CAT reporter gene. Several small molecules where investigated, to study the regulation of the late gene expression.

The results of the study was that a regulation of the late gene expression could be seen when pBELMCAT was treated with TPA, TA and RA where TPA gave the highest increase in the late gene expression. TA/RA combined with TPA increased the expression even more.

As a conclusion it seems possible for small molecules to be used in treatments for cervical cancer that is caused by HPV 16, to upregulate the late gene expression and maybe be able to eliminate the infection before serious damage and disease can develop.

KEYWORDS

BELMCAT, CAT assay (ELISA), Cell culture, Cervical cancer, Transfection
INTRODUCTION

The Human Papillomavirus is the most common known cause of cervical cancer. Around 99.7% of all cervical cancers are caused by this virus. There are over 100 known sub-types of the HPV virus. Of the 100 different sub-types, about 30 can cause infections in human genitals and of those, at least 13 have been discovered to be of a high risk, e.g. have the ability to develop into cancer due to an infection. External factors can increase the risk for the HPV infection to later develop into cancer. These factors are for example smoking, high number of pregnancies, use of birth control pills for an extended period of time or having a reduced immune system, for example people suffering due to an HIV infection. To get an HPV infection in the first place, having many sexual partners and young age are also risk factors.

In many cases, an HPV infection does not cause any symptoms or other problems, this is in regard to both high and low risk forms of the virus. The body can often get rid of the virus on its own, in some cases the infection might not even had been noticed in the first place. Sometimes cell abnormalities occur but the body is still able to get rid of the infection and the cells then return to normal. In an infection caused by high risk types of HPV that go on for several years, abnormalities may become more serious and without treatment they can also develop into cancer. In other cases the virus infection may cause warts, especially HPV 6/11 are highly associated with the development of warts around genital areas. These warts are not often the cause of cervical cancer, meaning that the type of viruses that causes the warts are low risk forms of HPV.

HPV type 16 is one of the forms that are highly related to many cases of cervical cancer caused by the HPV virus, this makes it a high risk form. Together with HPV 18 it causes up to 70% of the total number of cervical cancer cases. HPV 16 and 18 are also major contributing factors for other types of genital cancers such as anal and vaginal cancer were the virus also is responsible for around 70% of the cancer cases. These high risk types of the virus have also been found to play a significant role for the development of vulvar and penile cancer were it can be accounted for around 30-40% of the cancer cases [1]. HPV 16 is not only the cause of genital cancer but has also discovered playing a significant role in the development of oropharyngeal cancer, which is increasing. For other cancers as non-melanoma skin cancer

2 http://www.webmd.com/sexual-conditions/hpv-genital-warts/cervical-cancer-hpv-what-women-girls-should-know?page=1
3 http://www.who.int/mediacentre/factsheets/fs380/en/ 03/15
4 http://www.nlm.nih.gov/medlineplus/cervicalcancer.html 03/15
and cancer on the conjunctiva, the HPV virus can play a small role in the development in some cases [1]. This means that some types of HPV such as HPV 16 causes infections that can attach to the body’s mucus membranes, which means moist surfaces that works as a barrier to separate the body and its organs from the external side such as genital and oral parts of the body 

HPV is a sexually transmitted virus that can be transferred between humans through direct skin contact during oral, vaginal and anal sexual activities were penetrative sex is not required to cause an infection. The virus infects the basal cell layer of epithelial cells. It is possible that the infection even depends on that the virus infects epithelial stem cells [1]. Once the virus has infected the cells, it produces proteins that can disrupted the cells basic functions, the cell for example starts avoiding apoptosis and the differentiation of the cells get thrown in disarray. If the immune system isn’t able to stop the infection, the infected cells grow and mutate, the cells can after some time become tumorous and cancer can develop 

Cervical cancer is one of the more common forms of cancer that affects women. The cancer develops when cells in the cervix, the lower part of the uterus, start to grow in an uncontrolled manner. Symptoms of cervical cancer can be seen but they are generally not present in early stages of the disease. A few examples of symptoms are unexplained bleeding from vagina, pains in the lower stomach area and pain during sexual intercourse.

Like several types of cancer, it is important to detect cervical cancer at an early stage to be able to give successful treatment and increase chance of survival. Progression of cervical cancers can be divided into four stages, 1-4, where in stage 1 the survival is 80-99 % after 5 years and in stage 4 only 20 % have survived after the same amount of time.

Because of that this type of cancer takes long time to develop after infection and that it is mainly caused from a sexually transmitted HPV infection, women are rarely affected at young age. The long development from infection to cancer also gives time to find abnormalities before cancer is even developed by regular screenings. This has contributed in fewer cases, since treatment can focus on preventing cancer to develop. In screening pre-cancer lesions that are both non-invasive and not yet malign can be found, these are also called CINs (Cervical Intraepithelial Neoplasia’s). Depending on how developed and how severe the dysplasia of the CINs are there are 3 different stages where CINs stage 1 are the mildest and

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CINs stage 3 are the most severe or even carcinoma in situ\(^1\). Recently a vaccine against the two most high-risk forms of HPV (16 and 18) has been developed to decrease the risk for cervical cancer.

There are two types of cervical cancer, squamous cell cancer and adenocarcinoma, the difference between the two is the type of cells in the cervix that becomes cancerous. The more common of the two are squamous cell cancer (70-80 %), squamous cells are cells that cover the outer regions of the cervix. Adenocarcinoma is caused by cells called adenomatous cells and they are the cells that produce mucus. These cells are gland cells that can be found in the endocervical channel which is a passageway between the cervix and the womb\(^9\).

The HPV 16 genome has a late gene expression of L1 and L2 that are viral capsid genes. Where L1 is the major structural protein of the HPV-16 capsid, L1 has an ability to form virus like particles also called VLPs. L1 is also the most common antigen that is used in development of prophylactic vaccines [2]. Both the L1 and L2 genes are transcribed from a late promotor, that are located in the late region of the HPV genome therefore they are called late genes. By inhibition of L1 and L2 gene expression after an infection, in the early stages of the virus life cycle, the endurance of an infection may change\(^1\). The late gene expression for HPV 16 is only expressed in differentiating cells where the expression of L1 and L2 in the early layers of the epithelium when infected are inhibited. It is suspected that it may be a way for the virus to prevent being detected by the infected individual’s immune system [3]. This means higher expression of these genes in the early layers of infected cells could lead to that an infection dies out. The L1 and L2 genes express highly immunogenic proteins.

Late gene expression in HPV 16 can be regulated, and it has been shown that different small molecules such as TPA (12-O-Tetradecanoylphorbol-13-Aacetate) can increase the expression in the generated cervical cancer cell lines\(^1\). It is known since earlier that retinoids, which is related to or are different forms of vitamin A, can regulate both cell growth and cell differentiation. For example it regulates the normal differentiation pathway for epithelium cells [4]. Studies also show that under the right circumstances and combined with other inhibitors RA (Retinoic Acid) can help inhibit HeLa cell growth, increase apoptosis and effect protein expressions [5]. TA (Tannic Acid) has earlier known properties that include antitumor activities, antibacterial and antioxidant qualities, together with a chemotherapeutic agent TA has shown to have an inhibitory effect on HeLa cells [6].

In the project cell lines where the L1 gene has been replaced by a CAT (Chloramphenicol Acetyltransferase) reporter gene is used, it has earlier been shown that reporter genes such as CAT gave a good reflection of the expression of the L1 gene from the used plasmids [3]. Two different cell lines that each has a sub genomic HPV-16 plasmid (pBEL or pBELM) had earlier been generated and was used in the project. The late gene expression was possible to detect by use of a CAT ELISA\(^1\).

The purpose with the project was to see which molecule had the largest effect and if the expression may increase if combined with other small molecules. The molecules that were interesting to test the effects on the late gene expression were TPA, VPA (Valproic Acid), TA, and RA. It was done to find the best combination to get an optimal amount of molecule to treat different cervical cancer cell lines to get the highest late gene expression. Using the cell lines generated in the earlier study could therefore help to determine small molecules different effect and regulation on the late gene expression for HPV 16 [3].
MATERIAL AND METHODS

Cell Culture

The medium in the full flask (cell confluence about 80 %), was removed and the cells were washed with 10 ml PBS, after swirling it around the flask it was removed. One to three ml trypsin was added and swirled around, the flask was incubated in 37°C with 5 % CO₂ until the cells had detached themselves from the bottom of the flask, approximately 5-10 min. To be sure the trypsinization was complete the cells was studied under a light microscope were their movability was viewed. In cases where the trypsinization wasn’t complete the flask was put back in the incubator and/or the flask was tapped on the side to get them to detach. After the trypsinization was completed the trypsin was deactivated by 10 ml complete medium (RPMI (SIGMA-ALDRICH Life Science, RPMI 1640 MEDIUM, RNBD4664) with 10 % Fetal Bovine Calf Serum, 1% L-Glutamine and 1% Penicillin-streptomycin) being added. Two ml cell suspension from the flask was transferred to both of the new flasks (SARSTEDT TC Flask T75, 4024901) that had previously been marked. Ten ml medium was then added to each of the new flasks. The flasks were then incubated and let to grow to confluency. When the flasks were full again they were once again split until used in further experiments.

When experiments were done were a specific amount of cells were needed the cells were also counted in a hemocytometer to determine the concentration of cells in the flask.

The cell-lines used were cervical cancer cell lines pBELCAT-67, pBELMCAT-31 and C 33 a. The pBELCAT and pBELMCAT was HPV induced and had been generated from HeLa cells, generated cells had been frozen down for use in scientifically approved experiments. No other human material was used in the project.

CAT-ELISA Assay

Preparation of plates and insert of small molecules

The cell-lines pBELMCAT-31 and pBELMCAT-67 was used in this part of the project. The amount of cells in each well that was plated were 6·10⁵. After counting the cells for both cell-lines a calculation for the volume to put into each well of a 6 well plate was made. The calculated volume of cell suspension and 2 ml of complete medium was added to the wells. The plates were then incubated for 24 h in 37°C with 5 % of CO₂.
The next day the small molecule TPA (1 mg/ml) was inserted in duplicates of different concentrations to the plates with the 2 cell-lines. The medium was replaced by 2 ml of new complete medium with different concentrations of TPA. Two wells on each plate were inserted without any TPA, 2 wells with 400 ng/ml and the last 2 wells with the concentration 800 ng/ml. The plates were then once again incubated in 37°C with 5 % of CO₂ for another 24 h. The CAT expression and how the molecule influenced the expression were then analysed with a CAT-ELISA assay. After the first CAT ELISA analysis pBELMCAT-31 extracts that had been frozen down was diluted 1:10 and 1:100 before a new CAT ELISA was done in duplicates. Because of promising results from the second CAT ELISA a new assay was made for pBELMCAT-31 with a dilution of 1:100, this time the assay was done in triplicates.

The same procedure was made for VPA where the concentration that was provided was 300 mM. Going from the results from the earlier studies you could expect an increase of CAT expression around 0.5 mM VPA for pBELMCAT-31\(^1\). The VPA was diluted into the concentrations 0.1, 0.25, 0.5 and 0.75 mM, and induced to a 6 well plate were 6·10\(^5\) pBELMCAT-31 cells had been plated the day before. A well with 800 ng/ml TPA and one without drugs were also plated. The plate was incubated in 37°C with 5 % CO₂ over night before a CAT ELISA was performed. A second ELISA was performed with a wider range in concentrations (0.1, 0.5, 1 and 5 mM) of VPA.

Other small molecules that were analysed were TA and RA were 2.9 mM TA and 100 mM RA was provided. A 6 well plate was plated for both drugs respectively with 6·10\(^5\) pBELMCAT-31 cells. The next day after the plates had been incubated in 37°C with 5 % CO₂ the drugs were diluted to the concentrations of 0.1, 1, 10, 50 and 100 µM. The drugs were then induced to 6 well plates, a well without any drug and one well with 800 ng/ml TPA were included. The plates was incubated over night before the CAT expression was analysed using the CAT ELISA method. Both molecules showed promising results and therefore triplicates of the concentrations 0.1 to 50 µM was made in a new assay.

Going from earlier assays the small molecules with promising results was combined. TPA was combined with TA and RA respectively. Four hundred ng/ml TPA was combined with either 10 µM RA or 1 µM TA. The preparation of the plates with plating of cells and induction of the small molecules was performed as in earlier experiments. Both TA and RA combined with TPA were then done in triplicates after showing promising results. With the assay a well without any drugs and a well with 400 ng/ml TPA were included.
**Assay and detection**

For the Assay a CAT-ELISA kit was used (Roche, 10475800). The components of the kit was prepared and stored as instructed from the company. The method used was for cell extracts that was prepared after the instructions for adherent cells.

The medium was removed from the wells before they were washed 3 times with 5 ml of chilled PBS. After removing the PBS 1 ml of Lysis buffer was added to each well and the plate was then incubated in room temperature for 30 min. The Cell extract was then centrifuged for 10 min in a microfuge on a speed of 10 000 g at 4°C. The cell extracts that was not immediately used for the CAT ELISA was stored in a -80°C freezer. Two hundred µl of cell extract from each well of the 6 well plates were put into wells of a coated microplate. The microplate was covered and incubated in 37°C for 1 h. The solution was removed before the wells were washed 5x250 µl with a Washing buffer for 30 sec per wash. After the last wash 200 µl Anti-CAT-DIG (Chloramphenicol Acetyltransferase-Digoxigenin) working dilution was added to the wells. The microplate was then once again covered and incubated for 1 h at 37°C. The solution was removed and the washing of the plate was done as previously. Two hundred µl of Anti-DIG-POD (Anti-Digoxigenin-Peroxidase) working dilution was then added to the wells and the plate was covered and incubated again for 1 h at 37°C. The solution was removed and the wells were washed as before. When the wells had been washed POD-substrate was added into the wells. The plate was then incubated in room temperature until a change to a green/blue colour appeared (approximately 30 min). Detection of CAT expression was then done by measuring the absorbance in an ELISA reader (Labsystems Multiskan Plus) for 96-well plates at 450 nm.

In the case of a too high or too low CAT expression for the detection the extracts could be diluted or an enhancer could be added to the substrate to increase the expression.

**Data analysis**

The results from the CAT ELISA assays were after detection statistically evaluated. All raw data from the triplicates analysis for each drug was used to determine a mean value for every concentration and drug. Error bars depending on the range of the results were made based on the standard deviation, the error bars were calculated and shown in each staple of the respective graph. A T-test was also performed to determine if the results were statistically significant (P<0.05) in relation to untreated cells for every ELISA experiment.
Plasmid Isolation for transfection in C 33

Transformation of E.coli BL21 and isolation of plasmid DNA

250 ml LB (Lysogeny Broth), 2x250 ml LB agar and 200 ml of 0.1 M CaCl₂ was prepared and autoclaved. After autoclaved the agar was placed in a 50°C water bath to cool down. To one of the flasks of agar 250 µl Ampicillin (100 mg/ml) was added to get the concentration 100 µg/ml. The flask was then rolled when the temperature on the agar had decreased to 50°C. Five plates of both LB agar and LB agar with Ampicillin was poured and left to set.

One colony of BL21 was taken from the fridge and inoculated in 1 ml LB broth to be incubated in 37°C while being shaken under approximately 7 h. On a plate of LB agar the cell suspension with BL21 was streaked out with a sterilized loop to be grown in a 37°C incubator overnight. One colony was taken from the LB agar plate and suspended in 3 ml LB broth to be incubated overnight in 37°C. One ml of the cell suspension was then diluted with 25 ml of LB broth. The diluted suspension was let to grow to mid log (Absorbance approximately 0.45 (between 0.2 and 0.6) at 600 nm) the absorbance was analysed using a spectrophotometer (biochrom Libra S22). The suspension was in ice water for 15 min before it were centrifuged down to a pellet for 10 min at 3000 rpm. The supernatant was discarded and 10 ml of cold 0.1 M CaCl₂ was added, it was then mixed until the pellet had been resuspended. The solution was centrifuged down to a pellet and resuspended as before. After centrifuged to a pellet a third time it was resuspended in 500 µl CaCl₂. The solution was incubated on ice for 1 h before 100 µl of the cell suspension was added to an eppendorf tube with 1 µl plasmid (1ng). A negative control was also made were no plasmid was added to the tube only 100 µl cell suspension. After the plasmid was added it was once again incubated for 1 h on ice. The solution was then put in a water bath at 42°C for a heat chock for 2 min. One ml LB broth was added and the solution was then incubated at 37°C for 1 h. Cells were then plated and spread on LB agar with Ampicillin. The plate was incubated overnight at 37° to let them grow.

To isolate the plasmid DNA a colony of the growth on the LB agar with ampicillin plate was picked and suspended in 3 ml LB broth with 3 µl ampicillin to create a starter culture. The starter culture was then incubated at 37°C under shaking overnight. The next day 2x1 ml of the starter culture was suspended in 2 bottles with 100 ml of LB broth containing 100 µg/ml of ampicillin and then the tubes were incubated at 37°C under shaking overnight.
Genopure Plasmid Midi Kit (Roche, 15397700) was used for the preparation of the plasmid, solutions was made after the instructions provided from the company. The procedure used was for the low copy number of plasmids. The 100 ml of the bacterial cultures were centrifuged for 10 min with 3000 x g at 4°C. The supernatant was removed and the pellet was set to air dry for a few min before gently resuspended in 8 ml Tris-EDTA Buffer with 0.1 % ribonuclease (20 µg/ml). The tubes were then mixed well before 8 ml of Lysis Buffer was added. The tubes were then mixed by inverting them 6-8 times before they were incubated in room temperature for 2-3 min. After the incubation 8 ml of cold Neutralization Buffer was added to the solution it was then mixed by inverting the tubes as before. The tubes were then incubated for 5 min on ice before clearing the lysate. The lysate was cleared by filtration were the solution was filtrated through a moist filter by gravity flow and all the previously precipitates was removed and left was a clear flowthrough that was collected. The column was fixated in the collection tube by using a sealing ring before they were inserted into collection tubes and equilibrated by letting 2.5 ml of equilibrating buffer flow through them. When the columns had emptied the flowthrough was discarded. The cleared lysate were then loaded to the columns and let to flow through two times, after the second flow through the flowthrough was discarded. The column was then washed 3 times with 4 ml Wash Buffer, after each flow through the flowthrough was discarded. The columns were inserted in new collection tubes that could go through a high speed centrifugation. To elute the plasmid 2.5 ml 50°C Elution Buffer were added to the columns. The flowthrough were collected, the elution was done once again and the collected flowthrough of eluates were combined. After the elution 3.6 ml isopropanol were added to the tubes containing the plasmid eluate to precipitate the solution. The tubes were then centrifuged for 30 min with 15000 x g at 4°C. The supernatant was removed before the plasmid DNA was washed with 3 ml of 70 % cold ethanol. After the wash the plasmid DNA was centrifuged for 10 min at with 15000 x g at 4°C. The ethanol was then removed using a pipette tip and then the pellet was left to air dry for 10 min. The plasmid DNA was then dissolved again in 50 µl Tris-EDTA buffer.

The concentration and detection of the plasmid DNA was determined by using a nanodrop and running it through an agarose gel. The gel was made by mixing 1 g of agarose powder with Tris-Borate-EDTA buffer and bringing it up to a boil in a microwave. It was swirled and brought up to a boil once again before cooled down to about 60°C. Two µl gel red or 5 µl ethidium bromide was added and the solution was swirled and poured into a form with taped edges and a mould for wells. It was then left to set for at least 30 min. Samples of the DNA was loaded with 2 µl blue loading buffer and water, a total volume of 12 µl was loaded into
each well. A ladder was also present as a reference. The gel was run under a tension of 100 volts until the band had moved approximately 2/3 of the length of the gel (around 30-60 min). To make sure the DNA that was now confirmed in the sample was not chromosomal it was also cut by two restriction enzymes (EcoRI and XhoI) and run through the gel. The gel was studied under UV-light to see if the bands were what could be expected if it was the desired plasmid.

Transfection in C 33

*Plasmid transfection and selection*

C 33 cells were counted in a hemocytometer and 500,000 cells were plated in each well in a 6 well plate. After incubation overnight in 37°C and 5% CO₂ the transfection was performed with Gene Juice. It was plated in duplicates to the wells with a 0, 0.5 and 1 ng as transfection factor. The plasmid DNA had a concentration that was 0.1671 µg/µl and from that the amount added to each well could be calculated.

The next day the transfected cells were split to 100 mm³ plates. Two plates each for the different amount of plasmid. The cells in the 6 well plates were prepared as when splitting of flasks, after trypsinization 2 ml of complete medium was added to each well and then 1 ml was transferred to 2 plates, to the plates 10 ml of medium was added. The plates were incubated for 24 h at 37°C with 5% CO₂.

After the incubation the medium was removed and replaced by 10 ml new medium with 100 µl G418 solution (50 mg/ml, Roche 14548200) so that the concentration of G418 became 0.5 mg/ml. The plates were put back in the incubator. The medium was then replaced about once to twice a week with new medium with G418 solution. After 3 weeks the plates were studied for colony growth. Growth on all plates resulted in that a toxicity test was performed.

For the toxicity test C 33 was plated in 12 wells of two 6 wells plates and incubated overnight in 37°C with 5% CO₂. The next day the medium was replaced with 2 ml new medium containing different concentration of G418 solution. One well was with no G418 solution. The concentrations used were 0.5 mg/ml to 1.5 mg/ml. The concentration of G418 was raised with 0.1 mg/ml between each well. After inducing G418 the plates were once again incubated in 37°C with 5% CO₂. The survival of the cells was then studied throughout the following weeks before the medium was changed and new drug was added. After an additional week the wells and cell survival were compared between the concentrations.
Transfection with pCMV Sport β-galactosidase and in situ staining

C 33 cells were plated as previously done for the plasmid transfection. The plate was then incubated overnight in 37°C with 5 % CO₂.

Six eppendorf tubes were marked with 0, 0.25, 0.5, 0.75, 0.1 and 1.5. RPMI medium without additions was heated up and then 100 µl was added to the 6 Eppendorf tubes. Three µl of Gene Juice (Novagen, 70967-3) was then added to each of the tubes and the tubes were vortexed and incubated in room temperature for 5 min. The pCMV (Porcine Cytomegalovirus) Sport had a concentration of 0.15465 µg/µl. Different volumes of pCMV Sport were added to the tubes to get the 6 transfection factors wanted. The tubes were then incubated for 10 min in room temperature before added to the 6 well-plate (CELLSTAR greiner bio-one, 6 Well Cell Culture Plate, E10110MC) and put back into the incubator overnight.

To see if the transfection was successful an in situ staining for β-galactosidase activity was performed. The plates were taken from the incubator and the medium was removed before the wells were washed 2 times with 2 ml PBS each time. After the PBS was removed 1 ml glutaraldehyde (25 %) that had been diluted with water to a concentration of 0.25 % was added to each well and left for 15 min to fixate the cells. After the fixation the cells were washed 3 times in 2 ml PBS. Five hundred µl of X-Gal solution (2 % X-Gal with 2 mM MgCl₂, 5 mM K₄Fe(CN)₆·3H₂O and 5mM K₃Fe(CN)₆ diluted into PBS) was then added to each well and the plates were incubated in 37°C for 1 h, if no staining had appeared it was left in the incubator overnight to allow the solution to have effect. After the incubation the plates were studied in the microscope to check for a blue staining, activity indicating a successful transfection.
RESULTS

CAT-ELISA assay

The late gene expression was analysed to show that it could be increased by induction of different small molecules. The cell lines pBELMCAT and pBELCAT were used. The first thing that could be observed was that PBELMCAT naturally have a higher CAT expression then PBELCAT, which had also been the result of the study where the stable cell lines where generated [3].

Promising results, an increase in the expression depending on the dose of the drug, could be shown for pBELMCAT when treated with TPA, see figure 1, which effect had already been studied and evaluated in earlier studies\(^1\), the results in this study gave similar results were the late gene expression where more than 3 times higher for cells treated with 800 ng/ml TPA compared to untreated cells. While change in the expression for pBELCAT did not occur and further analysis was only made on pBELMCAT, the experiment was performed in triplicates.

Figure 1. pBELMCAT treated with different concentrations of TPA. The absorbance in relation to concentration of induced TPA in pBELMCAT. Error bars is for standard deviation error for each staple. * means that the results were statistically significant (* P>0.05) in relation to untreated cells.
Cells treated with VPA did not show much of an increase or decrease of the expression in relation to the untreated cells, no distinguished pattern could be observed, see figure 2. The experiment was performed in duplicates.

![Figure 2. pBELMCAT treated with different concentrations of VPA. The absorbance in relation to concentration of induced VPA in pBELMCAT. Error bars is for standard deviation error for each staple. * means that the results were statistically significant (* P>0.05) in relation to untreated cells.](image)

Further pBELMCAT cells were treated with TA, which showed a peak of the CAT expression at a concentration of 1 µM. Cells treated with a higher concentration of TA gave a decrease of the expression, see figure 3. The experiment was performed in triplicates.
The last molecule that was used to evaluate the effect of small molecules on the CAT expression was retinoic acid, RA gave an escalating increase of the expression up to the concentration of 10 µM, higher concentrations gave a decreasing effect of the expression, see figure 4. The experiment was performed in triplicates.

Figure 3. pBELMCAT treated with different concentrations of TA. The absorbance in relation to concentration of induced TA in pBELMCAT. Error bars is for standard deviation error for each staple. * means that the results were statistically significant (P>0.05) in relation to untreated cells.

Figure 4. pBELMCAT treated with different concentrations of RA. The absorbance in relation to concentration of induced RA in pBELMCAT. Error bars is for standard deviation error for each staple. * means that the results were statistically significant (P>0.05) in relation to untreated cells.
Taking the results from the assays with the cells treated by individual drugs in consideration the concentrations that gave the highest increases of the expression was combined with TPA and then compared with cells only treated with TPA. The combined assay showed that the small molecules together gave a higher expression than on their own, see figure 5. The experiment was performed in duplicates.

![Graph showing absorbance with different small molecules combined with TPA](image)

Figure 5. pBELMCAT treated with combinations of small molecules. The absorbance in relation to cells treated with different combination of small molecules. Error bars is for standard deviation error for each staple. * means that the results were statistically significant (P>0.05) in relation to untreated cells.

Plasmid Preparation and Transfection

The transfection with pCMV Sport into C 33 was unsuccessful, when no activity could be studied after in situ staining.

The plasmid preparation resulted in a small amount of isolated plasmid. The plasmid retrieved from the experiment was determined to be of the concentrations 0.5972 and 0.1671 µg/µl. Further analysis showed that it most likely was the desired plasmid DNA of the HPV genome, a gel electrophoresis indicated that the plasmid DNA was the length that was expected, see figure 6. Both samples of plasmid DNA seemed to clean for further use, when cleaved with restricted enzymes known to cleave the HPV genome the bands that could be expected appeared on the gel.
Figure 6. Gel electrophoresis where the bands from the first lane are a ladder and the 2 bands from lane 2 and 3 are the two samples from the retrieved plasmid.

The transfection with the retrieved HPV plasmid into the non HPV induced cervical cancer cell line C 33 did result in a high amount of colonies. However colonies also appeared on the plate where transfection without plasmid had been performed.

The toxicity test showed that G418 had a toxic effect to the C 33 cells if compared to untreated. No specific amount could be studied to have an effect however that resulted in complete death of all cells.
DISCUSSION

An increased amount of late gene expression in cervical cancer cells are thought to be favourable and to help the immune system to easier get rid of the virus infected cells. To estimate effects of small molecules that could induce a higher expression, maybe in the future the knowledge can be used in treatments to prevent disease and death of a highly spread condition. All the molecules in the study are known to have an effect on cells or diseases, such as different cancers or virus infections, they were therefore a good choice to use in the study. A few had been tested on the cell lines earlier and shown promising results but further evaluation and conformation of the effect was desired and to go forward with new drugs and combinations.

TPA has in other high risk types of the HPV virus shown to be an efficient regulator of the late gene expression, it has for example shown to increase the expression in HPV 31b [7]. Also in the experiments leading up to this study it gave a high increase of the CAT expression. TPA gave the best increase of the late gene expression of all the molecules used in the study and is therefore the best choice to work further with and use in combinations to further the effects and help the immune system when having an infection due to the virus.

That VPA didn’t give much of a result was not what could be expected. VPA have in many cases of other types of cancer shown to be a good alternative to use to induce differentiation and as an in chancer to regulate gene expression, and it was therefore believed it would have an effect. For an example it has shown to have an increasing effect on the viral gene expression in tumours cells infected by EBV, [8] and is also known to induce differentiation in leukemic, carcinoma and hematopoietic progenitor cells [9]. It is highly used to regulate brain cell activity such as with cases of epilepsy and in some cases treatment for conditions like ADHD. VPA have also shown promising effects when used in treatments for HIV. It was also observed to give a slight increase on the PBELMCAT late gene expression in earlier experiments done before this study. Treatments with VPA can have severe side effects and therefore I would rather chose different drugs for treatment if possible even if the results had been promising. When done in this study the results from both experiments where non successful when the drug where induced, a change in the late gene expression where not seen and therefore no triplicates of the assay was done and the small molecule was never used or combined in more experiments.

10 http://www.nlm.nih.gov/medlineplus/druginfo/meds/a682412.html 07/14
Both TA and RA showed that they had a rather specific range in dose where the response for the late gene expression gave an increasing result. Both high and low concentrations of the drugs gave either no change in expression, or a negative feedback from what was desired, in form of decreasing CAT expressions compared to untreated cells. Treatment with such molecules can therefore be less then optimal, hard to maintain and dosing when other outside factors also play a role in the optimal amounts to be taken for the desired effect. TA has shown to have an anticarcinogenic effect on cancers that were chemically induced, this may be the effect of that TA have an inhibitory effect on proteasomes in tumorigenic cells [10]. RA have been shown to be able to halt the progression of several types of cancer lesions before they have the chance to become malign, that includes lesions in the oral cavity, skin and in the cervix. When it comes to lung and head/neck cancer RA can prevent secondary tumours to develop [11]. RA has a reporter gene that also is a tumour suppressor gene, called RAR-ß2. RAR-ß2 is often silenced in a lot of cancers, this includes squamous cell carcinoma which is a type of cervical cancer [12]. With this in mind it may be so that lack of RA can be the cause of development of cervical cancer and by making the reporter gene active it may be possible to prevent cancer or treat cancer with RA.

When combined, both RA and TA worked well together with TPA and together they gave a stronger expression than on their own. Individually RA gave a slightly higher expression then TA did, while combined with TPA TA was more efficient and gave the highest increase. That they together gave a higher CAT expression indicates that maybe together all three of them will be even more effective.

CAT ELISA as a method to analyse the late gene expression worked well. As mentioned before the CAT reporter gave a god reflection of the expression from the L1 gene and was therefore a good choice to use in the generated the cell lines used to test the regulation of the late gene expression. The detection range of the method was limited, but by dilution of samples this was easy to work around. Triplicates of the assay resulted in similar results indicating that the method could be repeated and trusted to give reliable results.

It was planned that the analysis would be done of both the pBELMCAT and pBELCAT cell lines and exactly why the pBELCAT cell line didn’t give a change in the expression is hard to say, in the thesis this work is built upon it gave a slight increase when treated with TPA.

The transfection didn’t go as hoped. Even if the G418 was not as effective as expected, it should at least have made cells weaker in the plate that was without any plasmid, resulting in not as much colony growth. That all plates ended up looking the same probably means that
either the transfection didn’t work at all or that somehow there was contamination and plasmid ended up in the 0 plate as well as the others.

This project was a continuation from earlier studies and more analysis of the late gene expression can be done in the future. For example it would be interesting to analyse the effect TPA have together with both TA and RA. When generated several other cell lines was also made, these cell lines could also be treated with small molecules and analysed further for effects on the late gene expression. Testing other drugs with similar abilities may also be valuable, to see if they may have an effect on the regulation of the late gene expression in HPV 16. Generating cell lines for other high risk HPV such as HPV 18 and test if the drugs have the same effect on the late gene expression could also be something to look into in future studies.

The most important results where that it is possible to regulate HPV 16 late gene expression for pBELMCAT, that TPA gave the highest increase of the molecules and that TA and RA can increase the expression but only if the cells are treated with right amount of molecule. Combined the molecules work together and an even higher expression than for TPA could be seen, where TPA together with TA gave the best results. In conclusion it would seem that these small molecules could be an alternative to use in treatment for cervical cancer caused by HPV 16 to up regulate the late gene expression and help the immune system to get rid of the infection in an early state of the disease.

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REFERENCES


