Cape Peninsula
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THE EFFECT OF THE ORGANOSULFUR GARLIC COMPOUND bisPMB FOR HPV16 AND HPV18 PSEUDOVIRUS INFECTION *IN VITRO*.

By

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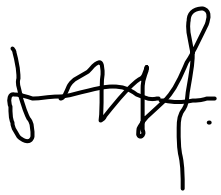
DECLARATION

I, Alda Nguefack, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed:  **Date: 15/10/2020**

ABSTRACT

Human papillomavirus (HPV) is the most frequent sexually transmitted infection known to be responsible for most cervical cancer occurrences worldwide, especially in less-resourced nations. HPV are small, double-stranded, non-enveloped DNA viruses mainly transmitted through skin-to-skin contact. Up to now, there is still no successful treatment for a progressing HPV infection, although there are highly efficient prophylactic vaccines. However, these vaccines confer only type-specific immunity, they are very costly, and their efficacy is limited in patients already infected by the virus. There is clearly a necessity to elucidate further cost-effective modalities and alternative means for the prevention of HPV infection and/or treatment. In the context of an Honours project in our laboratory in 2018, bisPMB, a synthetic analogue of the garlic compound ajoene was discovered for the first time to significantly reduce HPV16 pseudovirus (HPV16-PsVs) infection of the cervical cancer cell line HeLa. This thesis aimed to further characterize the effects of bisPMB on the most oncogenic HPV types, HPV16 and HPV18 for infection of cancerous and non-cancerous cell lines, with the long-term aim to aid in the conception of new approaches for blocking HPV infection.

SDS-PAGE, virus-encapsidated reporter gene (Gaussia luciferase) activity, PsVs infection-neutralization, and cell viability assays were used to assess the effect of bisPMB on HPV-PsVs infection. In this research, bisPMB inhibited cell proliferation at an IC_{50} concentration of 5.20 ± 2.15 µM in HeLa cells and 4.83 ± 0.50 µM in the normal keratinocyte cell line NIKS. Inhibition assays were then performed at sub-toxic concentrations to evaluate whether bisPMB could inhibit HPV infection. BisPMB was confirmed to decrease HPV16-PsVs (at 5 and 10 µM) and HPV18-PsVs (at 10 µM) infections in HeLa cells, with decreased cell viability at higher concentrations. We also observed that bisPMB decreased HPV16-PsVs infection (at 2.5, 5, and 10 µM) but increased HPV18-PsVs infection (at 5 and 10 µM) in NIKS cells. However, as inhibition of HPV-PsVs infection was at an already toxic concentration (10 µM) to the cells, bisPMB is unlikely to be used as prophylactic treatment.

Our results suggest that bisPMB might block HPV infection type-specifically, both in cancerous and non-cancerous cell lines, with HPV16 being more susceptible to bisPMB treatment than HPV18. Further investigations using other HPV strains and both cancerous and non-cancerous cell line models need to be carried out to support these observations.

Keywords: HPV infection; Oncogenic HPV; Cervical cancer; HeLa cells; NIKS cells; MTT assay; Ajoene compound; BisPMB; IC_{50} concentration.

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DEDICATION

I dedicate this thesis to my sister (Daïna Tiwa Tchiogouo Vannel), who has been my rock, and the rest of my dearest family for their unequivocal love and for believing in me throughout the years. I hope I have made you proud.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
APS	Ammonium persulphate
CIN	Cervical intraepithelial neoplasia
CO_2	Carbon dioxide
DAS	Diallyl sulphide
dH_20	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DS	Dextran sulphate
E	Early region
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EE	Early endosome
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
GAG	Glycosaminoglycans
HFK	Primary human foreskin keratinocyte cells
HIV	Human immunodeficiency virus
H_2O	Water

HPV	Human papillomavirus
HR	High-risk
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycan
IC_{50}	Half maximal inhibitory concentration
ICC	Invasive cervical cancer
IF	Intermediate filament
IPA	Isopropyl alcohol
L	Late region
LE	Late endosome
LMIC	Low- and middle-income country
LN_2	Liquid nitrogen
LR	Low risk
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NIKS	Near-diploid immortalized keratinocyte cell line
OD	Optical density
ORF	Open reading frame
OSC	Organosulfur compound
p53	Protein 53
PBS	Phosphate buffered saline
pGluc	Protein Gaussia luciferase
pRb	Protein retinoblastoma

PV	Papillomavirus
Rab	Rabaptin
RLU	Relative light unit
36S	3,6-*O*-sulfated chitosan
SA	South Africa
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
SSA	Sub-Saharan Africa
SUN	Stellenbosch University
TCID	Tissue culture infective dose
TEMED	N, N, N', N' Tetramethyl ethylenediamine
TRAPPC8	Transport protein C8
UCT	University of Cape Town
URR	Upstream regulatory region
USA	United States of America
vDNA	Viral deoxyribonucleic acid
VLP	Virus-like particle
WHCO1	Wits human carcinoma of the oesophagus
WT	Wild type

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Cervical cancer is one of the most significant threats to women's health. The disease is caused by human papillomavirus (HPV), the most common pathogen of the reproductive tract (Crosbie et al., 2013:889; World Health Organization (WHO), 2019). Cancer of the cervix ranks as the fourth most frequent female malignancy (following breast, colorectal, and lung cancers) and the eighth-most frequent cancer in both sexes combined globally; the fourth cause of mortality in women (behind breast, lung, and colorectal cancers) and the ninth in both sexes combined overall (Cecilia et al., 2017:11; Attipoe-Dorcoo et al., 2018:19; Bray et al., 2018:394; Cohen et al., 2019:169) **(Figure 1)**.

Over 500 000 women are diagnosed with cervical cancer every year and the disease gives rise to more than 300 000 deaths worldwide (Cohen et al., 2019:169). Contradictorily to developed countries, cancer of the cervix incidence and mortality rates are revealed to be significantly higher in low- and middle-income countries (LMICs), and sub-Saharan Africa (SSA) has the highest quotient (Attipoe-Dorcoo et al., 2018:19; Black & Richmond, 2018:1; Stern, 2019:1). Around 90% of all cervical cancer deaths in 2015 occurred in LMICs (Cohen et al., 2019:169). GLOBOCAN reported in 2018 that the incidence and mortality rates for the cancer of the cervix in SSA were 34.9 and 23.5 per 100 000 women every year, respectively **(Figure 2)**.

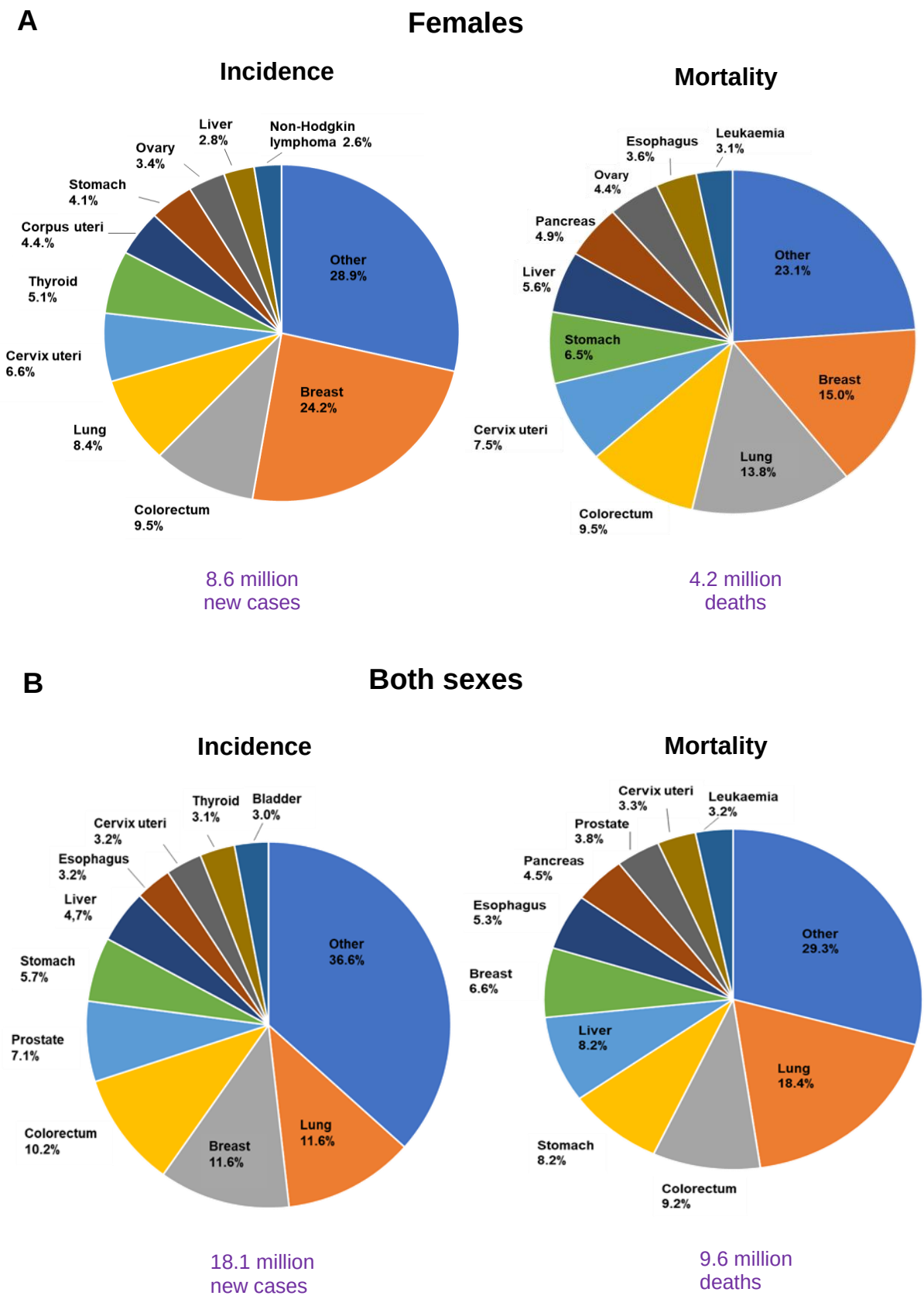


Figure 1: Charts present the worldwide approximated new cases and deaths for the 10 most prevalent cancers in 2018. Distribution in **A)** Women and **B)** Women and Men**.** The section of the pie chart indicates the percentage of the totality of cases or deaths. Figure adapted from GLOBOCAN 2018 (Bray et al., 2018:400)

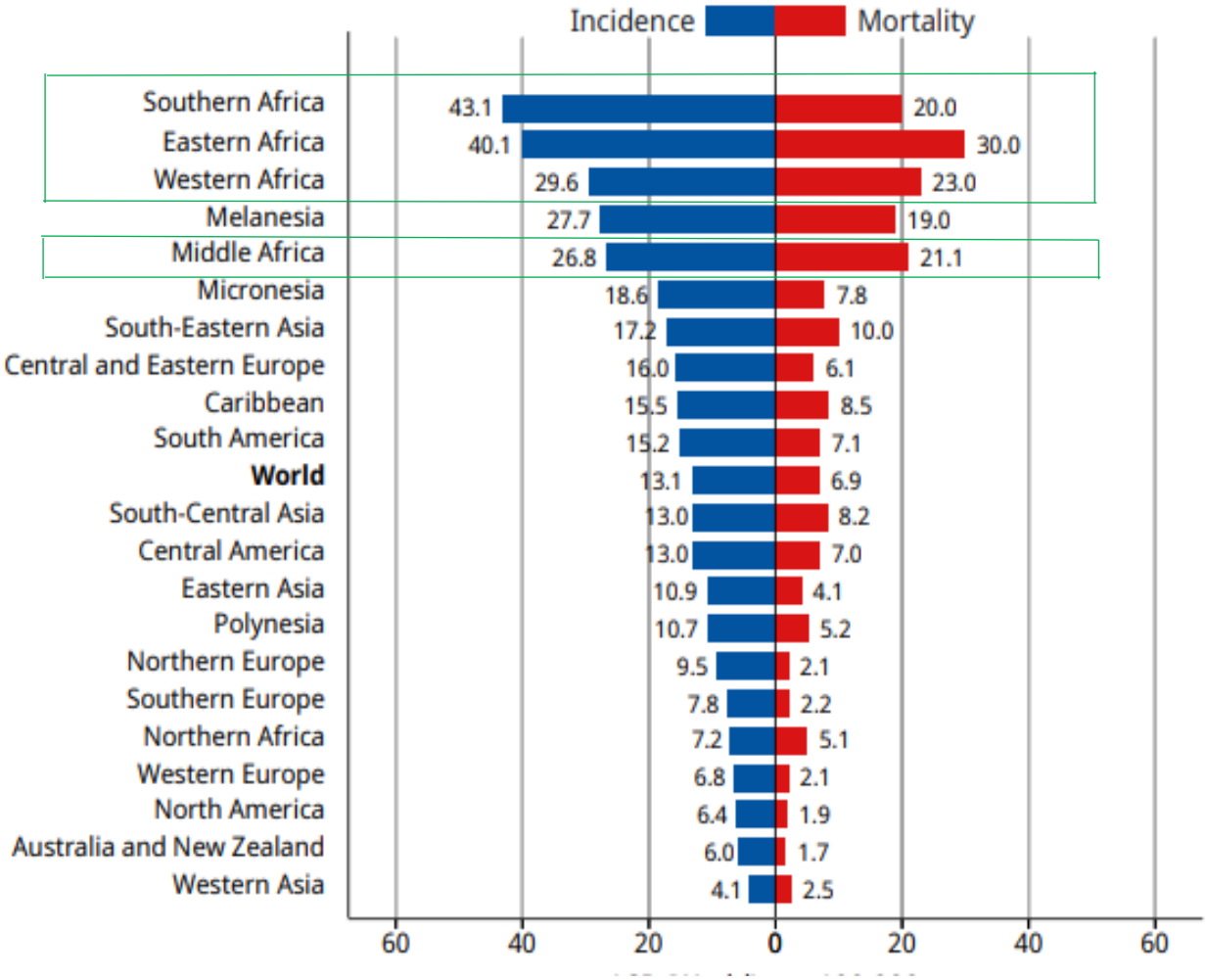


Figure 1: World age-standardized Incidence and Mortality rates of cervical cancer by world sub-regions. SSA countries (placed in the green rectangles) account in total for 34.9 of incidence and 23.5 of mortality. Rates per 100 000 women per year. Figure adjusted from GLOBOCAN 2018

(Ferlay et al., 2018a:2)

The latest facts from the International Agency for Research on Cancer (IARC) revealed that cancer of the cervix generally affects women aged 15–65 years globally, with the range of 40–64 years having the highest incidence and mortality rates (Bruni et al., 2019a:16) **(Figure 3)**. In SA, cancer of the cervix is the second leading female cancer (following breast cancer), the principal cause of cancer deaths among women, and major cancer among women aged 15–44 years (Attipoe-Dorcoo et al., 2018:19; Bruni et al., 2019b:6). The IARC recently also reported that in SA around 12 983 women are diagnosed with cervical cancer annually and 5 595 die from the disease (Bruni et al., 2019b:6,15; Women's Health, 2020). Importantly, successful oncogenic HPV infections cause virtually all cervical cancer cases (Villa et al., 2005:271).

Inadequate screening because of lack of infrastructures, expertise, economic and human resources challenging health needs, widespread poverty, and socio-cultural barriers is the cause of the high incidence of cervical cancer in LMICs (De Vuyst et al., 2013:1-2; Denny & Prendiville, 2015:S28; Bruni et al., 2019b:59). This growing burden could be minimized through organized interventions such as effective prevention, early-stage diagnosis, treatment strategies, and of course equal access to these programs (Bruni et al., 2019b:59; Cohen et al., 2019:169). However, although medical screening and other interventions (abstinence from sexual intercourse, barrier methods, monogamy) reduce the risk of cervical cancer, they do not prevent its development to precancerous lesions after HPV infection (Cohen et al., 2019:169).

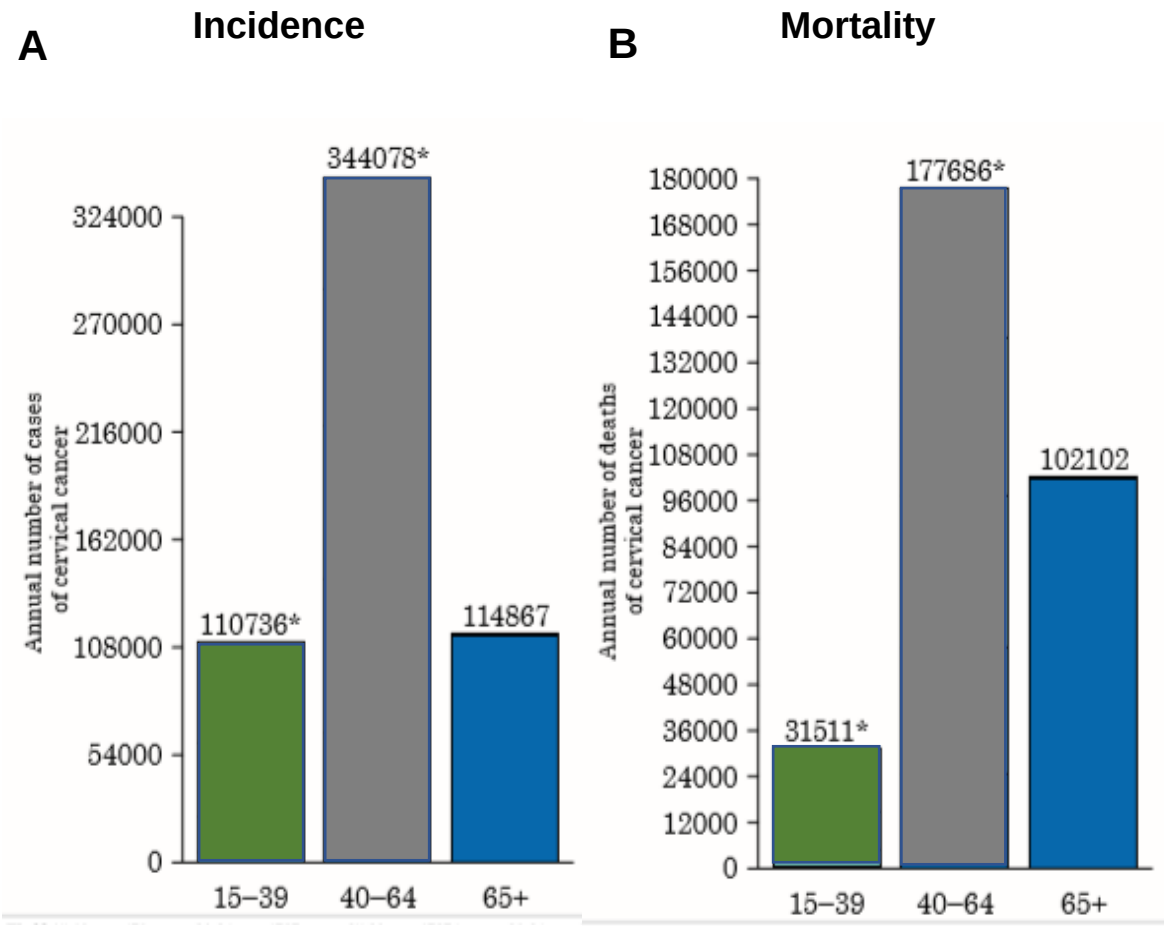


Figure 2: Annual total cases in relation to age, A) Incidence, and B) Mortality rates for cervical cancer worldwide. Rates per 100 000 women per year. Figure adjusted from GLOBOCAN 2018 (Ferlay et al., 2018b)

Despite being researched for decades, there is still no efficacious cure for an ongoing HPV infection. According to WHO, the initiation of HPV vaccines, consolidated with an extensive cancer of the cervix screening and treatment plan, is crucial to approach the burden of cervical cancer (WHO, n.d.). Three prophylactic vaccines against the most prevalent high-risk[1] (HR) types have recently been introduced; all of them protecting at least against the two most oncogenic strains HPV16 and HPV18. Indeed, cervical cancer significance in developed countries has considerably reduced since the implementation and sustention of population-based organized screening in addition to vaccination programs (Bonanni et al., 2015:3; Alsbeih, 2018:1425; Vu et al., 2018:457).

Since their usage, HPV vaccines are reported to provide excellent protection against at least 70% of cervical cancer occurrences and remarkably reduce the incidence of the highly oncogenic types infections, HPV16 and HPV18 (Brotherton et al., 2008:457, Lowy et al., 2008:1, 7; 460-461; Ciesielska et al., 2012:235, 240). Although effective against specific HPV infections, these vaccines are highly type-restricted in their protection and require greater than one dose relying on the age of the recipient, rendering them costly. Consequently, this further adds significance to the quest for more cost-effective and alternative means for prophylaxis and/or chemotherapy of HPV-associated diseases. Thus, the increasing interest for investigation on natural substances as prospective drug candidates with greater inhibitory activities against diverse HPV types in the pharmaceutical field is observed nowadays (Nicolaou, 2014:9128; Szychowski, et al., 2014: 9292). Indeed, previous research in the context of an Honours project at UCT in 2018 focused on screening a diverse range of natural compounds for their potential to inhibit HPV16-pseudovirions[2] (HPV16-PsVs) infection of HeLa cells. From the 100 compounds screened, bisPMB was found for the first time as a potential inhibitor of HPV16 infection (Chikanya, 2018:22) **(Figure 4)**. BisPMB is a synthetic counterpart of ajoene, one of the garlic organosulfur compounds (OSC) previously studied for its anti-proliferative activity against cancer cells (Hunter et al., 2008:5277-5278; Kaschula et al., 2011:262; Kaschula et al., 2012:239-240).

This Master's thesis focuses on assessing the effect of the newly discovered natural compound, bisPMB, on HPV16- and HPV18-PsVs infections, the major risk factors of cervical cancer, using both cancerous and non-cancerous cell lines, to identify alternative therapies to prevent HPV infection.

[1] **High-risk HPV:** HPV type capable of causing cancer of the cervix and other cancer types, namely oropharynx, vagina, penis, anus and vulva (Burd, 2003:1).

[2] **Pseudovirion:** synthetic virus used to introduce genetic material including RNA and DNA with precise traits into bacterial and eukaryotic cells (Li et al., 2018:1).

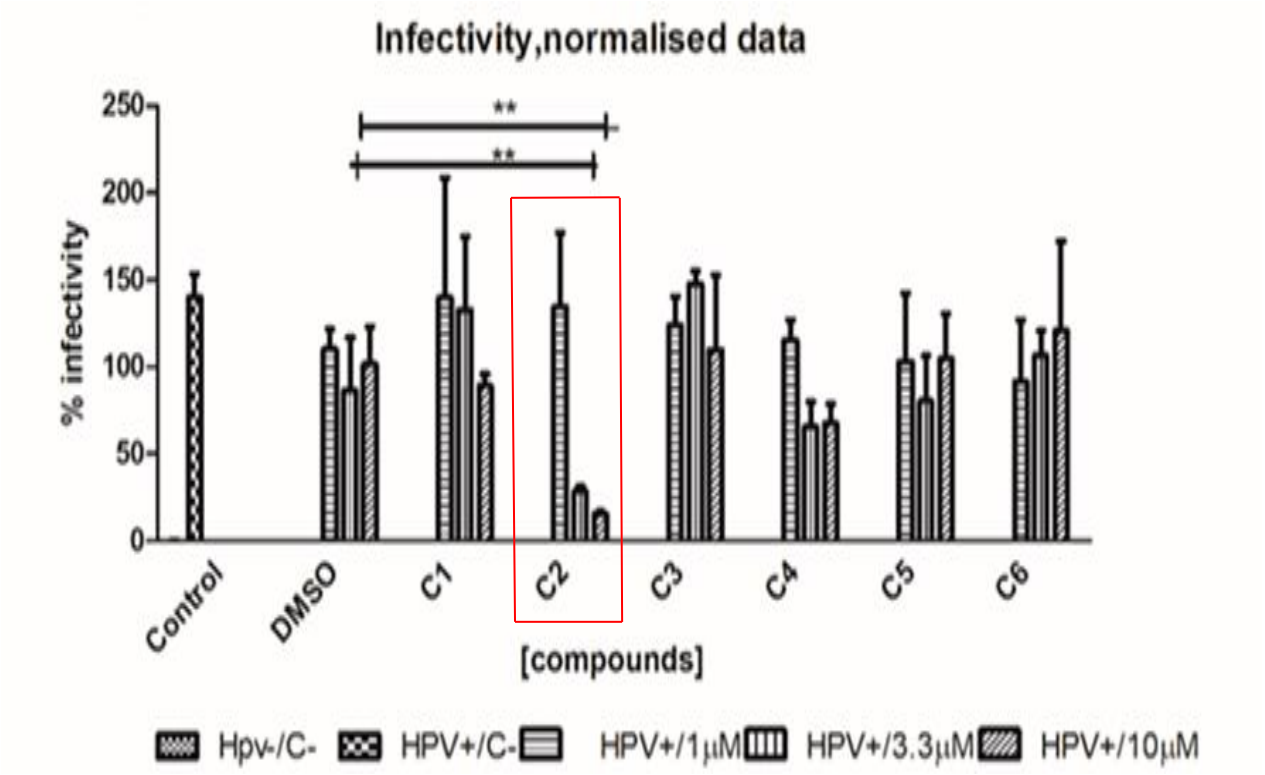


Figure 3: BisPMB represented by compound 2 (C2) decreases HPV16-PsVs infection of HeLa cells. HeLa cells treated with compounds C1 to C6 at 1, 3.3, or 10 µM concentrations as determined by MTT assay: C1 (Z-ajoene); C3 (Phenylethyl isothiocyanate); C4 (Flavokawain A); C5 (Silibinin) and C6 (Fisetin).

(Chikanya, 2018:21, 34-35)

1.2 Review of literature

1.2.1 Human papillomavirus

The evolution of diverse human cancers is well known to be associated with viral infections, the foremost being the link between HPV and cervical cancer (Buck et al., 2008:5190; Zur Hausen, 2009:260-261). HPVs are small non-enveloped double-stranded circular deoxyribonucleic acid (DNA) viruses with a virion size of 55–60 nm in diameter that can be responsible for benign diseases, precancerous lesions, and invasive malignancies in the mucosal epithelia (Lamprecht et al., 2016:1; Graham, 2017:1). Cancer of the cervix is mostly an HPV-driven disease that infects the genital mucosal epithelia (Münger et al., 2004:11451), with certain estimates that it causes more than 99% of cases (Bosch & De Sanjosé, 2003:3). HPVs belong to the Papillomaviridae family and the individual viruses are known to have an extremely strict host tropism (animals and humans) (Zheng & Baker, 2006:1; Doorbar et al., 2016:2). All HPV genotypes recognized to be cervical carcinogens are part of the alpha genus (Mirbahari & Sadeghi, 2018:113-115; Flores-Miramontes et al.,2020:1-2). At present, there are over 200 HPV genotypes classified into two major groups, high and low-risk[3] (LR) HPVs according to their oncogenicity[4] (Faridi et al., 2011:2, Mirbahari & Sadeghi, 2018:113; Flores-Miramontes et al., 2020:2).

Most of the HPV-linked cancers' aetiology is known to be linked with oncogenic mucosal HR HPVs types including HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82 (Muñoz et al., 2006:S1; Schiffman et al., 2009:1-6; Wang et al., 2014:2020). Among the large group of HR HPVs known up till now, HPV16 and HPV18 are the main subtypes as they are responsible for most HPV-associated cancers, accounting for roughly 70% of cancers of the cervix (the most frequent (WHO, 2020)), vagina and anus and approximatively 30-40% of cancers of the oropharynx, vulva, and penis (Zur Hausen, 2009:260-263; Ferlay et al., 2018b:1952). HPV16 is the most significant, in that it can generally be detected in 55 to 60%, while HPV18 only in about 10 to 15% of all cervical cancers globally (Muñoz et al., 2004:279-284; National Health Service (NHS), 2012; Mirbahari & Sadeghi, 2019:113, 116).

LR HPVs, including HPV6, 11, 12, 13, 15, 32, 34,40, 42, 43, 44, 53, 54, 61, 70, 72, and 81 have been associated with benign epithelial lesions (NHS, 2012; 2014; Wang et al., 2014:2020). HPV6 and HPV11, are responsible for about 90% of genital warts (condylomas acuminata) and additionally cause recurrent respiratory papillomatosis[5], which rarely develops into cancer (Aubin et al., 2008:610, 612-614; Egawa et al., 2015:3877-3878). Genital warts generally present as flat, papular, or pedunculated growths on the genital mucosa (Centres for

[3] **Low-risk HPV:** HPV type that does not causes cancerous diseases (Burd, 2003:1).

[4] **Oncogenicity:** the capacity of inducing tumour formation (Faridi et al., 2011:2).

[5] **Recurrent respiratory papillomatosis:** disease described by reccurent wart-like growths on the surface of the larynx or tissue around the vocal cords (National Institutes of Health (NIH), 2017).

Disease Control and Prevention (CDC), 2014:1). They are very contagious and usually diagnosed just by a look at the genital infected area. About 3.2% of the general SA women population harbour cervical HPV16/HPV18 infection at some point and 64.2% of invasive cervical cancers (ICCs) are linked to HPV16 or HPV18 (Catalan Institute of Oncology (ICO)/IARC Information Centre on HPV and Cancer, 2019:1).

1.2.2 HPV genome and structure

Papillomaviruses (PVs) replicate and assemble exclusively inside the nucleus of the infected cells (Zekan, et al., 2011:597-598). HPV genome is circular and has a size of about 8 000 base-pairs (8 kbp) (Münger et al., 2004:11451). The HPV genome can be separated into 2 regions: Upstream Regulatory Region (URR) and Open Reading Frames (ORFs). The URR does not code for any proteins yet contains cis-elements indispensable for the regulation of gene expression, replication of the genome, and its packaging into particles. The ORF can be segmented into two regions: Early region (E) and Late Region (L). The E genes encode for 6 proteins (E1, E2, E4, E5, E6, and E7) and the L genes for 2 (L1 and L2) (Stanley, 2010:S7; Zekan et al., 2011:596) **(Figure 5)**.

Proteins of the E genes are non-structural regulatory proteins of the viral genome, primarily expressed in undifferentiated or intermediately differentiated keratinocytes (Zekan, et al., 2011:596). E1 and E2 proteins are necessary for basal DNA replication and transcriptional regulation. E2 takes part in the control of low copy repeats transcriptions and reduces the expression of E6 and E7 (Zekan et al., 2011:596). E4 is reported to be associated with the release of the viral particle (Münger et al., 2004:11452). E5 reduces intercellular communication and insulates the transformed cells, corporates with the growth factor's receptors, and promotes cellular proliferation (Moody & Laimins, 2010:551). The oncogenic proprieties of HR HPVs lays in E6 and E7 which stimulate the growth and transformation of the host cell by inactivating, protein 53 (p53) and protein retinoblastoma (pRb), two cellular tumor-suppressors (Moody & Laimins, 2010:551-558; Egawa et al., 2015:3867-3870). E6 inhibits p53, by stimulating genomic instability which is essential for the evolution of cancer (Pinidis et al., 2016:49). E7 attaches to pRb, therefore allowing the release of E2F, a transcriptional factor promoting the entry of the cell into the S-phase of the cell cycle, thus indirectly generating hyperproliferation of the cell (Doorbar et al., 2012:F61; Pinidis et al., 2016:49-50).

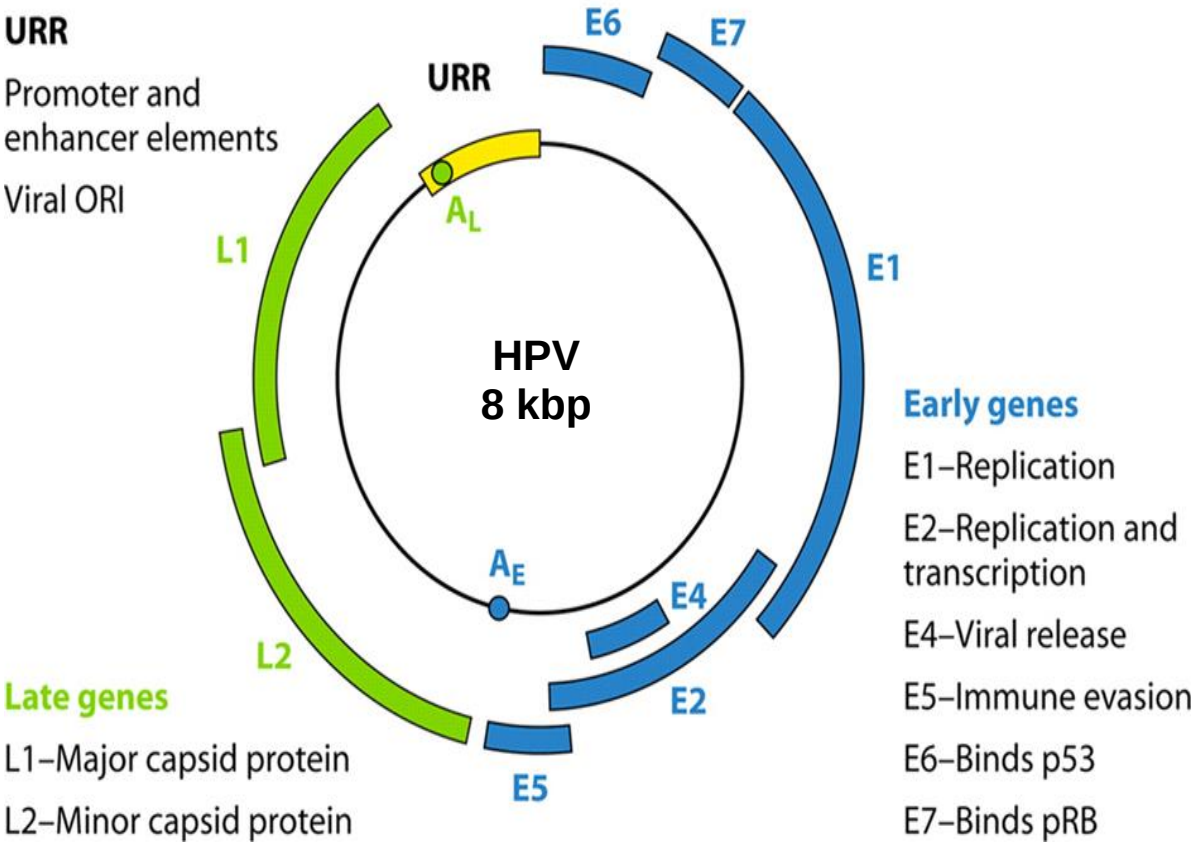


Figure 4: Illustration of the genomic arrangement of a classical mucosal HR HPV. The non-coding region is the URR, and the ORFs encode the E and L viral proteins.

(Stanley, 2012:216)

Within the L region, L1 and L2 are structural capsid proteins expressed in keratinocytes undergoing terminal differentiation, which are indispensable for virion formation, transmission, and spread (Cerqueira et al., 2016:2; Stern, 2019:1). L1, the major component of the viral capsid forms 72 pentamers which make the virus outer shell and can self-assemble into icosahedral virus-like particles (VLPs) that nearly look like the original structure of PV virions (Kirnbauer et al., 1993:6929; Buck et al., 2008:5190-5191). Wang and Roden (2013:1-4, 8-12) demonstrated in a study aimed at developing the PVs vaccine that icosahedral VLPs could consist of only L1 when expressed as eukaryote recombinant protein. L2 codes for the minor capsid protein which is comprised of up to 12-72 molecules and is hidden inside the capsid underneath the L1 pentamers except for a small N-terminal section (Buck et al., 2008:5190; Young et al., 2019:2). Although L2 is not necessary for capsid formation, it is however thought to act in the incorporation of HPV viral DNA (vDNA), and play several crucial roles in the infection establishment (Schiller et al., 2010:1-2; Cohen et al., 2019:2).

1.2.3 Machinery of HPV uptake

HPV undertakes three key steps to set up an infection: attachment, internalization, and intracellular trafficking to the nucleus.

Efficient infectious entry by HPV requires the glycosaminoglycan (GAG) chains of the heparan sulphate proteoglycans (HSPGs) (e.g., syndecan-1 (Sdc-1), the main epithelial HSPG for the binding to either the extracellular matrix (ECM), basement membrane, and/or keratinocyte plasma membrane (Dasgupta et al., 2011:2617; Day & Schelhaas, 2014:2; Young et al., 2019:5). Previous researchers suggest that not only HSPGs but additionally laminin-332 (formerly known as laminin-5) can act as an attachment receptor to the ECM for cultured human cells (Broutian et al., 2010:531, 534-535; Young et al., 2019:5), while in *in vivo* mouse vaginal models, HSPGs are principally used for the attachment of HPV to the basement membrane (Kines et al., 2009: 20458). This interaction is facilitated by L1 only (Schiller et al., 2010:1). The attached HPV-PsVs then undergo capsid conformational changes remodelled by host enzymes, liberated from the ECM or plasma membrane, and translocated to tetraspanin-enriched microdomains composing the tetraspanins[6] CD63 and CD151, α6-integrins, epidermal growth factor receptor, annexin A2t, and cytoskeletal associated adaptor proteins (obscurin-like protein OBSCL1 and syntenin-1) (Day & Schelhaas, 2014:3; Florin & Lang, 2018:2-3; Young et al., 2019:5, 7). This exposes L2 N-terminal amino acids by isomerization of cyclophilin B **(Figure 6)**. The exposed L2 N-terminal amino acids are split by furin convertase provoking the virus to have a lower affinity for HSPG (Day et al., 2008:12565). This permits the delivery of the virus permitting it to interact with a specific receptor (which has not been elucidated yet). Once the binding is accomplished, internalization commences.

The endocytic pathways implied in the internalization and intracellular trafficking of HPV virions in host cells have been extensively investigated. Several of the publications show conflicting results even though our knowledge of HPV entry mechanisms has been extremely advanced since the establishment of powerful HPV production methods (Bousarghin et al., 2003:3846; Smith et al., 2007:9922-9923; Schelhaas et al., 2012:1-2). Viral uptake is believed to take place via a receptor-mediated endocytosis, throughout an approach similar to micropinocytosis[7], necessitating actin polymerization (Schelhaas et al., 2012:1-2). Most of these publications suggest a clathrin-mediated endocytosis for most of the HPV types, as they show blockage of internalization by biochemical inhibition of clathrin, chlorpromazine is an example of inhibitor

[6] **Tetraspanins:** also called 'master organizes', are a family of conserved membrane proteins that play the role of regulating cell morphology, motility, signalling, plasma membrane dynamics, and protein trafficking (Florin & Lang, 2018:1).

[7] **Micropinocytosis:** incorporation of specific macromolecules or other chemical substances into cells by membrane invagination and the pinching off, resulting in small vesicles in the cytoplasm (Boyce et al., 2020:2).

(Bousarghin et al., 2003:3847-3849; Day et al., 2003:1-6; Smith et al., 2007:9927). Abban et al. (2008:3-7) in a study using the human embryonic kidney (HEK-293T) cell line also revealed a clathrin-dependent route of HPV entry, which contradicts data acquired from HeLa and HaCaT cells, demonstrating a clathrin-independent mode of HPV (Schelhaas et al., 2012:4-8; Spoden et al., 2013:7766-7767). Some investigations have concluded that HPV31, which is closely linked to HPV16, can enter via a caveolin-mediated pathway (Smith et al., 2007:9922). Additionally, other receptors have been listed, namely cholesterol-, dynamin-, flotillin-, glycophosphatidylinositol-anchored protein-enriched endosomal compartment-, and lipid raft-independent (Smith et al., 2007:9926; Spoden et al., 2008:11). These discrepancies of the actual pathway taken by the virus may be dependent on the cell culture models used in these studies.

Most opinions suggest that HPV requires a low pH for successful infectious entry since it must go through an acidified endosome, this facilitates the viral DNA uncoating of the capsid (Kämper et al., 2005:760). L1 proteins are uncoated and transferred to the lysosome for degradation. Viral localization to early endosome (EE) is believed to be CD63 and rabaptin 5 (Rab5)[8] dependent and is coupled to the acidification of the EE. In the EE post-acidification Rab5 is switched to Rab7a, which results in capsid dissociation, releasing the viral genome in a complex with L2 and facilitates the conversion from the EE to late endosome (LE) (Kälin et al., 2016:34-35). The evolution from EE to LE is usually explained by the formation of multi-vesicular bodies and eventual fusion with the lysosome (Huotari & Helenius, 2011:3483). The non-existence of Rab7a blocks the fusion of LE to lysosomes, i.e. ablating function. L2 N-terminal exposure and penetration results in the retromer complex recruitment (Young et al., 2019:5, 7). Retromer and Rabs 7b and 9a take part in the trafficking of the L2/vDNA complex to the trans-Golgi network, then escaping lysosomal degradation (Young et al., 2019:8). An important point to state is that furin cleavage of L2 at the cell surface is essential for endosomal evasion (Day & Schiller, 2009:2, 3).

To utilize the cellular transcription and DNA replication machinery, the HPV L2/vDNA must exit the trans-Golgi network and enter the nucleus. This process is established by cell cycle progression and nuclear envelope failure during mitosis (Pyeon et al., 2009:3; Aydin et al., 2014:4-6). The L2/vDNA complex penetrates and stays in the cis and cis-medial Golgi until mitosis eventually takes place (Day et al., 2013:3865-3866). The transport protein particle C8 (TRAPPC8), a component of the guanine nucleotide exchange factor, controls small GTPases, impacts on HPV capsid endocytosis, and additionally has improved functions through

[8] **Rabaptins:** families of protein member of the Ras superfamily of G protein. Rab proteins contain the largest family of GTPases folds, which hydrolase enzymes that links to the nucleotide guanosine triphosphate (GTP) and hydrolyse it to guanosine diphosphate (GDP) (Kälin et al., 2016:34).

interchanging with the viral L2 protein post entry (Ishii et al., 2013:9-10). Intracellular communication between TRAPPC8 and L2 originates the Golgi dispersion and remodels normal TRAPPC8 roles in the endoplasmic reticulum (ER)-to-Golgi transport. This may then explain the trafficking of the vDNA to the nucleus (Ishii et al., 2013:13).

Furthermore, early viral transcripts, upon persistent infection are translated into early gene products, E2 included, which binds the HPV vDNA to mitotic chromosomes (Zheng & Baker, 2006:2-4; Zekan et al., 2011:599; Young et al., 2019:9). Location to the mitotic chromosome gives the virus DNA access to the cellular transcription and replication mechanisms and helps in its initiation and maintenance in the host dividing cells DNA. HPV episomal integration into the host genome has been revealed to occur in two possible ways. First, as a single genome integrated and secondly as multiple tandem repeats of the viral genome inside the cellular genome (Mc Bride & Warburton, 2017:1-3). Numerous insertion and breakage operations have been suggested for this process. One of the most believed models is the ‘looping’, which states that HPV integration is conducted by DNA replication and recombination, which may lead to DNA concatemers (Akagi et al., 2014:185). This could then result in the disturbance of genes implicated in tumorigenesis, oncogene amplification, inter- or intra-chromosomal reorganizations, and/or genetic variability (Akagi et al., 2014:186).

Although the mechanism of HPV virus entry is not fully understood as it is a tricky process implicating diverse protein complexes, these proteins imply potential targets for intervening in HPV infection.

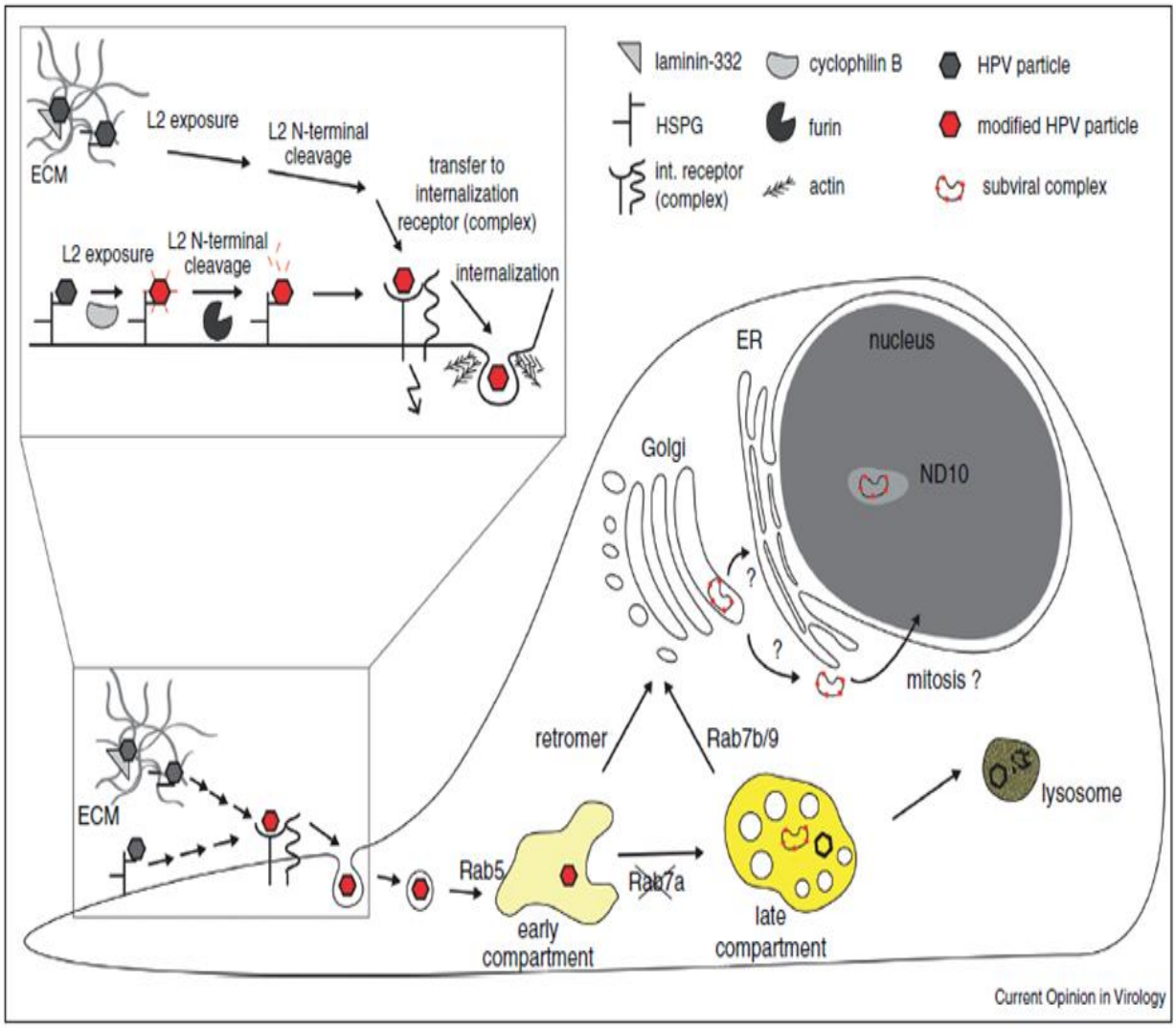


Figure 6: Schematic description of HPV infectious entry pathway into the host cells. The initial step involves attachment to HSPG in the ECM followed by a conformational modification which leads to the exposure of cleavable L2 N-terminal amino acids. Afterward, cleaved HPV binds to secondary receptor(s) initiating internalisation. Thereafter, the virus is trafficked to the nucleus via the trans-Golgi network.

(Day & Schelhaas, 2014:14)

1.2.4 HPV mode of transmission and risk factors

HPV transmission occurs via an abrasion, a cut, or a minor tear in the skin. HPV is primarily transferred by direct contact between infected skin to skin or mucous membranes. Genital HPV infection transmission occurs mainly through a sexual route. New-borns, subject to HPV during the perinatal stage can acquire the infection through vertical diffusion (Trottier et al., 2016:145-146, 148-149). Men are considered to be the principal reservoirs of genital HPV infection for women (Graham, 2017:2). Around 70% of sexually active women will become infected with HPV throughout their lifetime (Bosch & De Sanjosé, 2003:3).

In addition to oncogenicity (HPV type), many other factors influence HPV natural persistence and evolution from HPV infection to cervical cancer, especially: immunocompromised women living with the Human immunodeficiency virus (HIV), high parity, co-infection with other sexually transmitted agents (e.g. herpes simplex type 2, chlamydia trachomatis, and gonorrhoea), long term oral contraceptives use, tobacco smoking, certain dietary deficiencies (fruits and vegetables), early sexual debut and multiple sexual partners (Vanessa, 2012:2; Kim et al., 2018:v26-v27; Bruni et al., 2019b:56; Cohen et al., 2019:170; Lacey, 2019:1). Moodley et al. (2009:2-5) previously demonstrated in case-control research carried out in SA that HIV-positive women were about five times more susceptible to have HR HPV infection in comparison to HIV-negative women. Olorunfemi et al. (2018:2-6) recently showed in a study that the high prevalence of HIV in SA contributed to HPV high burden between 2001–2009. Contrary to other acquired immunodeficiency syndrome (AIDS)-defining diseases in which incidence reduces after the initiation of antiretroviral therapy, cervical cancer incidence remains constant due to chronic immunosuppression from HIV compromised immune system for virus-associated malignancies (Hawes et al., 2003:555-556; Strickler et al., 2005:577, 579-582; Brotherton & Bloem, 2015:223). Besides the fact that HIV and HPV share the same spreading route, the interaction between both diseases has a negative impact, namely T-cell dysfunction, higher risks of neutropenia and dormant infections reactivation throughout systemic treatment, complications in staging because of non-cancer-related lymphadenopathy, and HIV-associated thrombocytopenia which can intensify difficulties of chemotherapy and surgery (Ghebre et al., 2017:102-106).

1.2.5 HPV signs and symptoms

Most infections with HPV are clinically latent until the progression to precancerous lesions in later years (Baseman & Koutsky, 2005:S19-S20; Howell-Jones et al., 2012:3868). This is because HPV lacks a bloodborne phase, and there is minimal cytokine response causing minimal initial immune recognition (Stanley, 2012:215). As cancer progresses, the most frequent signs and symptoms include pelvic pain, abnormal vaginal bleeding or discharge, pain

during sex, bleeding after going through menopause, weight loss, fatigue, or nausea (MedecineNet, n.d.)**.**

1.2.6 HPV infectious cycle and development to cervical cancer

The incorporation of the HPV genome in the host chromosome is among the key events of HPV-induced carcinogenesis. Typically, the virus infects the primary basal keratinocyte cell layer (Otter et al., 2019:81) after minor abrasions or tears during sexual intercourse. This layer constitutes the unique cell layer in an epithelium that is effectively dividing (Münger et al., 2004:11452; Young et al., 2019:2). Nonetheless, high levels of viral gene expression plus viral protein making, and virus assembly take place exclusively in the upper differentiated layers of the squamous epithelia **(Figure 7)** (Chow et al., 2010:424-425). HPV utilizes the replication machinery within the nucleus of its host cell since it is a DNA virus that does not bring in its replication factors.

HPV infection and the complete replicative cycle can only occur upon the complete keratinocyte cell differentiation (Zekan et al., 2011:597; Stanley, 2012:216; Young et al., 2019:2). This explains the virus's strict host/cell tropism as well. Following infection of wound basal cells, E1 and E2 expressed proteins secure that the virus keeps a low viral copy number of around 50–100 copies per cell. Infected cells are believed to then exit this primary section and enter the epithelium transit-amplifying proliferative section, where there is a phase of plasmid or episomal conservation (Stanley, 2012:216). At this point in the viral life-cycle of HR HPVs, the expression of the potent oncogenes E6 and E7 is under strict regulation, and their transcripts in the proliferating section of the epithelium not easily detectable (Zekan et al., 2011:598; Stanley, 2012:216). However, as the cells start moving to the upper layer of the epithelium named the midzone, viral amplification of the viral genome copy amount increases to over 1000 copies per cell, and then E6 and E7 are expressed **(Figure 8)**. These oncoproteins are necessary for the preservation of the episome[9] and keep the differentiating cells in a replication-competent state. E6 of HR HPVs sticks and targets p53 for ubiquitination while E7 binds to the unphosphorylated form of the pRb, overriding the G1/S checkpoint of the cell (Zekan et al., 2011:599-600; Stanley, 2012:217). Moreover, the late promoter's activation leads to L1 and L2 gene expression, the cause of capsid proteins formation. Finally, the infected cells shed up even more to the superficial region and deliver the fresh viruses, which can start a new infection. The PV life cycle parallels cervical keratinocyte differentiation from the basal to the most superficial epithelium (at least 14–21 days) (Stanley, 2012:216; Crosbie

[9] **Episome:** a segment of DNA that can exist and replicate either autonomously in the cytoplasm or as part of a chromosome (Pediaa, 2019).

et al., 2013:889). Furthermore, specific premalignant lesions will eventually lead to cervical cancer.

Cervical cancer cases are entirely due to an untreated premalignant cervical intraepithelial neoplasia (CIN) also termed ICC, which is a change of the normal cells on the surface of the cervix, called dysplasia (Schiller et al., 2018:1). CIN is of 3 types according to its severity, CIN-1 (low-grade), CIN-2, and CIN-3 (high-grade). Patients with CIN-2 and CIN-3 are at higher risk of developing cervical cancer (within several years), while about 60% of CIN-1 will regress to normal after one year **(Figure 8)** (Stanley, 2012:215; Mello & Sundstrom, 2020:2). The relation between HPV and cervical carcinogenesis is linked to the HPV phenomenon of viral latency[10]**.** HPV infections can incite an antibody-mediated immune reaction, which is believed to get rid of the virus from the body, i.e. about 70 to 90% of all cases are cleared within two years (Graham, 2017:5). Briefly, newly released viral particles are recognized, and that immune response can clear the infection and protect the host against a new HPV infection (Stanley, 2012:217). Women aged 20–24 years are more readily able to clear the infection to an undetectable level (within approximatively 8 months) (Mello & Sundstrom, 2020:2), while those over 30 years are more susceptible to have a persistent infection (Massad et al., 2013:S2). In some cases, if the preceding infection was caused by an HR HPV type, the infection may become latent, which could then turn to be persistent and may develop into high-grade precursor lesions and eventually invasive cancer. This is noted in 10% of infected individuals within 10-30 years (Zekan et al., 2011:599; Crosbie et al., 2013:895).

[10] **Viral latency:** capability of a pathogenic virus to lie dormant within a cell, without producing more virus (Field & Vere Hodge, 2008:144).

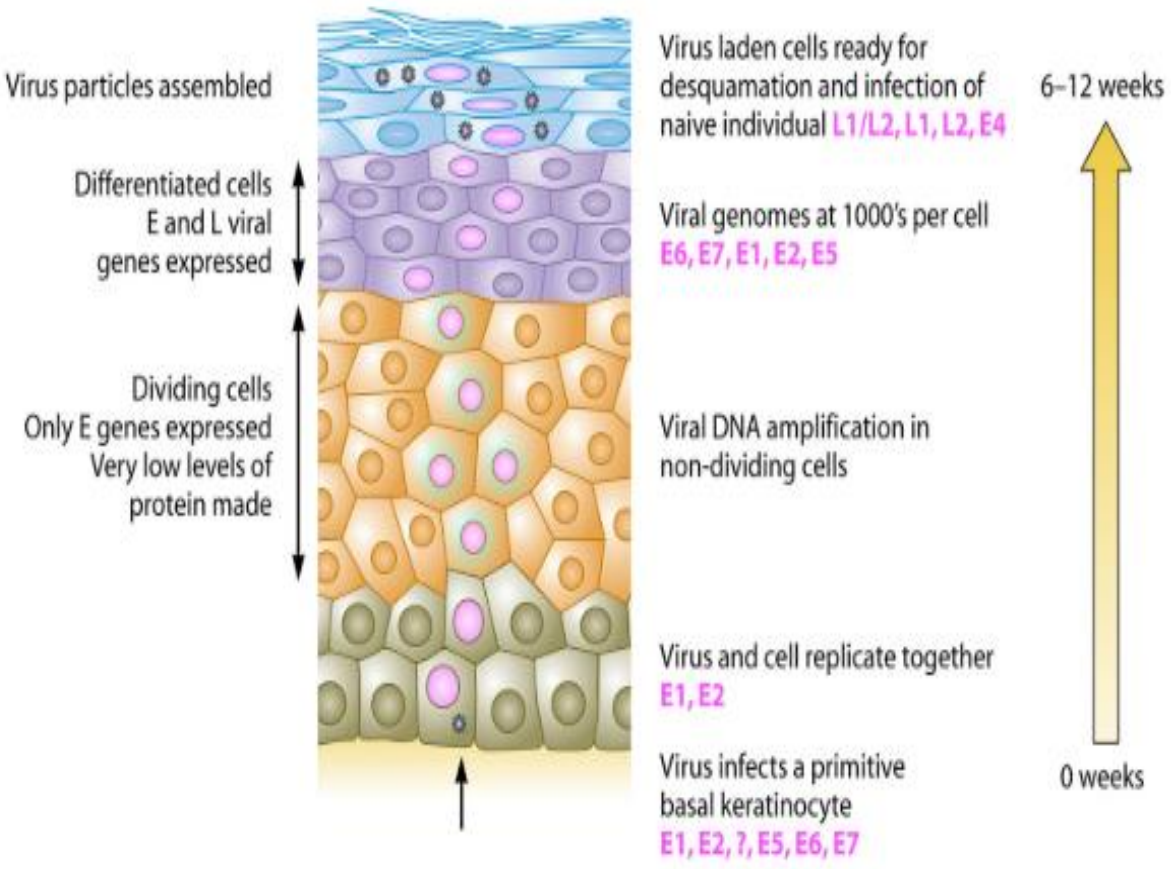


Figure 7: HPV life cycle is linked to keratinocyte differentiation
(Stanley, 2012:216)

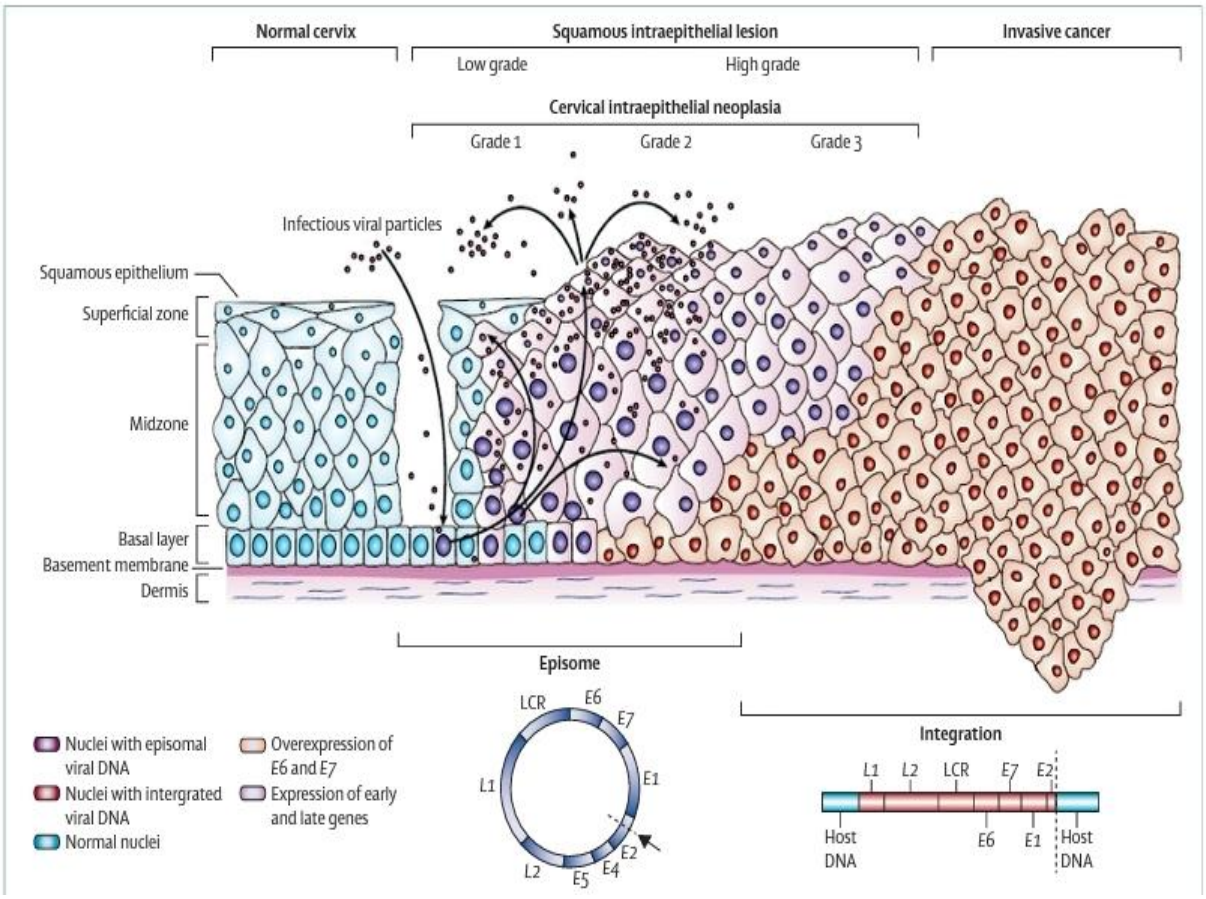


Figure 8: Infectious cycle and development of cervical cancer. Exposed basal cells permit the establishment of infection. As the basal cells proliferate so does the virus (episome) and eventually, infected cells move to the superficial zone.

(Crosbie et al., 2013:892)

After successful HPV infection and development into cervical cancer, five stages are observed: stage 0, I, II, III, and IV well explained in the table below **(Table 1)**.

Table 1: Outline of the different stages of cervical cancer

Stage	Description
0	The abnormal cell in the innermost lining of the cervix: carcinoma in-situ
I	Cancer limited to the cervix only
II	Cancer extension past the cervix, yet not to the pelvic wall or lower the one-thirds of the vagina
III	Cancer extension to the pelvic wall or lower the one-thirds of the cervix, and/or kidney problems exist
IV	Cancer extension to the bladder, rectal mucosa, other organs, or lymph nodes

1.2.7 Cervical cancer prevention

1.2.7.1 HPV vaccines and challenges

Previous studies have shown that cervical cancer prevention through the usage of condoms does not offer 100% protection (Buck et al., 2006:0672). Initial prevention from cervical cancer takes reliance on vaccination against HPV. HPV prophylactic vaccines are made from VLPs (which are highly immunogenic), produced by recombinant technology of virus surface protein L1 (Harper et al., 2004:1757)**.** The utilization of L1 VLPs in HPV vaccines originates from pre-clinical research in mice**,** which shows that injection of these particles can promote high levels of particular antibodies and very good protection (Suzich et al., 1995:11553-11555; Dupuy et al., 1997:220). The vaccines are non-infectious since they do not comprise any live biological material or DNA but entirely contain HPV capsids (Harper et al., 2004:1761). The body recognizes these particles as foreign and in response to that, makes antibodies against the various HPV types present in a specific vaccine. They are very effective only in young individuals who have not yet been sexually active (i.e. they are not therapeutic and have limited use in patients already exposed to the virus) (Harper et al., 2004:1763; Garland et al., 2007:S31. S33).

The vaccines have limited cross-protection against other HPV types (i.e. they work against at least 95% of the specific HPV types the individual has been vaccinated for only, which is not necessarily what s/he is exposed to (Sankaranarayanan et al., 2013:F50; Saraiya et al., 2015:10; Lamontagne et al., 2017:1). These vaccines have also been shown to be 100% effective in preventing pre-cancerous changes of the cervix (Sankaranarayanan et al., 2013: F50; Lamontagne et al., 2017:8). However, only one research has shown that all available vaccines might also provide cross-protection against other HPV strains not targeted (Herrero, 2009:919-921). Furthermore, the duration of immunity offered by HPV vaccines has not yet been fully elucidated. Current evidence suggests that protection will last for a minimum of 6–8 years (Medscape Medical News, 2019). Nonetheless, sustained follow-up will be needed to evaluate if revaccination is still required (Medscape Medical News, 2019). At present, there are three prophylactic HPV vaccines: Cervarix®, a bivalent vaccine {GlaxoSmithKline; 2007} which targets HR HPV16 and HPV18 (Moodley et al., 2016:497; Tathiah et al., 2015:1); Gardasil®, a quadrivalent vaccine {Merck & Co; 2006}, targeting HR HPV16 and HPV18 and LR HPV6 and HPV11 (Garland et al., 2007:1928; Moodley et al., 2016:497; Guo et al., 2018:2); and Gardasil 9®, a second-generation nonvalent vaccine {Merck; 2014}, with activity in resistance to HR HPV16, 18, 31, 33, 45, 52 and 58 and LR HPV6 and HPV11 (Guo et al., 2018:2; Cohen et al., 2019:171). The three vaccines have shown comparable efficacy, immunogenicity, and effectiveness for the main cervical cancer-related types, HPV16, and HPV18.

The administration of the vaccines is set out as 3 injections over six months (0, 1–2, and 6) for all women and men of 15 years and older; and 2 doses (0 and 6–12) for those who initiate vaccination at 9 through 14 years (Schiller et al., 2012:3-4; CDC, 2019). WHO recommends that all 11 and 12-year-old boys and girls, and additionally young women between 13–26 years of age who have not yet had the vaccine to be vaccinated as a routine in all countries (WHO, n.d.). However, in most resource-constrained settings the vaccination programs include only females of these age ranges. Sinanovic et al. (2000:6197-6201) demonstrated in a SA study that the inclusion of HPV vaccination in an existing cervical cancer screening program would be an inexpensive intervention. The side effects of HPV vaccines reported involve short-term injection site reactions (namely redness, swelling, or pain), fever, nausea, mild headaches, and in some recipients brief fainting spells and dizziness.

The first national HPV vaccination program was implemented in 2007 in Australia, the first country to use Gardasil, the quadrivalent vaccine with over 70% coverage in girls aged 12–13 years (Garland et al., 2007:1928). Vaccination began in SA in 2014 through the initiation of a national school-based HPV vaccination program targeting Grade 4 girls of 9 years and older frequenting public schools (Tathiah et al., 2014:179). Two doses of Cervarix were given 6 months apart. In SA, only two vaccines, Cervarix and Gardasil are currently registered for the prevention of HPV-related diseases (Tathiah et al., 2015:2; Attipoe-Dorcoo et al., 2018:19). Nonetheless, the development of these vaccines helped in the reduction of cancer of the cervix-associated deaths, even though largely in high incomes nations. Furthermore, a previous systematic review and meta-analysis showed that HPV prevalence was significantly reduced when nations attained more than 50% vaccine coverage rates (Drolet et al., 2015:2-3). For example, in the USA cervical cancer cases decreased by 13% and 29% in women aged in the intervals of 25–34 and 15–24 years, respectively, between the term of 2011–2014 as compared to 2003–2006 (Guo et al., 2018:1).

However, the HPV vaccination strategy alone, even with the existence of a certain cross-protection level against other oncogenic virus types, cannot completely prevent cervical cancer.

In consideration of these challenges, it is urgent to identify alternative cost-effective drugs or therapies that are widely powerful HPV infection inhibitors.

1.2.7.2 HPV screening and challenges

HPV screening is an approach targeting the detection of cervical cancer precursors and a test of cure after treatment. Regular screenings with a Pap smear test combined with an HPV test (cytology, HPV DNA test or visual inspection with acetic acid (VIA) of biopsy, swabs, or scrapes

specimens from mucosal surfaces), in women aged 21–65 years, is the most effective way for early cervical cancer diagnosis (Alsbeih, 2018:1425; Bruni et al., 2019b:59; Sawaya et al., 2019:1, 5). Documentary cervical cytology has been the pillar of cervical cancer screening, where successful implementation has had a crucial impact on decreasing the burden of cervical cancer, but the requests of this test are over complex for several LMICs (Denny & Prendiville, 2015a:S28). Alternative tests to cytology have been in search these past years, to permit point-of-care testing, enabling women to be screened and treated in a unique visit, without necessitating complex and high-cost laboratory inspections (Denny & Prendiville, 2015a:S28). In LMICs, these studies have focused mainly on using VIA (which is easy and cheap) and testing the DNA of HR HPV types (which is expensive and requires sophisticated laboratory equipment).

1.2.8 Cervical cancer treatment

To date, there is no pharmacologic cure for cervical cancer, but the disease is treatable if caught early enough and managed effectively through surgery (hysterectomy), radiation therapy, vaginal trachelectomy, cisplatin-based chemotherapy, conization, brachytherapy, or immunotherapy (Denny et al., 2012:F171; Kim et al., 2018:v29; WHO, 2020; American Cancer Society (ACS), n.d.). Depending on the stage and type, treatment can be multimodal using the techniques previously mentioned. Although treatment combinations of cervical cancer are presently allowable, the survival rate after diagnosis is still low. The Cancer Research UK (2020) reported that 61.4% of women diagnosed with cervical cancer between 2013 – 2017 in England survived for five years or more. The American Society of Clinical Oncology (ASCO) recently reported a five-year survival rate accounting for 66% for all women in the USA (Cancer.Net, 2020). Nevertheless, this rate may vary depending on age, race, or ethnicity.

1.2.9 Potential alternative HPV inhibitors and cancer-related drugs

1.2.9.1 Plants as a source of cancer prevention/therapy

Early screening of precancerous lesions has greatly reduced patient deaths in developed nations. However, in LMICs, cervical cancer remains a major cause of death due to the high costs and socio-economic factors. Based on this, there is an urgent need to develop small molecules that will be widely potent antagonists of HPV infection, especially in developing countries.

Recently, investigation of the utilization of natural substances derived from plants, microbial sources, and animals for treatment or prevention of cancer has been growing. Plant-based foods particularly are a reservoir of compounds **(Table 2)** active both in cancer establishment and evolution inhibition (Surh, 2003:768-769; Parekh & Chanda, 2007:766). Ajoene, a derivative of garlic is part of this multitude of compounds and our interest in this study.

Table 2: Dietary plants extracted from natural compounds with cytotoxic activities on cancer cell lines *in vitro* and *in vivo*

Origin	Compound	Cancer Cell Lines	References
Turmeric	Curcumin	Skin, colon, myeloid leukaemia and multiple myeloma	(Singh & Aggarwal, 1995; Plummer et al., 1999; Bharti et al., 2003)
Ginger	[6]-Gingerol	Mouse skin	(Park et al., 1998)
Chilli Peppers	Capsaicin	Mouse skin tumour and human leukaemia cells, Jurkat and melanoma cells	(Han et al., 2001)
Green tea	Epigallocatechin-3-gallate	Human breast cells, mouse epidermal keratinocytes JB6 cell line, head and neck cancer cell lines	(Masuda et al., 2002; Afaq et al., 2003)
Grapes	Resveratrol	Rat and human pancreatic carcinoma cells, human breast cancer cells, human mammary epithelial cells, cervical HeLa cells	(Subbaramaiah et al., 1998; Subbaramaiah et al., 1999; Banerjee et al., 2002; Mouria et al., 2002)
Soybeans	Genistein	Human breast, prostate cancer cell lines, alveolar epithelial carcinoma cells, hepatocarcinoma cells	(Davis et al., 1999; Tacchini et al., 2000)
Garlic	Ajoene	Human promyeloleukamia, basal cell carcinoma, breast, oesophageal transformed fibroblast and cervical HeLa cells	(Dirsch et al., 1998; Antlsperger et al., 2003; Tilli et al., 2003; Kaschula et al., 2011)

1.2.9.2 Heparin and Marine Heparinoid Polysaccharides

Cost-effective HPV-blockage compounds like topical microbicides might be helpful to block HPV infection. Natural compounds are a promising approach of research to find inexpensive, broad-scale HPV infection inhibitors that would be a successful alternative, especially in LMICs. In this regard, polysaccharides derived from marine and other bioactive compounds have been demonstrated to be effective for the establishment of a new generation of HPV inhibitor therapeutics, that work better with fewer side effects (Wang et al., 2014:2019).

The sulphate polysaccharides like heparin and the heparinoid polysaccharides can block HPV uptake by intervening with the primary binding of the viral particle to the host cell (Wang et al., 2014:2023).

Heparin, part of the GAG family of carbohydrates constituted of varying sulphated repeating disaccharide units has previously been found to be a potential HPV inhibitor (Wang et al., 2014:2022). HR HPV16, 31, 33, and 39 strains have been demonstrated to be capable of using heparan sulphate (HS) as a low-affinity co-receptor on the cell surface (Giroglou et al., 2001:1565; Johnson et al., 2009:2068-2073). Additionally, some publications stipulate that HPV16 and HPV33 VLPs cannot infect the cell after heparinase action on the COS-7 (fibroblast-like cell line) or HaCaT (spontaneously human transformed aneuploid immortal keratinocyte cell line), thereby confirming that HS is needed for HPV infection of cells (Giroglou et al., 2001:1566; Drobni et al., 2003:163; Selinka et al., 2007:10970, 10979).

Marine heparinoid polysaccharides have GAG-like biological properties, which constitute ulvans, alginates, and their sulphated extracts, and also chitosan sulphate (also known as sulphate 3,6-O-sulfated chitosan or 36S) and dextran sulphate (DS) (Shi et al., 2017:4-5). A previous study demonstrated that the DS inhibition mechanism of infection included direct interactivity between the compounds and the infectious virions as well as a blockage effect at the cell surface with insignificant cellular toxicity (Christensen et al., 2001:3427-3429). Gao et al. (2018:332-333) recently demonstrated that 36S may inhibit HPV uptake by direct attachment to the viral capsid protein. Furthermore, after entry into the cell 36S can down-regulate the cellular PI3K/Akt/mTOR pathway which is linked to autophagy[11] (Gao et al., 2018:334-335). A multitude of past researches has shown that other marine heparinoid polysaccharides namely fucoidan and alginic acid could as well efficaciously inhibit HPV-PsVs infection just as heparin (Buck et al., 2006:0672-0674).

[11] **Autophagy:** a natural, regulated host defence mechanism that directly destroys intracellular pathogens and induces innate immune response when infection presents (Levine & Kroemer, 2008:1; Kudchodkar & Levine, 2009:1).

An important point to state is that autophagy has been previously shown to play important roles in the detection, degradation, and antigen presentation of many intracellular pathogens (Levine & Kroemer, 2008:1; Kudchodkar & Levine, 2009:1; Mack & Munger, 2012:204, 208-218). For example, a previous study, in HeLa cells, using electron microscopy analysis of autophagosomes over HPV internalization showed that HPV16-PsVs particles contained autophagosomes (Ishii, 2013:385, 387-388). These outcomes suggested that autophagosomes are formed from the plasma membrane. Furthermore, the biochemical inhibition of autophagy experiments performed showed enhanced HPV16-PsVs infectivity (Ishii, 2013:387). Griffin et al. (2014:1) also recently demonstrated that HPV16-PsVs infection in epithelial cell lines is blocked by host autophagy. These results support autophagy's participation in intrinsic host cell protection from HPV infection. Moreover, blockage of autophagy retards HPV16 capsid proteins deterioration in the course of virus trafficking, indicating that host autophagy induced by HPV16 infection limits the efficiency of HPV infection of the natural host cell (Griffin et al., 2013:3-5).

The family of l-fucose-composing sulphated polysaccharides found in brown algae is termed fucan (Dietrich et al, 1995:144). Some algal fucans possess significant pharmacological effects like anti-inflammatory, anti-coagulant (Albuquerque et al., 2004:167, 170), anti-proliferative (Szychowski et al., 2010:9294), and anti-viral activities (Li et al., 2008:1). For instance, studies have reported that the polysaccharide-rich extracts derived from *Sargassum filipendula C. Agardh* have an important anti-proliferative action on HeLa cell proliferation (Costa et al., 2010:24-25). Furthermore, fucoidan made from brown algae was additionally reported to possibly block HPV-PsVs infection (Buck et al., 2006:0672-0674).

Another even more potent anti-HPV is carrageenan, a naturally derived sulphated polysaccharide from marine red algae (Buck et al., 2006:0672; Weiner, 2014:2). Carrageenan is widely commercially used as a thickener in a diversity of cosmetic and food items, ranging from sexual lubricants to infant feeding formulas (Weiner, 2014:1-2). A previous study revealed that carrageenan, particularly i-carrageenans act primarily by preventing HPV-PsVs from binding to the cell surface. This discovery is highly agreeable as carrageenan looks like HS, an HPV cell-attachment factor **(see section 1.2.3)**. I-carrageenan also inhibits HPV infection via a second, post-attachment HS–independent action. Furthermore, certain milk-based products, which constitute carrageenan, were revealed to have the potential to inhibit HPV infection *in vitro*, even after million-fold dilutions (Buck et al., 2006:0676-0678). Moreover, carrageenan has been demonstrated to block HPV genital spreading in a murine cervico-vaginal model (Roberts et al, 2007:857-859).

To date, several inhibitors for HPV infection have been under investigation, however, our present research focuses on the organosulfur garlic compound bisPMB.

1.3 Research outline

BisPMB, an ajoene synthetic analogue has previously been shown to protect against HPV16-PsVs infection of HeLa cells (Chikanya, 2018:22). This Master's thesis builds on this finding to confirm these observations both in cancerous HeLa as well as in non-cancerous NIKS cells using both HPV16-PsVs and HPV18-PsVs.

1.3.1 Hypothesis

BisPMB inhibits oncogenic HPV16- and HPV18-PsVs infections in HeLa and NIKS cells.

1.3.2 Aim

To elucidate the inhibitory effect of bisPMB on oncogenic HPV infection.

1.3.3 Research Objectives

From the research problem as articulated, the main research objectives were planned as follow:

1. To determine the half-maximal inhibitory concentration (IC_{50}) value of bisPMB of HeLa and NIKS cells using cytotoxicity assays.
2. To assess HPV16- and HPV18-PsVs infections in the presence of bisPMB using HeLa and NIKS cells.

1.3.4 Contribution of the research

This study focuses on investigating a worldwide major health problem, cancer of the cervix, as a result of infection with HPV. If bisPMB is identified as an alternative method of preventing HPV-PsVs infection, perspectives in the make-up of drugs based on the molecule structure are conceivable. These drugs could then be incorporated into inexpensive condoms or lubricants. They could function as a broad-spectrum topical microbicide intended to sexually transmitted HPV infection prevention, thus serving as beneficial adjuncts to HPV vaccination programs.

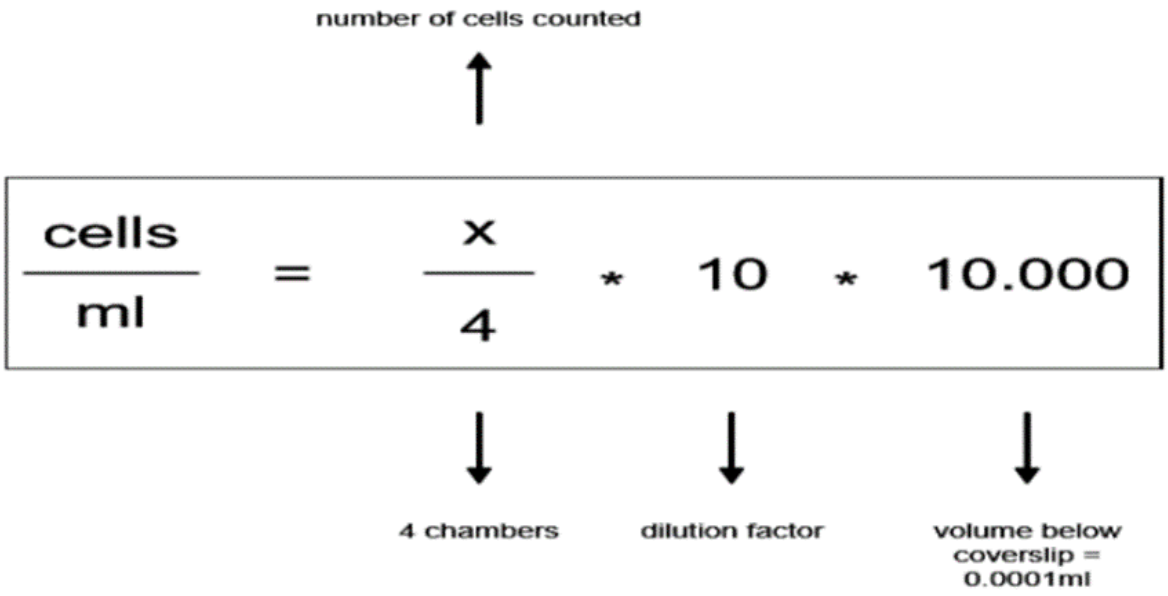
CHAPTER 2
MATERIALS AND METHODS

2.1 Cell Culture

HeLa, human epithelioid cervix carcinoma, the most ancient and commonly studied human cell line (Rahbari et al., 2009:1), was maintained in growth medium Dulbecco's Modified Eagle's Medium (DMEM, Gibco Life Technologies). The media was supplemented with 10% Foetal Bovine Serum (FBS, Sigma-Aldrich), and 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma). The non-cancerous Near-diploid Immortalized Keratinocyte skin (NIKS) cell line, representative of "normal" infection conditions (Mattmiller, 2000) was grown in F-MEDIUM containing 75% Ham's F-12 K medium, 25% DMEM (Gibco Life Technologies), 5% FBS (Sigma-Aldrich), 1% penicillin (100 U/mL) / streptomycin (100 mg/mL) (Sigma), 0.4 µg/mL hydrocortisone (Calbiochem), 5 µg/mL insulin (NovoRapid) , 8.4 ng/mL cholera toxin (CT, Sigma), 10 ng/mL Epidermal Growth Factor ((EGF), Life Technologies) and 24 µg/mL adenine (Sigma). DMEM and F-MEDIUM were kept at 4 ºC.

The cells were split by the addition of 5 mL trypsin solution (0.025% trypsin/0.01% Ethylene diamine tetraacetic acid (EDTA, Sigma)) in 1X Phosphate buffered saline (PBS), for a couple of minutes at 37ºC. The trypsin solution was kept at 4 ºC. 5 mL complete DMEM or F-MEDIUM was added to the 75 cm^2 cell culture flask to cease the trypsin reaction. The cell solution was transferred into a 15 mL fresh falcon tube and centrifuged at 3000 rpm for 3 minutes. The supernatant was carefully withdrawn, and the pellet was resuspended in 10 mL complete DMEM or F-MEDIUM. 2 mL of the cell suspension was added to a sterile 75 cm^2 cell culture flask containing 10 mL fresh DMEM or F-MEDIUM. All cells were conserved in a humidified incubator containing 5% CO_2 at 37 °C. HeLa cells were incubated for 2–3 days and NIKS for 4 days, or until confluent.

A volume of 10 µl of the resuspended HeLa or NIKS cells were transferred to 90 µL of 0.4% trypan blue (Sigma) in 1X PBS for staining in a 1.5 mL microcentrifuge tube. Cells were then counted by placing 10 µL of the solution on a haemocytometer (Neubauer chamber) before seeding. Unstained cells in the four quadrants of the haemocytometer were counted with a Nikon TMS microscope. The formula below was used for cell concentration calculation:



Based on the above-stated calculation, the proper number of HeLa or NIKS cells was added to the proper amount of complete DMEM or F-MEDIUM, respectively. For all experiments, cells were seeded in 96 well culture plates at a density of 5×10^3 cells per well (100 µL per well) and grown overnight. Wells for Dimethyl sulfoxide (DMSO, Sigma) controls and also untreated cells were additionally included.

2.1.1 Freezing cells

For durable storage, the cells were frozen in liquid nitrogen (LN_2), allowing the cell line to be preserved indefinitely. HeLa or NIKS cells were first split 1:5 into a sterile 175 cm^2 cell culture flask three or four days before the freezing. On the day of freezing, healthy and rapidly dividing cells were lifted, pelleted, and resuspended in 1.5 mL of freeze down media (90% complete DMEM or F-MEDIUM supplemented with 10% DMSO (Sigma)). It is recommended to count the cells on the day of freezing and have between $2–4x10^6$ cells per mL in a freezing vial (Merck, 2020). 1.5 mL of the resulting cell suspension was aliquoted in 2.0 mL sterile cryotubes. The aliquot tubes were then placed inside a cryogenic storage container containing isopropanol alcohol, which allows the cells to cool down slowly and kept at -80 °C overnight. The following day, the frozen vials were moved to the LN_2 tank.

2.1.2 Thawing cells

When needed, the frozen cryovials were taken out from the LN_2 storage and directly thawed in a 37˚C water bath. The cells were immediately moved to a 15 mL falcon tube containing an appropriate pre-warmed cell growth medium (10 mL/aliquot). The cell suspension was centrifuged at 3000 rpm for 5 minutes and the supernatant was removed. The cells were resuspended in 10 mL of appropriate fresh culture medium, placed in a sterile 75 cm^2 cell culture flask, and cultured as described above.

2.2 HPV-PsVs production

Investigations on the early steps of the PV life cycle have lately been made more comprehensible by the establishment of high-yield techniques for producing PV-based gene transfer vectors independent of epithelial cell differentiation, known as PsVs, using conventional monolayer cell lines (Buck et al., 2004:751; Buck et al., 2005a:2839-2840; Pyeon et al., 2005:9311, 9313-9315; Buck et al., 2014:445). These vectors are as well known as PsVs and have been important for studying PV assembly, entry, and neutralization, and possibly have future benefits as laboratory gene-transfer tools or vaccine vehicles[12] (Buck et al., 2014:445). Methods for *in vivo* production of PV virions or PsVs include production in keratinocyte raft culture (Buck et al., 2014:445), in cultured monolayers of mammalian cells following infection with recombinant vaccinia or Semliki Forest virus vectors expressing L1 and L2 (Hagensee et al., 1994:4503-4504; Sapp et al., 2005:463-465; Buck et al., 2014:446), or in a tube after reassembly of capsomers in the presence of plasmid DNA (Roden et al., 1996:5877; Unckell et al., 1997:2934-2935; Touzé et al., 1998:112-115). A Previous research by Touzé et al. (1998:111) reported variations in the yield of VLP, suggesting that more than one strain of each HPV genotype must be investigated to obtain the high-level VLP production, useful for the development of HPV vaccines or serological tests.

HPV16- and HPV18-PsVs encapsidating the Gaussia luciferase reporter gene plasmid pGaussia luciferase (pGLuc, Promega) were kindly generated in the virus packaging cell line originated from human embryonic kidney, HEK-293TT[13] by Dr. Georgia Schäfer. The pGluc is a light producing-enzyme naturally obtained from Gaussia princeps[14] and is commonly used to assess the transcriptional activity in cells (Verhaegen & Christopoulos, 2002:4378-4379; Wille et al., 2012:3). HEK-293TT cells were co-transfected with either HPV16- or HPV18-pXULL plasmids (which co-express both codon-optimized HPV16-L1/L2 and HPV18-L1/L2, respectively) and pCMV-GLuc2 following previously published procedures (Buck et al., 2005b:450-451; Marušič et al., 2012:10; Schäfer et al., 2013:3). The HPV-PsVs preparations were stored at -80 ºC.

[12] **Vehicles:** inert media or carriers used as solvents in which a medicinally active agent is formulated and/or administered (Titi Tudorancea, n.d.).

[13] **HEK-293TT:** to generate 293TT cells, 293T cells are transfected by the introduction of SV40 DNA into HEK-293 early mRNA that encodes small t antigen with an SV40 Large T Antigen cDNA expression cassette (plasmid pTIH).

[14] **Gaussia princeps:** a mesopelagic small crustacean found in temperate and tropical waters globally (Yu et al., 2018:1509).

2.2.1 HPV-PsVs preparations quality controls

2.2.1.1 SDS-PAGE and silver staining

To evaluate the purity of the virus stock, proteins (L1 and L2) in the HPV-PsVs preparations were separated using Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and visualized to identify possible contaminants by subsequent staining of the gel with Pierce™ Silver Stain Kit (Thermo Scientific).

Briefly, the 1.5 mm spacer glass plates were securely clipped together on a Casting frame. 12% resolving gel **(Appendix A)** was prepared in a 50 mL falcon tube and poured in between the two glass plates. The level of the gel was defined by placing the comb on the glass-plates, leaving about 1 cm space below the comb (a permanent marker was used to mark up the line). While the gel was let to polymerize for about 30 minutes, a 3% stacking gel solution **(Appendix A)** was prepared in another 50 mL tube. The stacking gel was loaded on top of resolving gel in between the glass plates and the comb inserted immediately after loading. The stacking gel was then let to solidify for about 30 minutes and the comb was vigilantly removed after that. The formed gel and buffer dam were tightly secured on the Casting chamber. The 1X running buffer solution **(Appendix A)** was then poured into the running module. The samples were prepared in 2 mL microfuges tubes, consisting of 3 µL of loading buffer supplemented with 0.1M dithiothreitol (DTT), which reduces any disulphide bridges[15] (S-S Bridges) present between the polypeptides of the proteins, and 3 µL of HPV16- and HPV18-PsVs preparations. The samples were heated at 95 ºC for 5 minutes and pulse centrifuged for 5 seconds. 6 µL of the colour protein standard (ladder) and each HPV-PsVs sample were carefully loaded in the wells using a micropipette. The lid of the tank was closed, and the electrodes were properly inserted in the power supply (red wire connected to the red slot and black wire to the black slot). Voltage was first turned at 100 V for about 15 minutes, then to 200 V for another 50 minutes (or until proteins seated at the bottom of the gel).

Following the electrophoresis, a gel releaser was carefully used to release the gel from the glass plates and the stacking gel trimmed off before proceeding to stain with Pierce Silver Stain Kit (Thermo Scientific). In short, SDS-PAGE gels were washed using a shaker in distilled water (dH_20) (2 × 5 minutes), then fixed in 30% ethanol: 10% acetic acid (i.e., 6:3:1 water: ethanol: acetic acid) solution (2 × 15 minutes). Further washings with 10% ethanol (2 × 5 minutes), then dH_20 followed (2 × 5 minutes) was done. Furthermore, gels were sensitized for staining using a sensitizer working solution (provided in the kit) for 1 minute, then by 2 × 5 minutes washes

[15] **S-S Bridges:** bonds find between two thiol groups which are of major importance in the stabilization of the tertiary and/or quaternary structures of proteins (Rajpal & Arvan, 2013:1721).

with dH_20. Then using a stain working solution (provided in kit), the gel was stained for 30 minutes. The gel was washed in dH_20 (2 × 20 seconds), then by developing with the developer working solution (provided in kit) for 2–3 min until the bands appeared. Lastly, when the desired band intensity was reached, the reaction was stopped with 5% acetic acid for 10 minutes.

Pictures of the gel were taken with the Biospectrum™ 500 Imaging System (Ultra-Violet Products, UVP) and the L1/L2 protein molecular weights (MWs) evaluated using a Colour Pertained Protein Standard, Broad Range (112**–**45 kDa, New England Biolabs).

2.2.1.2 Neutralization assay

To differentiate and evaluate the quality of the two PsVs preparations (HPV16- and HPV18-PsVs), inhibition assays for virus infection were performed. HeLa cells were seeded in triplicates in 96 well culture plates at a density of 5×10^3 cells per well and grown overnight. The following day, neutralization was done by incubation of the HPV16- and HPV18-PsVs with the neutralizing antibodies H16.V5 (positive control) or H18.J4 (negative control) for HPV16 and H18.J4 (positive control) or H16.V5 (negative control) for HPV18 in siliconized tubes for 1 hour at 4 °C (**Table 3**). The two PsVs preparations concentrations were not comparable as they were two parallel performed experiments.

Neutralized HPV-PsVs were then added to the cells (2 µL in each well) and incubated at 37ºC for 24 hours. Infectivity was measured by transferring 10 µL of supernatant from each well into a new white 96 well-plate and assessed for secreted pGluc activity with the GloMax® Explorer (Promega) Microplate Luminometer **(see section 2.4.3 below)**.

Table 3: Preparation of samples used for the neutralization assay

A

HPV16-PsVs neutralization	Tube 1 (untreated)	Tube 2 (non-type specific neutralizing antibody)	Tube 3 (type-specific neutralizing antibody)
	1 µL HPV16-PsVs	1 µL HPV16-PsVs	1 µL HPV16-PsVs
	5 µL H_20	4 µL H_20	4 µL H_20
		1 µL H18.J4	1 µL H16.V5

B

HPV18-PsVs neutralization	Tube 4 (untreated)	Tube 5 (non-type specific neutralizing antibody)	Tube 6 (type-specific neutralizing antibody)
	1 µL HPV18-PsVs	1 µL HPV18-PsVs	1 µL HPV18-PsVs
	5 µL H_20	4 µL H_20	4 µL H_20
		1 µL H16.V5	1 µL H18.J4

For all *in vitro* experiments, cells were always seeded in triplicates for each condition.

2.3. Cytotoxicity assays

2.3.1 MTT assay

The MTT assays were used to indirectly measure cell proliferation at various stages of the experiments by quantifying the cell viability. MTT reagent was prepared using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefore (MTT, Sigma) dye stock of 5 mg/mL in PBS. Briefly, 50 mg of the MTT stock was added to 10 mL of 1X PBS to make a final concentration of 12 mM, filter sterilized, wrapped with aluminium paper for light protection, and stored at 4 ºC. MTT assay is a colorimetric assay that measures the conversion of yellow 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide by mitochondrial succinate co-enzyme Q reductase into insoluble blue formazan crystals (dark purple) (Larkin & Hastie, 2011:397). Cell viability is characterized by the level of activity which can only be measured in metabolically active cells. To evaluate cell viability, 10 µL of the prepared reagent was added to the wells of a 96-well plate containing 90 µL of old culture medium and incubated for 4 hours at 37 ºC until a dark purple precipitate was visible. The old medium was replaced with 100 µL of solubilization reagent (DMSO, Sigma) and incubated for 30 minutes at 37 ºC. The resulting colorimetric intensity was determined using the GloMax® Explorer (Promega) Microplate Luminometer at a wavelength of 600 nm.

2.3.2 Determination of the IC_{50} concentration of bisPMB

To identify non-toxic concentrations of bisPMB for subsequent HPV infection assays, MTT assays were performed in both HeLa and NIKS cells. BisPMB, in solid-state, was dissolved in DMSO to make up a stock concentration of 100 mM and stored at -20 ºC. The IC_{50} of bisPMB of both cell lines was determined using concentrations ranging from 0–100 µM (successively halved), cells were then subjected to 4 hours of treatment. Upon removal of the old treatment medium, a fresh culture medium was added, and cells were incubated for 48 hours. MTT assay was then performed. The cell viability values (absorbance) obtained were plotted against the log-transformed concentration of bisPMB and fitted onto a nonlinear regression log (inhibitor) vs. response equation to construct a sigmoidal dose-response curve using GraphPad Prism version 5.04 software.

2.3.3 Micrographs of cells

To visualize the effect of bisPMB on cell morphology and viability, images were taken of HeLa and NIKS cells after 48 hours of treatment with bisPMB at concentrations between 0–100 µM. Phase-contrast images of the cells in 96 well plates were taken with the Zeiss Axiovert 200M Fluorescent Microscope (Carl Zeiss, Jena, Germany) at 100x magnification.

2.4 Infection and inhibition assays

2.4.1 Treatment with bisPMB

To assess the inhibitory effect of bisPMB on cells, HeLa and NIKS cells were treated with bisPMB concentrations according to the IC_{50} value obtained from the cell viability assays. BisPMB stock of 100 mM concentration, when used in the experiments was then further diluted down to 1 µM, 2.5 µM, 5 µM and 10 µM with corresponding DMSO control concentrations (0.001%, 0.003%, 0.005% and 0.01%).

Cells were either left untreated or treated with bisPMB or the corresponding DMSO control and incubated for 4 hours at 37 ºC (100 µL in each well).

2.4.2 Infection with HPV-PsVs

To determine whether bisPMB could inhibit infection by HPV-PsVs, bisPMB or DMSO composing the culture medium was removed and fresh culture medium containing HPV16-PsVs or HPV18-PsVs encapsidating the Gaussia luciferase plasmid pGLuc (New England Biolabs) at a tissue culture infectious dose (TCID) of 2 pg HPV virions per cell (10 ng per well) was added for 48 hours at 37 ºC. pGluc is a reporter gene vector expressing secretable Gaussia luciferase. During the transcriptional process, the enzyme luciferase is excreted out from the cells into the medium.

2.4.3 Measuring Gaussia luciferase

Successful infection of HeLa and NIKS cells with HPV-PsVs (pGLuc) was measured by transferring 10 µL of supernatant from each well into a new white 96 well-plate and assessed for secreted pGluc activity on the GloMax® Explorer (Promega) Microplate Luminometer using Gaussia Luciferase Assay Kit (Thermo Fisher Scientific). Enough Gaussia Reagent solution was prepared containing 50 µL Gaussia buffer and 0.5 µL Gaussia substrate per well (plus 700 µL for loading the machine), wrapped with aluminium paper for light protection, and stored at -20 ºC. Luminescence was measured at the following conditions: 50 µL injection volume, 2 seconds lag time and 10 seconds integration time. Raw luminescence readings were noted down as Relative Light (luminescence) Units (RLU).

2.5 Final data analysis

To determine comparable levels of HPV-PsVs infection, luciferase readings were normalized to cell viability values. One-way ANOVA and post-hoc Tukey statistical tests (GraphPad Prism version 8.4.3 software) were used to perceive the statistically significant differences between the control and treated samples. P values are recorded significant where * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

CHAPTER 3
RESULTS

3.1 HPV-PsVs preparations quality controls

For the performance of our infection experiments, HPV16- and HPV18-PsVs were prepared and tested before being used. The PsVs preparations are made up of the capsid proteins L1/L2 of HPV16 or L1/L2 of HPV18 respectively, both encapsidating the reporter gene plasmid pGLuc. pGluc, when transcribed and translated, is secreted into the cell culture supernatant, allowing the measurement of successful HPV-PsVs uptake into the cells.

3.1.1 SDS-PAGE and Silver staining

SDS-PAGE of our PsVs preparations followed by silver staining were performed to visualize the Major (L1) and Minor (L2) capsid proteins and assess the purity of our HPV16- and HPV18-PsVs (pGluc) preparations. Both HPV16-PsVs (pGluc), as well as HPV18-PsVs (pGluc), did not have any impurities (contaminants), as exclusively the L1 and L2 protein bands were detected by the SDS-PAGE **(Figure 9)**. Both HPV16-PsVs L1 and HPV18-PsVs L1 proteins have an apparent MW of about 55 kDa in size. While the L2 protein is shown to slightly vary between the two genotypes as expected: HPV16-PsVs L2 protein has an apparent MW size of approximately 80 kDa and HPV18-PsVs L2 protein is approximately 60 kDa.

3.1.2 Neutralization assay

We then tested the inhibition of infection by the PsVs preparations to assess their functionality via neutralization assays. As seen in **Figure 10**, HPV16-PsVs infection in HeLa cells was completely blocked when pre-incubated with the HPV16-specific neutralizing antibody H16.V5, while the HPV18-specific neutralizing antibody H18.J4 had no effect. The opposite was observed when cells were infected with HPV18-PsVs: the HPV18-specific neutralizing antibody H18.J4 neutralized the infection while the HPV16-specific neutralizing antibody H16.V5 had no effect.

These assays guaranteed high purity and quality of the different HPV-PsVs preparations that were utilized for all subsequent experiments.

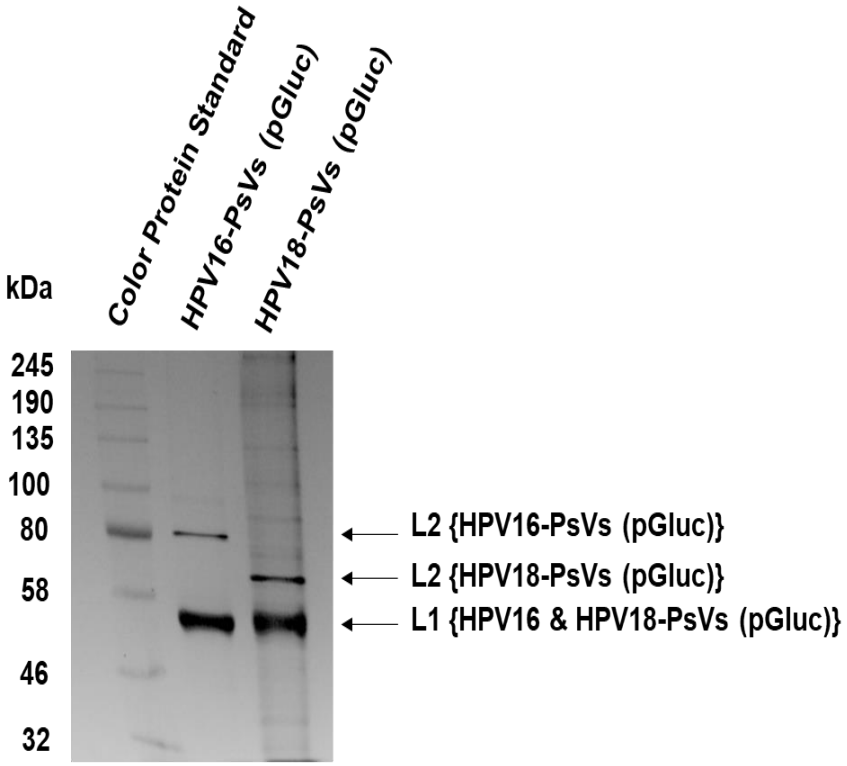


Figure 9: SDS-PAGE followed by silver staining to analyse the purity of the HPV-PsVs encapsidating the reporter gene (pGluc) preparations. The L1 protein of both HPV16- and HPV18-PsVs stocks have an observable size of 55 kDa; L2 protein size apparently differs in the two HPV strains.

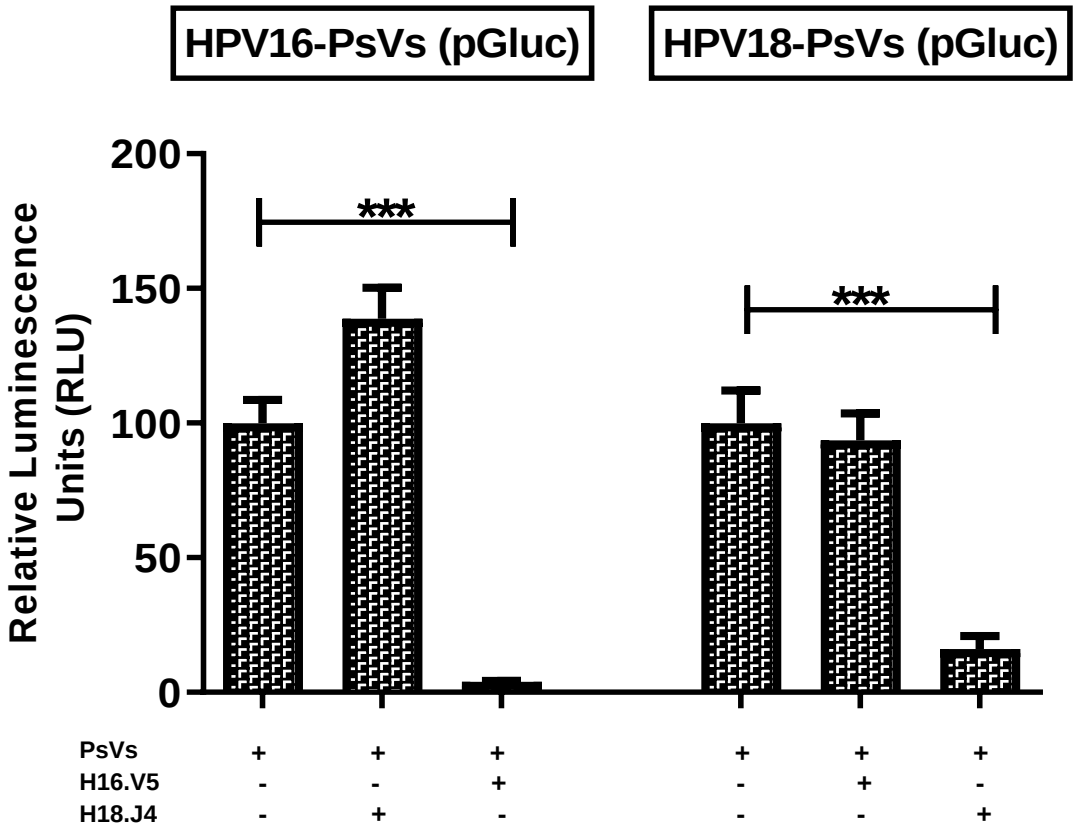


Figure 10: **HPV-PsVs (pGluc) neutralization assay to test the functionality of the preparations.** Control HeLa cells were infected with either HPV16 or HPV18-PsVs (pGluc). Neutralization was done by incubation of each PsVs preparation with antibodies against HPV16-L1/L2 (H16.V5) and HPV18-L1/L2 (H18.J4) as indicated. Secreted Gaussia luciferase activity was measured 24 hours post-infection using the GloMax® Explorer (Promega). Each bar represents the average reading from triplicates samples ± standard deviation (SD) and is representative of one experiment. One-way ANOVA and post-hoc Tukey tests were performed using GraphPad Prism version 8.4.3 and statistical analysis *** indicated $p < 0.001$.

3.2 The effect of bisPMB on HeLa and NIKS cell viability

To characterize bisPMB effect on the human cervix carcinoma HeLa and normal keratinocytes NIKS cell viability, we first performed cytotoxicity assays (MTT), which allowed us to determine the IC_{50} of bisPMB. The MTT assay quantifies cell proliferation by quantitating the formation of an insoluble blue formazan dye produced from the MTT tetrazolium salt cleavage by the mitochondrial dehydrogenase in viable cells.

As treatment with bisPMB at a concentration that inhibits HPV infection should ideally be performed in the sub-toxic range, we then determined the IC_{50} to know at which concentration the compound could show toxic effects on the cells. Both cell lines were treated for 4 hours with bisPMB concentrations ranging from 0–100 µM, successively ½ dilutions were done.

3.2.1 HeLa and NIKS cells IC_{50}

Based on the dose-response curves, absorbance was measured at 600 nm and plotted against log [bisPMB], the IC_{50} value was calculated for HeLa and NIKS cells **(Figure 11)**. Our results demonstrated that cell viability was reduced with increasing bisPMB concentration. The average IC_{50} values determined after 48 hours of treatment of the six independent experiments obtained for HeLa and four for NIKS are presented in **Table 4**. As can be observed, the bisPMB IC_{50} concentration of HeLa cells was found to be 5.20 ± 2.15 µM and that of NIKS cells was 4.83 ± 0.50 µM. Interestingly, bisPMB was found to be marginally more active in NIKS compared to HeLa cells, which display a slightly higher IC_{50}.

Table 4: **Summarized cell viability experiments with bisPMB concentrations, obtained using the MTT assay.** The standard error (SE) and 48 hours average IC_{50} values (µM) with SD were calculated from the dose-response curves of **A)** six HeLa and **B)** four NIKS independent biological experiments.

A

BisPMB concentration	SE	Average $IC_{50} \pm$ SD µM
2.45 µM	1.10	5.20 ± 2.15
2.70 µM	1.34	
7.70 µM	1.17	
6.53 µM	1.08	
5.67 µM	1.30	
6.20 µM	1.29	

B

BisPMB concentration	SE	Average $IC_{50} \pm$ SD µM
4.72 µM	1.07	4.83 ± 0.50
4.40 µM	1.10	
5.56 µM	1.05	
4.64 µM	1.14	

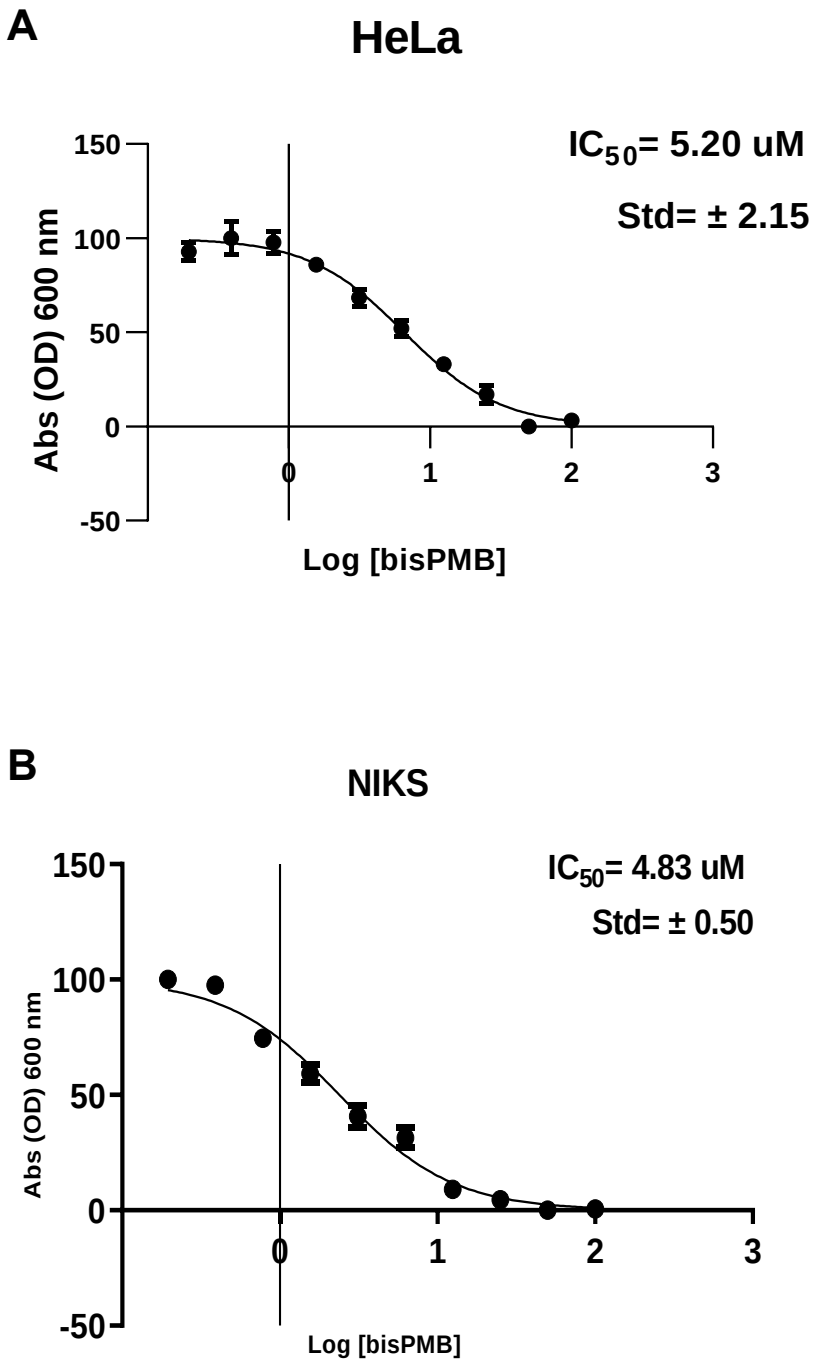


Figure 11: Dose-response curves used for 48 hours IC_{50} determination in A) HeLa and B) NIKS cells. The cell lines were treated and incubated with an array of bisPMB concentrations between 0 to 100 µM for 4 hours and subjected to an MTT assay. Each dot represents the mean cell viability of triplet samples for each concentration from **A)** six independent experiments in HeLa and **B)** four in NIKS. Absorbance was measured at 600 nm using the GloMax® Explorer (Promega). BisPMB concentration (µM) was log-transformed and plotted on the x-axis using nonlinear regression on GraphPad Prism version 5.04.

3.2.2 Effect of bisPMB on HeLa cell morphology

To assess the effect of bisPMB on cell morphology, HeLa cells were used as a representative cell model. The cells were captured after 4 hours of treatment with bisPMB. As shown in **Figure 12**, bisPMB results in a higher number of dead HeLa cells the higher the compound concentration. The untreated cells (control) displayed no change in the initial morphology as well as cell viability, whereas treatment at cytotoxic concentrations leads to rounded morphology and cell death (the original number of viable cells stuck to the plate decreased). Indeed, we observed the same change in morphology and cell death for NIKS cells (data not shown). This can be explained by the swell-up and ultimately burst of cells, when in the incapacity of regulating the in- and out-flow of liquid during necrosis (Selinka et al., 2002:281). This eventually leads to the release of their contents into the neighbouring tissue.

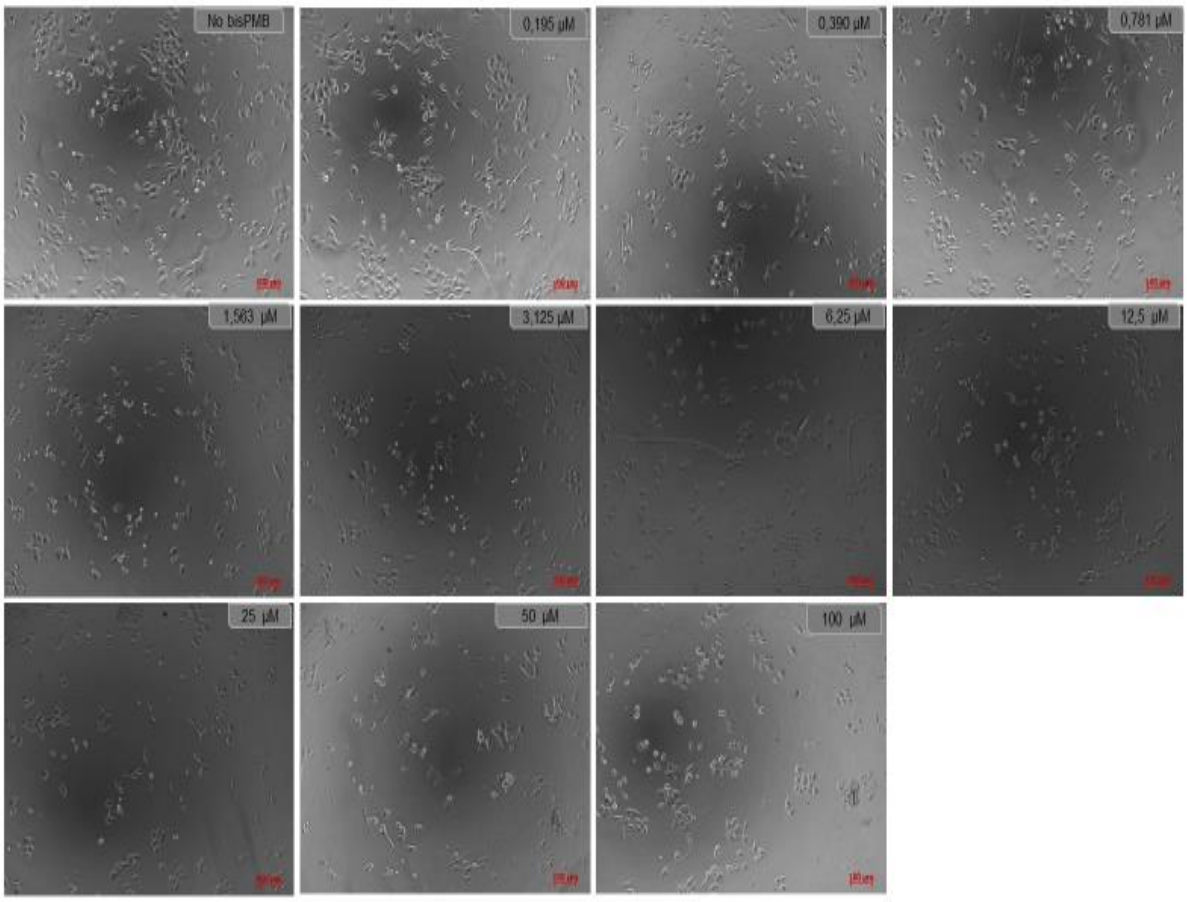


Figure 12: **Morphology of HeLa cells and viability decrease upon treatment with increasing bisPMB concentrations.** The cells were seeded in 96 well culture plates at a density of 5×10^3 cells per well (100 µL per well) and grown overnight. The cells were then treated with the different bisPMB concentrations successively halved (from 100 to 0.195 µM) for 4 hours. ‘’No bisPMB’’ was included as a control and morphology observed under fluorescent microscopy at 100x magnification. The red writing (not visible) is the scale bar that makes all images comparable.

3.3 Infection and inhibition assays

To assess the effectiveness of bisPMB in blocking oncogenic HPV-PsVs infection, *in vitro* tissue culture experiments were performed in HeLa and NIKS cell lines. The experiments were carried out using both HPV16- and HPV18-PsVs strains.

For this study, HeLa and NIKS cells were treated with four bisPMB concentrations (1 µM, 2.5 µM, 5 µM, and 10 µM) deduced from the IC_{50} experiments or corresponding DMSO controls (0.001%, 0.003%, 0.005%, and 0.01%) for 4 hours before the addition of the viral particles for 48 hours. These concentrations provided insight into the molecular response of cervix carcinoma and keratinocyte cells to different cytotoxic concentrations of bisPMB.

All infection and inhibition experiments data were analysed as shown in **Figure 13**. An HPV16-PsVs experiment in HeLa cells is shown for the demonstration of analyses. As represented in **Figure 13 A**, the RLU in the cell culture supernatants (raw data) correspond to the level of HPV-PsVs infection under the different treatment conditions. The first condition represents cells not treated with bisPMB but infected with the virus (control), signifying maximal viral infection. As expected, we observed no effect of the four different DMSO concentrations (0.001%, 0.003%, 0.005%, and 0.01%) on infection as it is the bisPMB 'drug vehicle', with RLU values always almost similar as that of viral infected but untreated cells. In comparison to their corresponding DMSO controls, the four bisPMB treated conditions seem to have a dose-dependent reduction in infectivity as seen by lower RLU.

Cell viability assays (MTT) were performed in parallel to exclude the probability that the changes in RLU values was due to the compound's toxicity rather than an effect on HPV-PsVs infection. As exemplified in **Figure 13 B**, the measured absorbance values represented the number of living cells for each bisPMB and DMSO treatment conditions. As expected, we observed no effect on cell viability by the four DMSO concentrations, with the optical density (OD) values almost the same as the control. Furthermore, the four bisPMB treated conditions in comparison to the DMSO controls showed to have a dose-dependent decrease in cell viability the higher the bisPMB concentration, as seen with low OD values, particularly at 10 µM.

To detect HPV-specific effects, the luciferase readings were normalized to the cell viability readings. By normalizing our data, comparable levels of infection were obtained in only live cells, as seen in **Figure 13 C**.

For a successful inhibition of HPV-PsVs infection, we anticipated seeing a reduction in Gaussia luciferase readings with minimal or no change in cell viability. Statistical differences between the control and treated conditions were identified using One-way ANOVA and post-hoc Tukey tests.

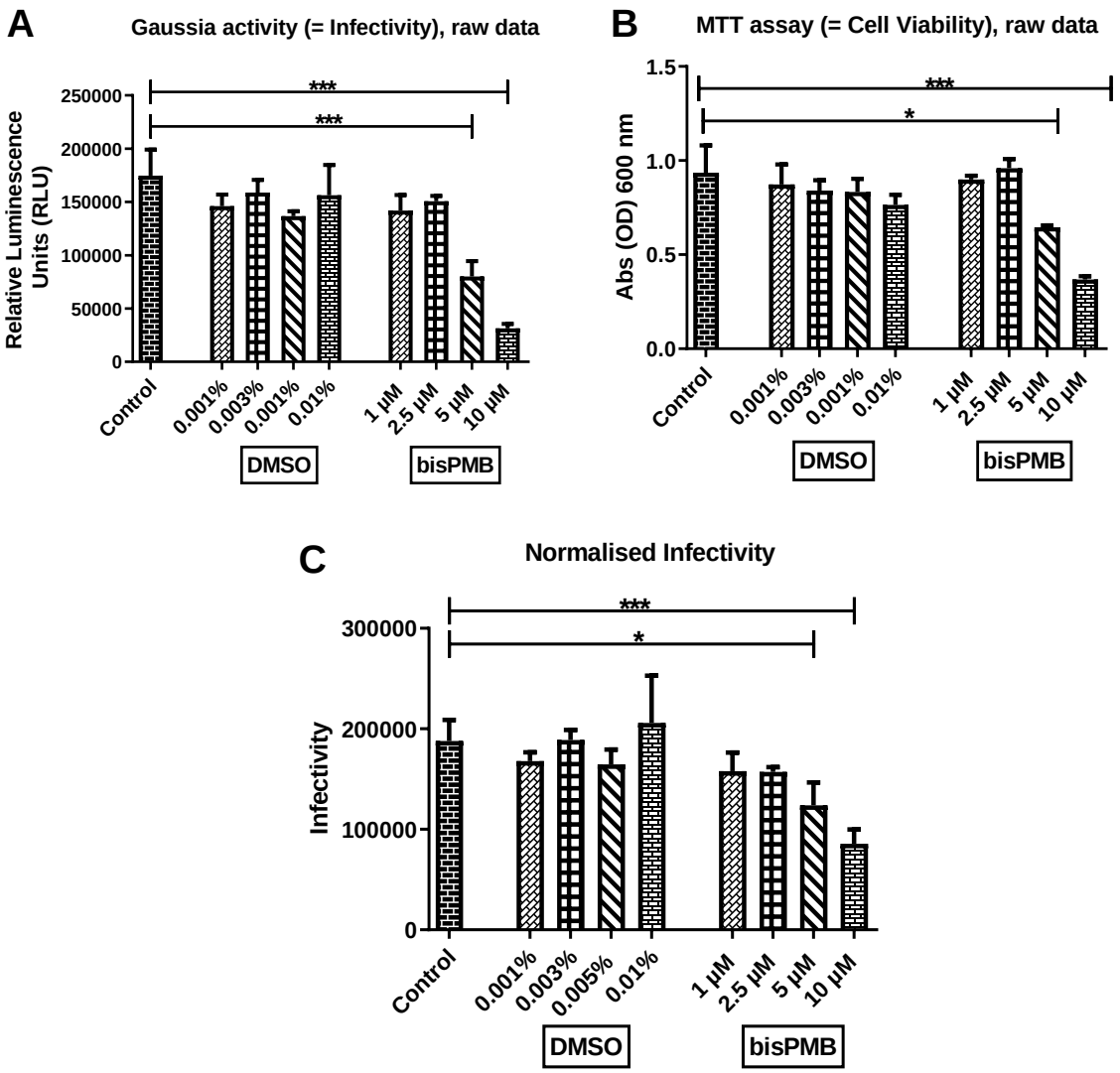


Figure 13: **Data analysis procedure of HPV-PsVs infection and inhibition assays in HeLa and NIKS cells. A)** Shown are the results of a representative experiment conducted in HeLa cells monitoring HPV16-PsVs (pGluc) infection in the absence (control) or presence of bisPMB at 1; 2.5; 5 and 10 µM concentrations. Cells were infected using a TCID of 10 ng virions per well. Luminescence was assessed 48 hours post-infection, RLU is presented. **B)** Average cell viability with bisPMB treatment at 1, 2.5, 5, and 10 µM concentrations in the presence of control. Raw OD values measured at 600 nm absorbance are presented. **C)** Infectivity values as presented in **A)** were normalized to cell viability shown in **B)**. Each data point represents the average ± S.D. from three technical replicates samples. Significances were calculated by means of One-way ANOVA and post-hoc Tukey tests using GraphPad Prism version 8.4.3. (*, $p < 0.05$; ***, $p < 0.001$) indicate a statistically significant difference between the control and treated samples.

3.3.1 BisPMB inhibits HPV16-PsVs but not HPV18-PsVs infection in HeLa cell line

To evaluate the effect of bisPMB on HPV16- and HPV18-PsVs infections in cancerous HeLa, cells were subjected to 4 hours treatment with four bisPMB concentrations (1 µM, 2.5 µM, 5 µM, and 10 µM) or corresponding DMSO controls (0.001%, 0.003%, 0.005%, and 0.01%) before the addition of the viral particles for 48 hours.

HeLa cells infected with HPV16- or HPV18-PsVs and untreated with bisPMB were used as a control and represent maximum infection, i.e. set at 100%. By normalizing the luciferase readings to the cell viability data, the control showed high RLU compared to bisPMB treated conditions as expected **(Figure 14)**. Furthermore, the four DMSO concentrations did not have any effect on the cell viability, showing that DMSO which is used in the preparation of bisPMB has no effect on infection nor inhibition.

As seen in **Figure 14 A**, relative to their DMSO controls, bisPMB at 5 and 10 µM significantly reduced HPV16-PsVs infection, whereas HPV18-PsVs infection was significantly reduced at 10 µM **(Figure 14 B)**. BisPMB at higher concentrations causes cell death, but when normalized (i.e. toxicity took into consideration), there is still an effect on HPV infection. Our normalized data results showed a repeatedly significant reduction of HPV16 and HPV18 infections at 10 µM which may be indicative of another mechanism than simply cell death. We decided to repeat these experiments using NIKS, to compare bisPMB's effect on normal cell line model, as they are representative of "normal" infection conditions (not an already HPV-infected cell line like HeLa).

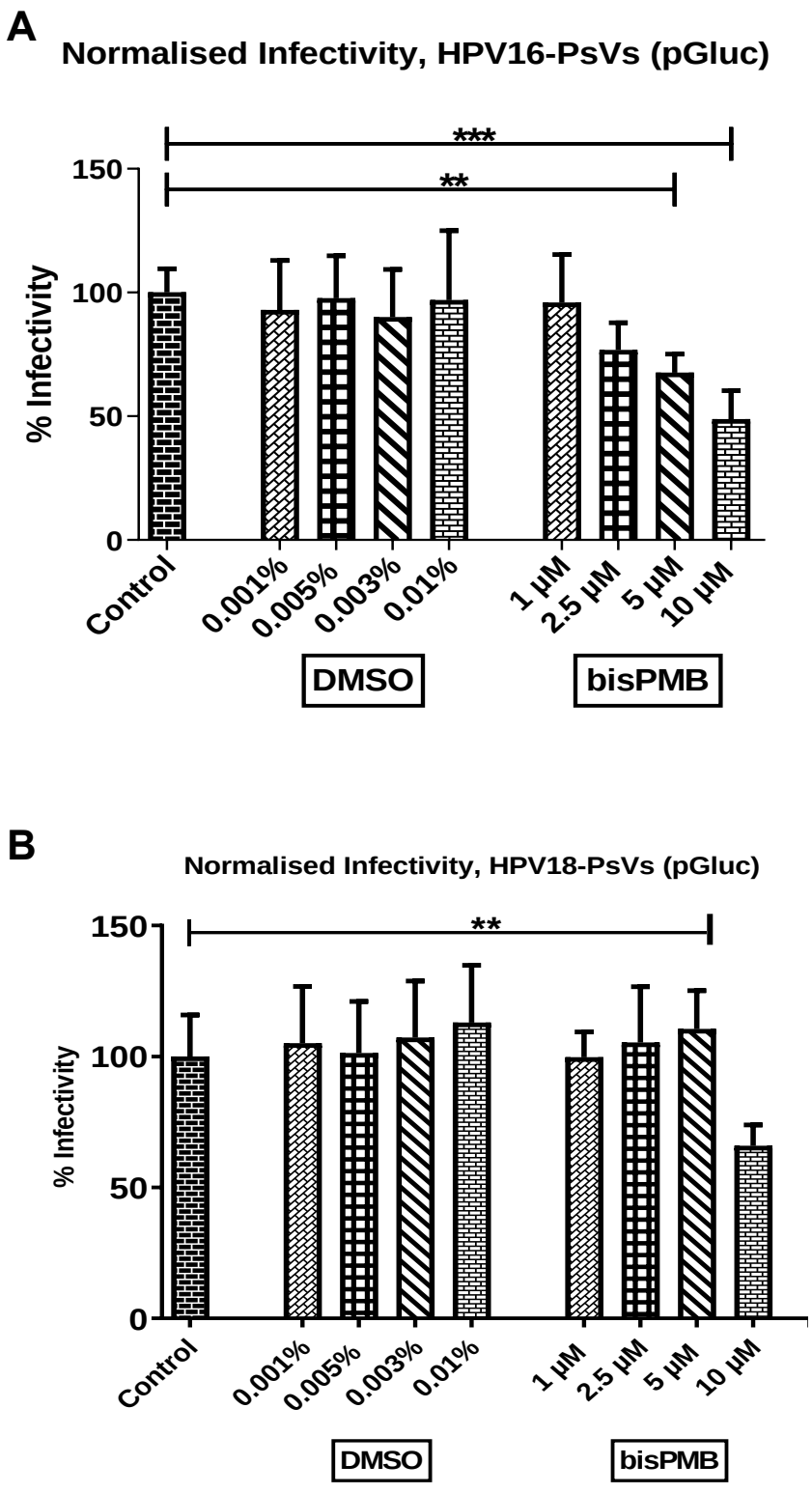


Figure 14: **Effect of bisPMB on A) HPV16-PsVs and B) HPV18-PsVs infections in HeLa cells.** Infectivity values of each experiment were normalized to cell viability and are presented as 100% relative to the control. Cells were infected with HPV16-PsVs or HPV18-PsVs (pGluc) using a TCID of 10 ng virions per well. Luciferase activity was assessed 48 hours post-infection. Shown are representative graphs of three combined experiments for each HPV-PsVs strain in the HeLa cell line. Each bar represents the average values ± SD from triplicate samples. Significances were evaluated using One-way ANOVA and post-hoc Tukey tests (GraphPad prism 8.4.3). (**, $p < 0.01$; ***, $p < 0.001$) indicates a statistically significant difference between the control and in the presence of bisPMB.

3.3.2 BisPMB inhibits HPV16-PsVs but increases HPV18-PsVs infection in NIKS cell line

To assess the effect of bisPMB on HPV16- and HPV18-PsVs infections in non-cancerous NIKS, cells were subjected to 4 hours of treatment with four bisPMB concentrations (1 µM, 2.5 µM, 5 µM, and 10 µM) or their corresponding DMSO controls (0.001%, 0.003%, 0.005%, and 0.01%) before the addition of the viral particles during 48 hours.

Untreated NIKS cells infected with HPV16- or HPV18-PsVs, respectively, were used as a control and represent maximum infection, i.e. set at 100%. By normalizing the luciferase readings to the cell viability data, the control showed high RLU relative to the control as expected **(Figure 15)**. As expected, the four DMSO concentrations did not affect the cell viability, i.e. the DMSO which is used in the preparation of bisPMB has no effect on infection.

As seen in **Figure 15 A**, relative to their DMSO controls, HPV16-PsVs infection showed to be significantly reduced by bisPMB at 2.5, 5, and 10 µM. Surprisingly, HPV18-PsVs infection was significantly increased at bisPMB concentrations of 5 and 10 µM **(Figure 15 B)**.

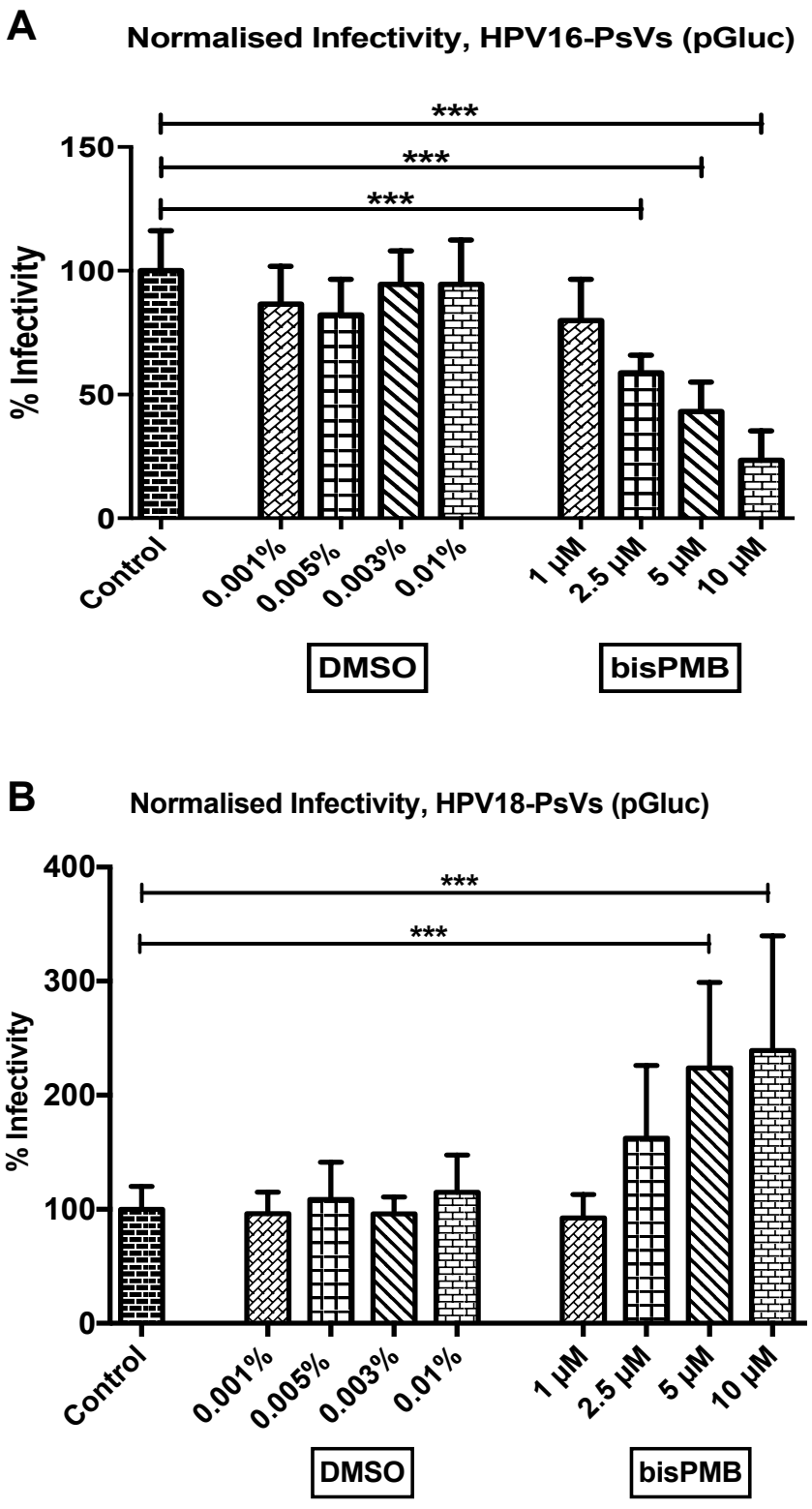


Figure 15: Effect of bisPMB on A) HPV16-PsVs and B) HPV18-PsVs infections in NIKS cells. Infectivity values of each experiment were normalized to cell viability and are presented as 100% relative to controls. Each graph is representative of three combined experiments for each HPV-PsVs genotype in the NIKS cell line. All data points are representative of the average of values ±SD from triplicate samples. Cells were infected with HPV16-PsVs or HPV18-PsVs (pGluc) using a TCID of 10 ng virions per well. Luciferase activity was assessed 48 hours post-infection. One-way ANOVA and post-hoc Tukey tests statistical analysis were done with GraphPad Prism 8.4.3. (***, $p < 0.001$) represents a significant difference between the control and in the presence of bisPMB.

CHAPTER 4
DISCUSSION, CONCLUSION, AND FUTURE WORK

This research focused on investigating a worldwide major health concern predominantly in LMICs, cervical cancer. Although secondary prevention by way of national screening programs has considerably helped in the reduction of cervical cancer incidence and mortality estimations in high-income nations, limitations to implement and sustain comparable programs in SSA have restricted the efficacity of screening in SSA. The reasons for this being related to weak healthcare systems rendering the initiation and/or sustainment of cervical cancer screening programs unattainable and lack of access to the current therapies (Jemal et al., 2012:4372; Denny & Prendiville, 2015:S28). The burden of cancer of the cervix in SSA is of considerable importance, and improved prevention is highly needed to reduce this sickness morbidity and mortality rates.

Cervical cancer primary prophylaxis via HPV vaccination is currently being implemented in numerous LMICs, including SA. Although the available prophylactic vaccines present great efficacity against certain HPV types, they are not therapeutic (i.e. they require the recipient to be uninfected). This being a huge number of sexually active women already infected with HPV still existing. Furthermore, more than one dose is essential for these vaccines based on the recipient's age, making them too costly for general use worldwide particularly in LMICs, plus their protection is limited to a few numbers of oncogenic HPV strains (Harper et al., 2004:17-20; Villa et al., 2005:277). This clearly shows the need to identify affordable and efficacious novel alternatives to prevent HPV infection.

Even though it is believed that medicinal plants will go aside in human life gradually, people progressively figure out the difficulties of chemical medicines use (due to their broad side effects) and considerations return to the medicinal plants (Bayan et al., 2014:1). Medicinal plants are a reassuring route for research and can be looked at as an alternative to vaccination (De Wit & Cook, 2014:494). Dietary plant-derived compounds have been exhibited to have inhibitory effects on the initiation of cancer infections **(Table 2)**. For this purpose, numerous previous investigations have stated the importance of cost-effective anti-virals by a wide range of HPV strains, viz. marine-derived compounds, and carrageenan (Huang et al., 2012:5-7)**.**

Our present study focused on bisPMB, a synthetic analogue of ajoene derived from garlic OSC. BisPMB is known to have selectivity for cancer cell lines (Kaschula et al., 2011:262). We investigated in more detail its cytotoxic mechanism against HPV infection using HPV16- and HPV18-PsVs, well known as clear and powerful carcinogens of the woman cervix in the cancerous HeLa and non-cancerous NIKS cell lines. We aimed to elucidate whether bisPMB

could have anti-viral effects for the prevention of HPV infection, the main pathogen responsible for cervical cancer.

If eventually shown as an effective anti-HPV, bisPMB should be used for the prevention of HPV-PsVs infection rather than treatment where localization to the basement membrane and basal keratinocytes seems very complex. Approaches in the make-up of drugs based on the molecule structure are then conceivable. Oral formulations of the drug would be problematic, as localization to the cervix is not likely and topical application might as well be problematic. These drugs could then be incorporated into inexpensive condoms or lubricants for use by individuals. They could function as a broad-spectrum topical microbicide intended to sexually transmitted HPV infection prevention.

4.1 The anti-cancer activity of garlic organosulfur compounds (OSC)

4.1.1 Composition of OSC in a garlic clove and its chemo-preventative activity

Garlic is possibly the most broadly reported plant with medicinal capacities known in publications. Member of the Allium genus including onions, shallots, leeks, chives, and garlic (*Allium sativum* L.), have been utilized for ages for their health-improving benefits in traditional medicine (Schäfer & Kaschula, 2014:233; Nicastro et al., 2015:1-2; Siyo et al., 2017:1). When crushed, the garlic clove releases a group of about 33 sulphur-composing polysulfanes termed u OSC aimed at chemical defence from the pathogen invasion (Gebreyohannes & Gebreyohannes, 2013:401; Schäfer & Kaschula, 2014:232-233; Siyo et al., 2017:1-2; Kaschula et al., 2019:2). Garlic has the highest collection of OSC than any other Allium species (Gebreyohannes & Gebreyohannes, 2013:401). These crushed cloves are the operative principles accounting for the bioactivity of garlic (Schäfer & Kaschula, 2014:232; Nicastro et al. 2015:2). Allicin (S-allyl cysteine sulfoxide) is the most abundant and biologically operative compound of garlic and is attributed to its strong odour as well as most of its anti-microbial properties (Gebreyohannes & Gebreyohannes, 2013:402; Burian et al., 2017:849). In the full unbroken cloves, the enzyme alliinase and its substrate alliin are isolated into partitions. When the clove is eventually broken, previously compartmentalized alliinase and alliin then associate and that enzymatic reaction result in the production of allicin (Yi & Su, 2013:363; Kaschula et al., 2019:2). This unstable allicin is promptly capable of undertaking thiosulfate/disulphide interchange or decomposing into a collection of more stable oil and water metabolites constituting the second OSC generation of which the principal components include diallyl sulphide (DAS), diallyl disulphide, diallyl trisulfide, and ajoene **(Figure 16)** (Kaschula et al., 2010:80; Kaschula et al., 2017:3).

Epidemiological studies have shown that raw garlic derivatives decrease the levels of (blood pressure, blood sugar, and blood cholesterol) (Gebreyohannes & Gebreyohannes, 2013:402).

Garlic increases blood coagulation time as well, along with the treatment of microbial infections and various diseases among which cardiovascular disorders (Block et al., 1986:7045-7046; Gebreyohannes & Gebreyohannes, 2013:402-404). Garlic is known as a natural immunity booster, as OSC induce higher immune system activity which permits killer cells or macrophages to be more active, resulting in the sustainment of well-being (Lau et al., 1987:140; Abdullah et al.,1988:439). As an example, in the USA, trials in HIV/AIDS patients have revealed the raise of natural killer cell action with the use of garlic extracts. Moreover, it is an effective anti-oxidant helping in the protection of cell destruction by free radicals in the body and also stimulates the lymphatic system to accelerate waste product evacuation from the body (Gebreyohannes & Gebreyohannes, 2013:406; The Garlic Farm, 2020). Another fortunate ability of garlic is the reduction of oxidative stress, protecting the cells from its bad effects which might result in affecting the neuroendocrine and autonomic nervous systems (Gebreyohannes & Gebreyohannes, 2013:406). Garlic is additionally used for drug toxicities and 'pharmacokinetics'[16]. In this sense, OSC can prevent the reduction of glutathione levels, a compound required for the liver to ease the detoxification of substances (Gebreyohannes & Gebreyohannes, 2013:406). Scientific investigations state that garlic use in multi-drug resistant tuberculosis patients allows successful management because of its cost-effectiveness and the non-existence of toxic effects (Catia et al., 2011:465-465).

Garlic is able to elicit both preventative and therapeutic potentials. In prevention, garlic has been reported to possess anti-microbial properties. Garlic has been demonstrated to knock out multiple common cold and flu viruses as well as a wide number of gram-positive and negative bacteria, intestinal parasites, fungus, and yeast (Shams-Ghahfarokhi et al., 2006:321; Burian et al., 2017:849-851). Ruddock et al. (2005:329) at the University of Ottawa previously tested the effectiveness of 19 natural health products containing garlic and additionally five fresh garlic extracts against *Neisseria gonorrhoeae, Enterococcus faecalis,* and *Staphyllococcus aureus*. The most effective products at eliminating these bacteria were the ones with the highest allicin composition, then confirming the infection-fighting capacity of garlic. Bakri and Douglas (2005:645, 648-649) demonstrated that garlic extract inhibits disease-causing bacteria in the mouth and may be relevant in fighting periodontitis[17]. Furthermore, allicin can be used to prevent yeast infections or ameliorate present conditions, particularly when it's used along with a regimen like the Candida diet or medication (Healthline, 2020). A study in 2006 tested garlic against 18 Candida strains and findings revealed that garlic may be a promising avenue in reversing the effects of fungus growth (Shams-Ghahfarokhi et al., 2006:322). Lu et al. (2012:1918-1920) demonstrated that the garlic-derived

[16] **Pharmacokinetics:** branch of pharmacology dedicated to the study of the movement of drugs administrated throughout the body (Merck Sharp & Dohme Corp, 2019).

[17] **Periodonititis:** a severe gum infection that damages the soft tissue and bone that support the teeth (Bakri & Douglas, 2005:646).

compound DAS was 100 more active than the antibiotics erythromycin and ciprofloxacin at fighting *Campylobacter jejuni*, a common type of bacteria causing food poisoning. Moreover, garlic extract has been demonstrated to have inhibitory effects on the infectious bronchitis virus (IBV) (a coronavirus) in the chickens embryo (Shojai et al., 2016:458).

Many epidemiological investigations have shown a link between regular garlic intake and reduced cancer risks specifically of the digestive tract, namely stomach, oesophagus, and colorectal cancers (Fleischauer & Arab, 2001:1034S-1037S; Gebreyohannes & Gebreyohannes, 2013:403-404; Zhou et al., 2020:1, 7). For instance, Gao et al. (1999:614, 618) demonstrated that high amounts of Allium vegetable consumption were linked with the reduction of the stomach and oesophageal cancer risks in the province of Jiangsu in China. Some studies have additionally revealed that garlic intake reduces the prevalence of strokes, coronary thrombosis, and atherosclerosis, as well as oesophageal, skin, colorectal, colon, and lung cancer-associated deaths (Dai et al., 2010:6-7; Turati et al, 2014:174-176; Zhou et al., 2020:1). Fresh garlic OSCs have been demonstrated to protect from carcinogenesis in culture as well as mouse xenograft models through constructing the carcinogen-DNA adduct and blocking cell proliferation and inducing apoptosis and tumorigenic genes, along with altering the xenobiotic metabolism[18] and reorganizing the cell-cycle (Shukla & Kalra, 2007:173-174; Powolny & Singh, 2009:2-4; Altonsy & Andrews, 2011:1104; Siyo, 2015:39-40). As an example, garlic extracts are reported to inhibit the activation of the mutagens such as aflatoxin B1 and 4-nitroquinoline-1-oxide in *Salmonella typhimurium* and *Escherichia coli* (Tadi et al., 1991:2037; Kweon et al., 2003:2520-2522). Moreover, the garlic antiseptic effect has been demonstrated in the treatment of mild intestinal infections such as diarrhoea and additionally lung infections namely bronchitis (Shojai et al., 2016:458).

Nevertheless, garlic has also been reported to have some adverse effects. The major side effect of garlic intake is offensive breath odor, precisely in case of usage of the plant raw form (Rana et al., 2011:61; Gebreyohannes & Gebreyohannes, 2013:406). Vomiting, diarrhea, headache, dizziness, insomnia, tachycardia, flatulence, and nausea have been reported as well (Rana et al., 2011:61-65). Ingestion of high doses of raw garlic on an empty stomach can induce modifications in the intestinal flora and gastro-intestinal upset (Piasek et al., 2009:153-155; Batiha et al., 2020:12). Furthermore, blisters dermatitis, and burns have been observed from raw garlic local applications (Piasek et al., 2009:153-155). Previous *in vivo* studies demonstrated that long-term feeding with raw garlic in high doses caused weight loss and anemia due to red blood cell lysis, while administration of 5 mL/kg of raw garlic juice led to stomach injury that finally resulted in death (Mathew & Biju, 2008:30).

[18] **Xenobiotic metabolism:** also called drug metabolism, is the biological process of transforming less polar compounds into more polar so they can be easily excreted (Mc Ginnity & Grime, 2017:35).

Although restricted in their capacities to evaluate Allium intake, the vast majority of the epidemiological researches conducted suggest a protective and curing role of garlic consumption against different cancer diseases.

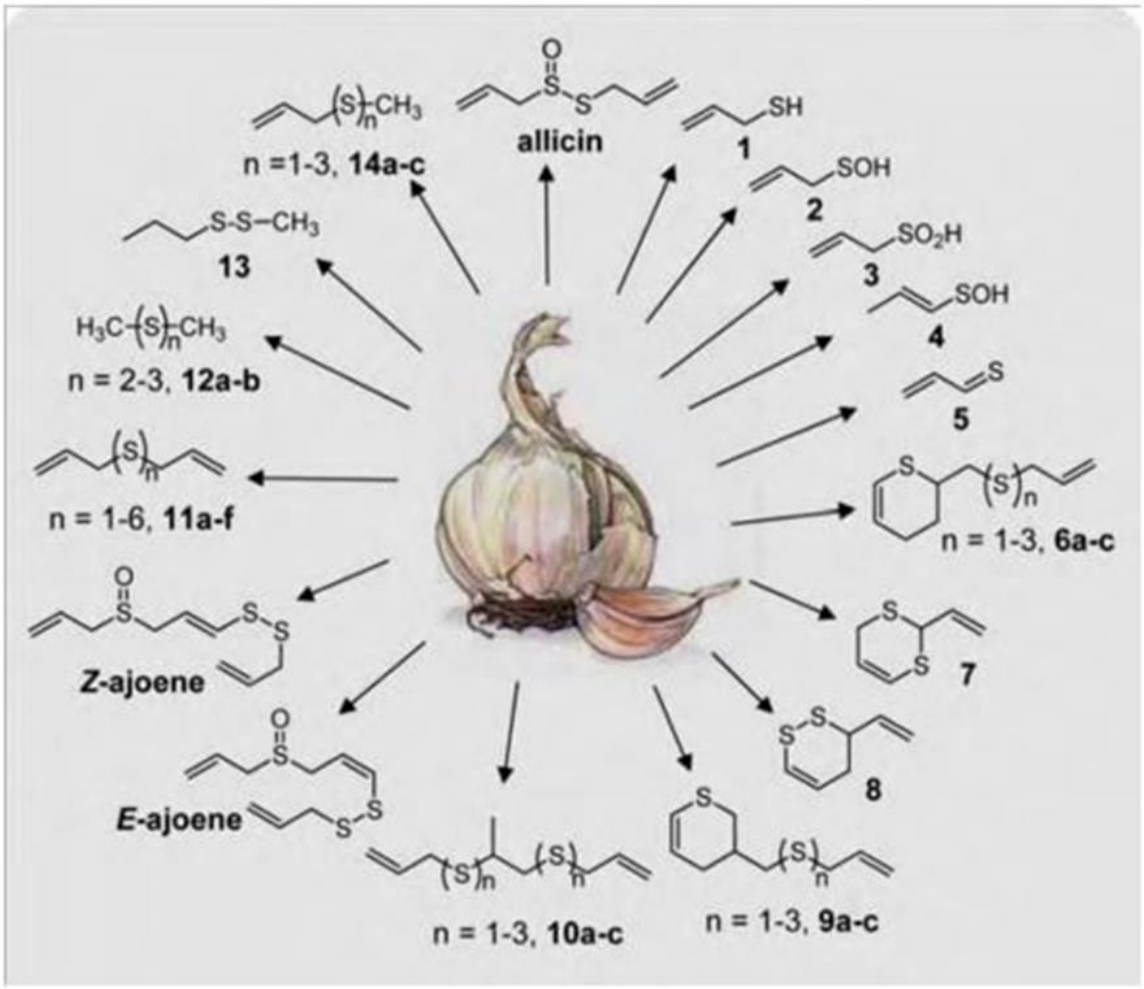


Figure 16: Chemical structures of Allicin degradation products obtained from macerated garlic extracts

(Kaschula et al., 2010:79)

4.1.2 Ajoene and its analogue compound bisPMB

Ajoene, with its vinyl disulphide-sulfoxide rarely found in other natural products (Siyo et al., 2017:2), is part of the phytochemicals[19] released during heated garlic cloves crush (Li et al., 2002:573; Kaschula et al., 2011:260-261). Ajoene and its associated family members have been hypothesized to interfere with tumour biological processes by inhibiting its initiation through the action of S-thiolating reactive cysteines in targeted proteins (Hunter et al., 2008:5278-5279; Schäfer & Kaschula, 2014:234-235; Siyo et al., 2017:3) being cytotoxic for tumour cells *in vitro* in the low micromolar range (Dirsch et al., 2002:75; Antosiewicz et al., 2004:123; Taylor et al., 2006:300). Garlic components can decrease fibrin formation and are more potent than aspirin in the reduction of fibrin existing in the blood (Fukao et al., 2007:89). Ajoene has been related to this anti-clotting activity.

Ajoene has as well been demonstrated to have an anti-proliferative activity against cancer cell lines, and this effect is related to its ability to block the initiation of G2/M cell cycle arrest and activation of the mitochondrial-dependent caspase cascade during apoptosis (Shukla & Kalra, 2007:175-176; Powolny & Singh, 2008:2-3). For instance, some publications report that ajoene inhibits the cell cycle development of the human leukaemia HL-60 and human lymphoma U937 cells at the G2/M phase (Xu et al., 2004:171, 176-178; Ye et al., 2005:516, 518-519). A recent study demonstrated the capability of ajoene to covalently attach to Cys-328 of vimentin in cancer cells, provoking the condensation and disruption of the filaments, and also a decrease in the amount and size of the cells (termed shrinking) (Kaschula et al., 2019:1, 6). The study showed that cancer cells treated with ajoene are less capable of migrating and invading the ECM than untreated ones. Vimentin is a type III intermediate filament protein freshly discovered as a target of ajoene, being reported important in the maintenance of the structural integrity of normal cells and epithelial-to-mesenchymal evolution (Goldman et al., 1996:975-977; Sripada & Dayaraj, 2010:2-4). Furthermore, a previous study identified vimentin as a viral restriction factor preventing HPV16-PsVs infection (Schäfer et al., 2017:1, 6-8). The researchers in the study hypothesised that vimentin acts in modulating the HPV entry process, possibly by intercepting with the attachment of viral particles at the cell surface and uptake of molecules.

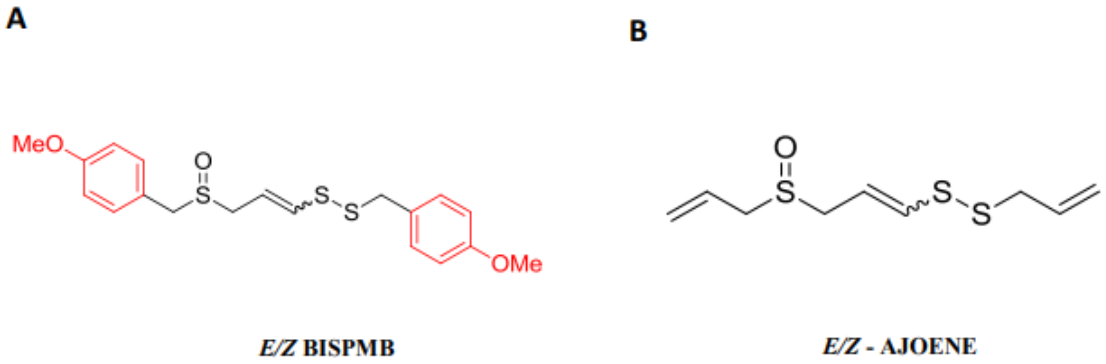
Structure-activity investigations showed that some ajoene analogues substituted with R^1 end-groups were more potent than their predecessor ajoene, those substituted with para-

[19] **Phytochemicals:** powerful chemicals compounds produced by plants (Thakur et al., 2020:341).

methoxybenzyl at both the R^1 and R^2 terminal ends being the most powerful ones (Hunter et al., 2008:5278-5279; Kaschula et al., 2011:239-240).

BisPMB is an analogue of ajoene, developed (and synthesized) by Dr. Catherine Kaschula and her research team from SUN (Kaschula et al., 2015:1217-1224). The bisPMB we have used in our study was generously prepared by Dr. Daniel Kusza at UCT. This compound bisPMB contains the same vinyl disulphide pharmacophore as its parent, ajoene. BisPMB **(Figure 17 A)**, an R^1 and R^2 para-methoxybenzyl substituted ajoene **(Figure 17 B)**, has previously been demonstrated to have a 12-fold enhanced anti-proliferative effect on diverse cancer cell lines namely cervical HeLa, transformed fibroblast CT1, breast MDA-MB-231 and oesophageal WHCO1 cancer cell lines compared to its natural parent, ajoene (Kaschula et al., 2011:262-263; Kaschula et al., 2012:245-246). Interestingly, bisPMB is known to have (2–3 fold) selectivity for cancer cells including the human prostate cancer PC3, nerve cancer CT1, breast cancer MDA-MB-231, and especially WHCO1 oesophageal cancer cells over the human diploid WI31, human prostatic PNT1A, mammary epithelial MCF12A and human oesophageal epithelial EPC2 normal cell counterparts (Kaschula et al., 2011:262). BisPMB best selectivity and activity have been exhibited against WHCO1 oesophageal cancer cells (Kaschula et al., 2011:262) (Siyo, 2015:33). BisPMB was demonstrated to promote a G2/M cell cycle arrest in WHCO1 cells in a concentration-dependent way. This cell cycle arrest action of bisPMB can be associated with the disturbance of the microtubule system which previously has been demonstrated for normal marsupial kidney Ptk2 cells upon Z-ajoene treatment (Li et al., 2002:573, 575-576).

Taking this into account, bisPMB is attractive as a potential inhibitor of HPV infection. Considering that bisPMB is an analogue of ajoene and the two compounds share a common pharmacophore (Hunter et al., 2008:5278; Kaschula et al., 2011: 260-261; Kaschula et al., 2012:237)**,** it is then likely that bisPMB acts through identical molecular mechanisms as its originator ajoene.



E/Z BISPMB

E/Z - AJOENE

Figure 17: **Chemical structures of A) E/Z bisPMB and B) E/Z ajoene in isomeric mixtures representations.** The substituted R^1 and R^2 p-methoxybenzyl end groups are represented in red.

(Siyo, 2015:29)

4.2 Project discussion

4.2.1 Cell lines used in the project

Among the important scientific discoveries of the last century was ‘’HeLa’’, the first immortal human cell line that revolutionized the research world. HeLa cells are the most widely cultured cell line by scientists for research. They are a relatively cheaper alternative to *in vivo* study model and can also divide indefinitely, contradictorily to normal human cells which divide around 40 to 50 times before death (Cram, 2020). Regarding this, HeLa cells were first selected for this research.

Human keratinocyte monolayer cultures present as well as an important model to study the early stages of HPV infection, as it only occurs in basal proliferating keratinocyte, and virus assembly and release uniquely take place in differentiated upper-layer keratinocyte (Griffin et al., 2014:2). Accordingly, NIKS, one of the three most widely recognized keratinocyte models for HPV infection study (including the primary human foreskin keratinocyte (HFK) and HaCaT cell lines) was additionally selected for this study (Ozbun, 2002:11291-11292; Griffin et al., 2014:2-3; Schäfer et al., 2017:1, 13). HFK is acknowledged as the most suitable native host cell line for HPV infection, however, it is rather difficult to isolate and grow, plus these cells are very complex to infect with HPVs (Day et al., 2008:12567; Griffin et al., 2013:6-7). NIKS and HaCaT have been previously demonstrated to be far more permissive to HPV infection compared to HFK (over 100- and 1000-folds greater respectively) (Hoffmann et al., 2000:452-454; Ozbun, 2002:11298; Griffin et al., 2013:3; Griffin et al., 2014:2).

4.2.2 HPV-PsVs preparations and quality controls

HPV-PsVs containing reporter genes provide convenient and quantifiable methods for the determination of relative HPV infectivity (Griffin et al., 2014:3). As several pathogens must be manipulated in biosafety level 3 or 4 containment laboratories, research, and development of vaccines or anti-virals against infectious diseases are sometimes disrupted (Li et al., 2018:1). In this sense, other possible approaches have been intensively investigated, more especially the use of PsVs instead of wild-type (WT) viruses (Li et al., 2018:1). PsVs have been found to be safe handling in biosafety level 2 laboratories as they lack some virulent gene sequences of their parent virus, eliminating the possibility that they could cause an active infection to an exposed individual (Welch et al., 2016:13-14). PsVs can replicate only once upon entry into susceptible cells compared to WT viruses that generally replicate many times (Zhang et al., 2017:1815). Most importantly, the conformational structure of these pseudoviral surface proteins presents a high resemblance to that of native viral proteins (Li et al., 2018:2), rendering it possible to carry out a mechanistic investigation on viral entry as well as evaluating potential neutralizing antibodies (Radoshitzky et al., 2007:92; Robinson et al., 2016:2). Moreover, data obtained from *in vitro* PsVs-based anti-viral experiments and *in vivo*

biodistribution assays have been reported to be very consistent with the results obtained when using WT viruses (Wright et al., 2008:2208-2211; Zhou et al., 2016:2730).

Because PsVs have been engineered to carry reporter genes, they are much easier to manipulate experimentally than live WT viruses (Liu et al., 2012:4; Sehr et al., 2013:10). Interestingly, the number of PsV-infected cells has been shown to be directly proportional to the reporter gene expression. In the context of our study, we used one type of luciferase reporter, pGluc to incorporate HPV16- and HPV18-PsVs, the most widely studied and best-characterized sexually transmitted oncogenic HPV genotypes (Robadi et al., 2018:693).

Quality controls of our HPV16- and HPV18-PsVs stocks using HeLa as control cells demonstrated their purity and good quality. Both HPV16-PsVs L1 (pGluc) and HPV18-PsVs L1 (pGluc) showed to be similar in MW sizes while HPV16-PsVs L2 (pGluc) and HPV18-PsVs L2 (pGluc) not. It has been previously found that L1 and L2 protein sizes vary moderately between HPV genotypes (Icenogle, 1995:1), meanwhile, there is still no conclusive explanation(s) yet to L2 divergent motility. More interestingly, the neutralization assays showed that the HPV16-specific antibodies were only able to block the HPV16-PsVs preparation, as well as HPV18-specific antibodies only blocked the HPV18-PsVs preparation, perfectly distinguishing our two HPV strains used in this research.

4.2.3 The effect of bisPMB on HeLa and NIKS cell viability

Firstly, the cytotoxicity assays (MTT) performed in both cell lines demonstrated that bisPMB increased cell death the higher the concentration. The IC_{50} of BisPMB in both HeLa and NIKS was assessed with 48 hours of treatment with the drug. NIKS cells were slightly more sensitive to treatment with bisPMB than HeLa cells although the difference was not significant. This observation is in contradiction to what we expected as referring to previous publications, bisPMB has been reported to particularly destroy cancerous cells (Kaschula et al., 2015:1225; Siyo et al., 2017:3-5)**;** this may be related to the OSC selectivity for dividing cells, rendering the fast proliferating cancer cells more exposed to blockage (Sakamoto et al., 1997:154; Li et al., 2002:577; Zhang et al., 2014:10). Our two cancerous and non-cancerous cell lines showed similar sensitivities to treatment with bisPMB. Previous researches carried out in a number of cell lines, HeLa included, demonstrated that the IC_{50} of bisPMB ranged between 2.1–6.5 µM (Kaschula et al., 2011:262), approving the IC_{50} value obtained in this investigation. At present, there is no literature on studies related to the use of bisPMB in NIKS cells.

4.2.4 Infection and inhibition assays

The primary aim of this research sought to elucidate whether bisPMB inhibited HPV16-PsVs infection in the cancerous HeLa (already infected with HPV18) and non-cancerous NIKS cell

lines without affecting cell viability. We conducted several *in vitro* infection and inhibition experiments to elucidate if bisPMB inactivates oncogenic HPV infection. As expected, bisPMB statistically significantly inhibited viral HPV16-PsVs infection in HeLa cells when normalized to cell viability at 5 and 10 µM concentrations (with $p < 0.01$ and $p < 0.001$, respectively), while viral HPV16-PsVs infection was statistically significantly inhibited in NIKS cells when normalized to cell viability at 2.5, 5 and 10 µM concentrations (with $p < 0.001$ in all three cases). Our results confirm that bisPMB has an inhibitory effect on HPV16-PsVs infection of HeLa and NIKS.

A previous study carried out by Chikanya in 2018, assessed bisPMB's role in the early events of HPV infection, namely its entry and inhibitory modes using viral internalization assays. It was found that bisPMB did not inhibit the internalization of the viral particles. This suggests that bisPMB has a post-entry effect**,** affecting intracellular events during HPV infection**;** still, further investigations are required. A previous investigation by Kaschula et al. (2017:6-7) showed that bisPMB interferes with the normal folding of proteins in the ER. The rough ER is an intracellular organelle and the location of the synthesis of secreted and membrane proteins. About 30 % of freshly synthesized proteins in normal eukaryotic cells undertake degradation, this is especially due to failed folding (Schubert et al., 2000:770; Goldberg, 2003:896). BisPMB interferes with the normal folding of proteins by assembling in the ER and thiolating freshly produced proteins, therefore, establishing a horde of S-alkylated proteins: this operation is termed 'ajoenylation'. The abnormal stack of these misfolded proteins leads to the establishment of protein aggregates which are extremely toxic to cells (Bucciantini et al., 2002:509-5010; Stefani & Dobson, 2003:690-691). In some cases, it may lead to what is called ER stress and finally cell death (Kaschula et al., 2017:9-10)**.**

HPV first needs to successfully access the host genome to establish infection and eventually result in cervical cancer**.** In this sense, HPV must proceed throughout the ER. This is the stage at which bisPMB has been reported to modulate biological operations in precedent researches. Taking this into consideration, it is feasible that bisPMB disturbs the folding of proteins implicated in either HPV replication or mitosis (the virus obtains access as the nuclear membrane breaks down during mitosis) or nuclear pore transport proteins. A result of this is the probability that once this process is disturbed the virus is incapable of accessing the nucleus.

Surely more investigations to elucidate bisPMB exact way of the virus inhibition are needed. This will allow an understanding of the exact molecular mechanisms guiding the effect of bisPMB.

The second question aimed to find out if bisPMB inhibited HPV-PsVs infection in HeLa and NIKS cells either type-specifically or in a broad manner using HPV18. Our results showed that bisPMB significantly inhibited viral HPV18-PsVs infection in HeLa cells when normalized to cell viability, at 10 µM concentration (with $p < 0.01$). But regarding the fact that 10 µM is already a cytotoxic concentration, it is not viable to use it as a treatment as it will be toxic to the cell we are trying to protect. We then conclude that bisPMB does inhibit HPV18-PsVs infection of HeLa cells but cannot be used as prophylactic as cell viability is also affected. We additionally found that viral HPV18-PsVs infection was statistically significantly increased upon bisPMB treatment in NIKS cells when normalized to cell viability at 5 and 10 µM concentrations (with $p < 0.001$ in both cases). Our normalized data persistently demonstrated a significant increase in HPV18-PsVs infection of NIKS cells which must have been caused by mechanisms not well known at the moment. These results demonstrate that bisPMB elicits type- and cell-specific effects on the infection of HeLa and NIKS cells.

Taking all into consideration, these outcomes suggest that bisPMB significantly lower only HPV16-PsVs infection at concentrations near its IC_{50} of both HeLa and NIKS cell lines, acting therefore in a type- and cell-specific manner for HPV infection. Indeed, more researches need to be executed in more healthy cell lines to have a better understanding of the mechanisms involved.

4.3 Conclusion

This thesis provided insights into the inhibitory effect of bisPMB for oncogenic HPV-PsVs infection. Our findings on bisPMB are in accordance with published facts on the cytotoxicity of ajoene in cancer cells. In this research, bisPMB displays a similar cytotoxicity effect in cancerous HeLa and normal NIKS cells, even though the mode of action and molecular mechanisms behind are still not clearly known. We found out that bisPMB successfully inhibited HPV16-PsVs infection in both cancerous and non-cancerous cell lines. Interestingly, HPV18-PsVs infection was inhibited in cancerous cells but not in non-cancerous cells. There is a possibility that some of the uptake steps, as well as the function of bisPMB, vary depending on HPV types. We then hypothesize that bisPMB may not act as a general viral restriction but instead as a cell type-specific factor for HPV-PsVs infection.

However, there are many other HPV types and to confirm our hypothesis, we would suggest testing a range of other types including non-oncogenic types (HPV6 and HPV11).

4.4 Future work

A few limitations should be mentioned in this present research. First, not many cell types and HPV strains were used in our experiments, restricting somehow our assessment of bisPMB

role in the inhibition of HPV infection. As the cancer-inhibitory mechanism remains unclear, we suggest future research in this sense using flow cytometry assays and confocal laser scanning microscopy. Finally, *in vivo* research should be performed mirroring *in vitro* tests, as cell culture approaches do not entirely illustrate the sequences of modifications the virus undertakes *in vivo*. We would therefore recommend *in vivo* studies using a mouse model to investigate bisPMB inhibition of HPV infection.

Despite the limitations, this study adds to our comprehension of the impacts of the garlic analogue bisPMB as a modulator of HPV infection.

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APPENDICES

APPENDIX A: REAGENTS AND VOLUMES REQUIRED FOR THE PREPARATION OF SOLUTIONS FOR SDS-PAGE

A

Reagents	12% Resolving solution	3% Stacking solution
Acrylamide/ bisacrylamide 29:1 (40%)	6	1.3
1.5 M Tris PH8.8	5	/
1 M Tris PH6.8		1.25
10% SDS	0.2	0.1
H_20	8.8	7.4
Tetramethyl ethylene diamine (TEMED)[20]	20 µL	20 µL
10% ammonium persulphate (APS)	100 µL	50 µL

B

Reagents	10X SDS-PAGE running buffer
Solid Tris	30 g
Solid Glycerine	144 g
Sodium dodecyl sulphate (SDS)	10 g
H_20	To 1 L

[20] **TEMED:** catalyses the polymerization of acrylamide and therefore must be added lastly. If added too early, the gel will set before the solution is ready to be pipetted in between the plates (Thermo Fischer Scientific, n.d.).

APPENDIX B: RECIPES

Developer working solution:
0.5 mL Enhancer was mixed with 25 mL Developer.

Sensitizer working solution:
50 µL of Sensitizer was added to 25 mL dH_2O.

Staining working solution:
0.5 mL Enhancer was added to 25 mL Stain.